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Penetration resistance and cell death regulation in interactions of cereals with the powdery mildew fungus

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2. General Introduction

1.1. The importance of basic molecular research for plant production

This work is supposed to contribute knowledge about the physiological and molecular processes that change in a cereal crop plant under attack from the parasitic fungus *Blumeria graminis* that causes the powdery mildew disease of wild grasses and cereals. Despite years of intensive research in plant pathophysiology and phytopharmacology, the tools available to farmers to control fungal diseases of their crops are still limited. This is one of the main reasons for the fact that yield losses caused by biotic stresses are estimated to reach approximately 30 % of the theoretically obtainable yield to date (Oerke et al. 1994). Against the background of a growing world population and a dramatic loss of arable landmass, this has to be recognized as a threat of human nutrition security and food quality.

During the last decade, plant biotechnology or green gene technology have provided novel crop plants that face this problem. For instance, maize or cotton plants expressing the *Bacillus thuringiensis* toxin have been proven to express resistance against insect pests (*Lepidoptera* larvae) in the field (discussed in Geldermann and Kogel 2002). Besides this, there are several approaches to improve plant resistance to biotic stresses by biotechnological tools. However, since many of these approaches led to promising results under controlled conditions but never met application, further research to uncover new tools against biological threats is urgently required. To develop such tools against plant diseases, one needs to know the biology of a certain plant-microbe interaction, which then should allow specific intervention. To further improve durable plant disease resistance, an exact knowledge about the molecular interactions allowing disease development is an essential prerequisite. This work aims to contribute to such kind of knowledge and to provide a basis for further development of ideas and technologies helping to safeguard plant production in future times.

1.2. Basic incompatibility

Plants are immobile and face a regularly changing environment. In general, this forces plants to cope with many kinds of stresses including pathogen attack. Nevertheless, plants are resistant to the majority of potential pathogens they are in constant contact with. The tremendous amount of microbial pathogens in the environment would otherwise kill every plant. This kind of plant disease resistance is also called basic resistance (basic incompatibility, if both plant and pathogen are implicated) or non-host resistance because the fact that a certain pathogen cannot grow on a certain plant is most often provoked by a specialization of the pathogen on a more or less narrow host range (Heath 1981). It is possible that this specialization is a consequence of co-evolution with plants that were constantly forced to improve their resistance qualities. Basic resistance is defined as resistance of all cultivars of a plant species to all races of a certain pathogen species. This definition excludes laboratory produced or non-durable mutant genotypes.

To date, it is not finally understood, how basic resistance is constituted. It works through several mechanisms and the degree by which they contribute to complete resistance might depend on the individual plant-microbe interaction. Major mechanism of incompatibility are non-recognition of a plant by a pathogen, missing tools of the pathogen to overcome preformed penetration barriers, and recognition of the inappropriate pathogen by the plant followed by effective defence reactions. The latter can be alternatively interpreted as a lacking ability of the pathogen to avoid recognition and to suppress plant defence. This interpretation became more accepted in the light of understanding that all pathogens invading a plant are accompanied by pathogenesis associated molecular patterns (PAMPs) that are recognized by the plant as non-self molecules. These PAMPs are believed to be conserved pathogen structures such as surface peptides and polysaccharides (Gomez-Gomez and Boller 2002, Nürnberger and Brunner 2002, Schulze-Lefert and Panstruga 2003). One can assume that a microbe either has to avoid recognition of its PAMPs or has to bypass recognition and to suppress subsequent signal transduction and defence to fulfil its life cycle on a certain plant that can then serve as a host.

1.3. Basic compatibility

Little is known about the way pathogens overcome basic resistance of their host plants. It is supposed that pathogens have evolved different strategies. Pathogens might avoid PAMP recognition by release of so-called suppressors that might derive from the plant or the pathogen. Such suppressors might directly interfere with PAMP recognition by competing with a PAMP for binding by a PAMP receptor. Once bound, such a suppressor would then not trigger subsequent downstream effects. Oligomers of galacturonic acid might be an example for endogenous suppressors in the interaction of cereals with biotrophic fungi (Moerschbacher et al. 1999, Vogel et al. 2002).

A second strategy of pathogenic microbes to achieve and maintain compatibility is apparently suppression of active host defence. Bacterial pathogens, for instance, inject effector proteins via a type III secretion system into the host cytoplasm. These effectors are suppressors of plant defence reactions as shown for *Pseudomonas syringae* AvtPtoB that suppresses the hypersensitive defence reaction (HR) in tomato (Abramovitch et al. 2003). Defence suppression by fungal pathogens was shown for tomato leaf spot fungus *Septoria lycopersici* that follows a double suppression program. *S. lycopersici* produces tomatinase, an enzyme that converts the preformed tomato phytoalexin saponin to less toxic compounds. The products of this reaction then inhibit the HR of tomato allowing pathogenesis (Bouarab et al. 2002).

Another prominent example for host-specific suppression of plant defence is given by the pea pathogen *Mycosphaerella pinodes* that provokes Mycosphaerella blight on peas. This fungus produces supprescins A and B, substances that are sufficient to suppress pea penetration resistance to *Alternaria alternata* but not non-host resistance of cowpea (reviewed in Toyoda et al. 2002).

Finally, the secretion of host-selective toxins by necrotrophic pathogens can be also interpreted as a strategy to suppress host defence since toxin-induced perturbations of host physiology should prevent coordinated defence (Wolpert et al. 2002).

1.4. Non-specific and race-specific resistance

Once a microbe has overcome basic resistance of a plant, basic compatibility is achieved and the virulent pathogen can amplify on the susceptible host. However,

normally not every host genotype is equally susceptible to a certain race of a pathogen. This is explained by non-specific mechanisms and race-specific recognition of invading pathogens and subsequent variable effective plant defence.
The aggressiveness of pathogen race and background resistance of a susceptible host plant together determine the severity of disease. Little is known about the molecular basis of these quantitative traits. Genetic tools have been developed to recognise the responsible genomic loci (quantitative trait loci, QTL) and to include these loci in plant breeding programs. However, it is believed that different degrees of residual basic resistance mechanisms or incomplete specific-recognition processes contribute to background resistance (e.g. Gebhardt and Valkonen 2001). Additionally, quantitative resistance trait loci appear to co-segregate with defence gene loci indicating a potential influence of general defence gene expression on background resistance (Geffroy et al. 2000, Ramalingam et al. 2003).
Background resistance to microbes is generally incomplete whereas race-specific resistance is usually complete. It depends on a corresponding pair of a plant major resistance (*R*) gene and a fungal avirulence (*Avr*) gene (Flor et al. 1971). If one of these factors is missing or altered by a mutation, compatibility is the result, which led the assumption that both components are involved in a specific recognition process. This would be most easily explainable if the *R* gene would code for the receptor of the *Avr* gene product. However, in spite of plenty *R* and *Avr* genes cloned to date, direct evidence for physical interaction of the gene products appears to be the exception. This paved the way for the so-called guard hypothesis according to which the R protein monitors certain cellular functions by interaction with multi-component protein complexes that are directly or indirectly targeted by microbial *Avr* gene products (Dangl and Jones 2001, Shirasu and Schulze-Lefert 2003).

1.5. Pathogenicity of *Blumeria graminis*

The fungal pathogen *Blumeria graminis* is the causal agent of cereal powdery mildew diseases. Thereby, specialised formae speciales infect an extremely narrow host range, meaning that e.g. *Blumeria graminis* f.sp. *hordei* (*Bgh*) infects only barley but no other cereals whereas *Blumeria graminis* f.sp. *tritici* (*Bgt*) infects only wheat. The powdery mildew fungi are biotrophic ectoparasites invading only epidermal cells. Nevertheless, powdery mildew disease changes the physiology of the entire plant.

The first prerequisite for fungal establishment after landing of a conidial spore on the host surface is attachment and penetration of the host cuticle and cell wall. The fungus builds two germ tubes, the second of which is infectious, while the first is needed for attachment on the leaf surface and water uptake (Thordal-Christensen et al. 1999). The secondary germ tube swells at its tip to build an appressorium that is the essential penetration organ of *B. graminis.* The fungus penetrates the host cell wall seemingly by means of both hydrolytical and mechanical power (Pryce-Jones et al. 1999). After breakdown of the cell wall barrier, the fungal haustorium is built. This organ invaginates the host plasma membrane whereby the invaded cell remains intact. During this process the host membrane surrounding the haustorium is changed to serve for nutrition of the invader. This so-called extra-haustorial membrane might even loose continuity with the rest of the host plasma membrane to fulfil its role in transport of nutrients across the membrane (Schulze-Lefert and Panstruga 2003). During penetration, the fungus has to cope with cell wall associated defence of the host. The fact that even in a compatible interaction only a certain portion of fungi succeeds in penetration, argues for either the speed of penetration or individual spore abilities to suppress host defence being crucial for access to host cells. Additionally, the different host cell types differ remarkably in their ability to prevent fungal penetration (Koga et al. 1990). Together, the outcome of a fungal penetration attempt on a susceptible host depends on both the fungal aggressiveness and the physiological state of the attacked cell. This provides the basis for the possibility to modulate plant resistance to *B. graminis* by pharmacological or biotechnological engagement in host physiology in front of a susceptible background.

Once *B. graminis* has established a haustorium in a host cell, it has to keep the penetrated cell alive until it has accessed enough nutrients to produce a new generation of conidial spores. Interestingly, *B. graminis* needs to penetrate only one epidermal cell to reprogram the host in the sense that it becomes a nutrient sink and supports fungal sporulation. This has been shown by microprojectile-mediated over-expression of the dominant *Mlo* cDNA in a resistant mutant *mlo*-background that subsequently became susceptible on the level of single-transformed cells (Shirasu et al. 1999a). Although it is self-evident that a biotrophic fungus needs a living host to maintain a compatible interaction, there is also molecular evidence for this essential

feature of the interaction of barley with *Bgh*. The barley MLO-protein is a central switch in the interaction with *Bgh*. In susceptible hosts, MLO expression enhances during attack by *Bgh* (Piffanelli et al. 2002), and the protein is essential for accomplishment of compatibility. It is therefore also called a host susceptibility factor. In mutants (*mlo*-genotype) not expressing the functional protein, fungal penetration is totally restricted (Schulze-Lefert and Panstruga 2003). Interestingly, *mlo*-mutants show also some pleiotropic effects under sterile conditions. This includes spontaneous formation of cell-wall appositions and an early senescence-like phenotype finally leading to leaf cell death reactions (Peterhänsel et al. 1997, Piffanelli et al. 2002, Wolter et al. 1993). Thus, MLO is both a host susceptibility factor and a cell death control element underscoring the link between host cell survival and susceptibility to the biotrophic fungus *Bgh*. However, the molecular basis of MLO abuse by *Bgh* is not understood (Schulze-Lefert and Panstruga 2003). An additional hint that leaf cell death and powdery mildew development are linked is given by the observation that *Bgh* induces a `green island effect´ on infected leaves (Schulze-Lefert and Vogel 2000). The leaf tissue surrounding a fungal colony remains green whereas the rest of the leaf shows chlorosis and senescence. This indicates semisystemic cell death suppression at infection sites, redefinition of invaded tissues as a nutrient sink, and early senescence of the rest of the leaf.

1.6. Plant defence against powdery mildew fungi

Effective plant defence against powdery mildew fungi is usually organised in different subsequent steps. Early defence prevents penetration, second line of defence inhibits nutrient uptake by haustoria.

Penetration resistance of cereals to *B. graminis* is normally achieved under formation of cell wall appositions (CWAs) that are believed to build crucial mechanical and chemical barriers against hydrolytic and osmotic pressure from fungal appressoria. CWAs are constituted of altered cell wall material that contains *inter alia* polysaccharides such as 1,3-glucans (callose), silicon, lignin-like material, and diverse cell wall proteins. The role of these constituents in penetration resistance is not fully understood. For instance, the polysaccharide callose is generally recognised as a resistance factor making the cell wall difficult to digest by fungal hydrolases. However the *Arabidopsis pmr4* mutant that is resistant to powdery mildew is affected

in glucan synthase 5, an enzyme responsible for callose deposition at sites of fungal penetration (Jacobs et al. 2003, Nishimura et al. 2003). Thus, callose formation acts in suppression of powdery mildew resistance instead of being essential for penetration resistance. It appears likely that *B. graminis* cannot dissolve lignin-like material. Blue and yellow autofluorescent material is generally integrated in CWAs and it was observed that such material is insensitive to saponification earlier in resistant *mlo*- than in susceptible *Mlo*-genotypes (von Röpenack et al. 1998). Additionally, protein cross-linking and immobilisation was observed in cell wall appositions induced by *Bgh* (Thordal-Christensen et al. 1997). Both lignification and protein cross-linking depend on H_2O_2 as an oxidant (Bradley et al. 1992, Olson and Varner 1993). H_2O_2 was detected in cell wall appositions built under appressoria of *Bgh* (Thordal-Christensen et al. 1997). Interestingly, H_2O_2 accumulation in cell wall appositions was much more frequently observed when CWA effectively prevented penetration when compared to penetrated CWAs. This was found in susceptible hosts but even more predominant in resistant *mlo*-barley where H_2O_2 accumulated in nearly all CWAs (Hückelhoven et al. 1999).

As an additional or second line of defence the host can prevent nutrient uptake by disturbing haustorial function. The most prominent way to achieve this is the HR including programmed cell death (PCD) of the attacked and/or a few surrounding cells. Although it seems sufficient to kill a cell that should otherwise support fungal growth, PCD is not the only central feature of HR. HR additionally summarises lignification-like accumulation of autofluorogens in the entire cell and the rapid formation and release of low molecular weight (phytoalexins) and protein defence compounds. These compounds are usually toxic to powdery mildew fungi such as the phytoalexin p-coumaroyl-hydroxyagmatine or the fungal cell wall degrading chitinase II (Oldach et al. 2001, von Röpenack et al. 1998). Similar to penetration resistance, H_2O_2 accumulates during execution of HR (Thordal-Christensen et al. 1997, Hückelhoven et al. 1999). H_2O_2 might contribute double to HR since it acts as a signal for PCD and as an antifungal compound (Grant and Loake 2000, Lamb and Dixon 1997). Indeed, the fact that HR involves PCD but comprises also other effective defence reactions leaves the question open, whether PCD is required to render HR effective. This question was highlighted when the *dnd1 Arabidopsis* mutant with functional *R*-dependent resistance to *Pseudomonas syringae* but

simultaneous loss of HR was isolated (Clough et al. 2000). The fact that *mlo*-mutants show penetration resistance to *Bgh* in young seedlings but spontaneous cell death only in adult and senescing leaves might be taken as an additional hint that PCD and full resistance to biotrophs can be phenotypically uncoupled in plants.

Post-penetration defence appears not to be restricted to HR. Quantitative resistance of mildew host plant genotypes is often observed as a slow disease phenotype or mild disease severity without leaf necrosis (e.g. Rubiales et al. 2001). The factors restricting fungal development in these cases are largely unknown since corresponding QTL have not been isolated yet. However, some laboratory derived *Arabidopsis* mutants including *pmr4* show also late incomplete resistance without HR (Vogel and Somerville 2000). Possibly, isolation of the corresponding genes will end up in identification of host susceptibility factors that are also influenced by some QTL.

1.7. Induced resistance against *Blumeria graminis*

As already mentioned, a susceptible cereal host is not completely defenceless to powdery mildew fungi but might even prevent infection at the majority of individual interaction sites. In spite of this, limited infection success of *B. graminis* is sufficient to result in a phenotype that is macroscopically judged as fully susceptible because it leads to development of typical white mildew pustules with many spores formed on green leaf tissue without any indications of plant defence. However, the fact that background resistance of cereals can be effective in stopping individual infection attempts provides the molecular basis to strengthen this kind of background resistance by influencing host physiology.

The phenomenon of Induced Resistance (IR) is defined as enhanced disease resistance of a plant that has been pre-treated with an inducing agent before challenge inoculation by a pathogen. Development of IR normally requires a certain gap of time between induction and pathogen challenge (Ryals et al. 1996, Sticher et al. 1997). IR can be understood as enhanced background resistance because, in induced plants, the same defence responses that are observed in control plants are more rapidly and stronger expressed. This led to the designation for induced plants being in a `primed` status (Conrath et al. 2002).

Besides other types of IR, one distinguishes local and systemic IR as well as chemically and biologically IR. While local IR is only efficient in the same plant organ

that was treated with the inducing agent, systemic IR protects also distant plant parts. Salicylic acid (SA) accumulates in many dicot plants during HR and subsequent establishment of IR. SA is a central signalling molecule in local and systemic IR because expression of a bacterial SA-hydroxylase (*NahG*) abolished the IR defence response in challenged plant parts (Gaffney et al. 1993). However, the mechanism of systemic signalling is not understood although a lipid transfer protein appears to be required for systemic IR in *Arabidopsis* (Maldonado et al. 2002).

Originally, IR was observed after biological plant induction by necrosis-inducing pathogens (reviewed by Ryals et al. 1996, Sticher et al. 1997). Alternatively, IR can be established by plant treatment with chemical substances. The most prominent chemical agents to trigger IR are SA and their functional analogues 2,6-dichloroisonicotinic acid (DCINA) and acibenzolar-S-methyl (synonym Benzo(1,2,3)-thiadiazol-7-carbothioic acid-S-methylester [BTH], Bion ®). Although other plant hormones such as jasmonates are efficient inducers of IR in dicots, highly reproducible effects in monocots were only reported for SA-analogues thus far (Görlach et al. 1996, Kogel et al. 1994, Kogel et al. 1995, van Loon et al. 1998).

Interestingly, evidence for biologically induced systemic resistance in monocots is sparse. Root treatment with chemical inducers protects the entire plant but it was not finally clarified whether this is based on systemic transport of the inducer or on an endogenous second messenger. Local IR is commonly seen in cereals after preinoculation of leaves with avirulent or inappropriate pathogens (Jørgensen et al. 1998). In case of powdery mildew this phenomenon was also called induced inaccessibility (e.g. Lynkjær and Carver 2000). Thereby, cells attacked by *B. graminis* and their neighbour cells are resistant to a second penetration attempt by *B. graminis* if the first attempt failed. Vice versa, a successful infection induces local accessibility to *B. graminis*. This could be explained by primed or suppressed defence reactions in the surrounding of inaccessible or accessible cells, respectively.

In barley, both biologically or chemically induced resistance are mainly characterised by penetration resistance to challenging *Bgh*. Additionally, HR of non-penetrated cells is frequently observed. The response of barley to *Bgh* is thereby reminiscent of the phenotype microscopically observed in *R*-gene mediated (*Mlg*-dependent) resistance. It was therefore called a phenocopy of *Mlg*-mediated resistance (Kogel et al. 1994). Interestingly, the subcellular accumulation patterns of H_2O_2 and $O_2^{\bullet-}$ were

also similar in chemically IR and *Mlg*-mediated resistance (Hückelhoven and Kogel 1998, Hückelhoven et al. 1999, Kogel and Hückelhoven 1999). The molecular basis of chemically IR in monocots is not understood. Differential gene expression analyses have identified sets of genes that are activated in barley or wheat, respectively, after treatment with DCINA or BTH (Besser et al. 2000, Görlach et al. 1996). Interestingly, most of these genes are not activated by *B. graminis*. Therefore their role in IR, extending their function as reliable marker genes, needs to be clarified. Although DCINA and BTH are recognized as functional analogues of SA, SA does not accumulate in barley after inoculation with either virulent, avirulent or inappropriate *B. graminis* (Hückelhoven et al. 1999, Vallelian–Bindschedler et al. 1998). The defence carried out in the interactions studied comprises penetration resistance, pathogenesis-related gene expression, HR and finally culminates in induced inaccessibility. Hence, one can assume that non of these processes neither relies on SA accumulation nor provokes it. Although this does not finally prove that SA is superfluous in barley defence to *Bgh*, this might be an important characteristic of constitutive and induced monocot resistance when compared to many types of resistance in dicots.

1.8. Genetics of barley resistance to *B. graminis*

In the last twenty years cereal powdery mildew resistance research was carried out mainly taking barley as a model host. The diploid barley genome together with the construction of near-isogenic lines bearing distinct powdery resistance loci opened up the possibility for comparative studies and genetic approaches (Collins et al. 2002, Jørgensen 1994, Kølster et al. 1986).

The number of dominant or semi-dominant race-specific resistance genes in barley was estimated to be approximately 85 (Jørgensen 1994). About 30 out of these are closely linked to the *Mla* locus on chromosome 5. The complex *Mla* locus was genetically and physically located within an interval of approximately 250 kilobases (Wei et al. 1999, 2002). All *R* gene homologues at *Mla* belong to the coiled coil-nucleotide binding-leucine rich repeat (CC-NB-LRR) class. Remarkably, the four isolated *Mla* genes *Mla1*, *Mla6*, *Mla12*, and *Mla13* code for deduced proteins that are approximately 97% sequence identical within the CC-NB domain and about 87% in the C-terminal LRR region. This suggests that they might be alleles of a single gene

(reviewed by Schulze-Lefert and Panstruga 2003). Besides the four *Mla* genes cloned to date, no further *Mlx* specificities have been isolated. *Mla12*-mediated resistance was further characterised by the recessive suppressor mutant genes *rar1* and *rar2* (required for *Mla12*-specified resistance, Jørgensen 1988). The latter was meanwhile found out to be a recessive *mla12*-mutation (Shen et al. 2003), whereas wild type *Rar1* encodes a potentially zinc binding protein containing two CHORD domains (CHORD = cysteine and histidine rich domain, Shirasu et al. 1999b). The RAR1 protein was found to interact physically with SGT1 that was also found to be required for *Mla*-mediated resistance (Azevedo et al. 2002). Although the MLA proteins are very similar, their dependency on RAR1 and SGT1 differs. Despite this, RAR1 acts in race-specific resistance mediated by non-*Mla* genes (Jørgensen 1996, Schulze-Lefert and Vogel 2000, Shen et al. 2003). Furthermore, both RAR1 and SGT1 are essential for several types of disease resistance in dicot plants (e.g. Austin et al. 2002, Peart et al. 2002). The molecular cooperation of MLA, RAR1 and SGT1 is not understood but they may work together in a recognition complex for pathogen effector molecules. Alternatively, RAR1 and SGT1 might be involved down-stream of recognition in removal of negative regulators of defence responses since SGT1 interacts with components of the SCF (SKP1-cullin-F-box) ubiquitin ligase complex that initiates substrate-specific protein degradation (Azevedo et al. 2002, Shirasu and Schulze-Lefert 2003).

The *Mlo*-Locus is perhaps the most important locus in regard to powdery mildew resistance of cereals. Barley homozygous for a recessive *mlo* null-mutant allele shows broad-spectrum resistance to all European field isolates of *Bgh*. This led to an extensive use of the *mlo*-genotypes in agriculture so that nowadays an increasing number of European spring barley cultivars bear *mlo* (Jørgensen 1994). The recessive nature of this resistance might explain why it is durable although it is in use since more than 20 years. The corresponding dominant *Mlo* gene was located on barley chromosome 4 and was isolated by Büschges and associates (1997). The gene encodes a deduced 60 kilodalton protein with seven transmembrane domains reminiscent of a G-protein coupled receptor (Büschges et al. 1997, Devoto et al. 1999). Despite of this topology, MLO function in susceptibility to *Bgh* appears to be independent of heterotrimeric G-proteins. Instead, MLO interacts Ca^{2+} dependently with calmodulin to fulfil its function in susceptibility (Kim et al. 2002).

Re-mutagenesis of resistant *mlo*-genotypes led to isolation of two independent mutant genotypes, *ror1* and *ror2* (required for *mlo*-specified resistance), that show partial loss of *mlo*-mediated resistance (Freialdenhoven et al. 1996). Together with other findings, the fact that *Bgh* growths on null-*mlo ror* genotypes proves that MLO is not simply a factor needed by *Bgh* to recognise its host but rather a negative regulator of defence responses.

Major *R* and *mlo*-mediated resistance are usually complete or close to it. Quantitative (syn. partial, horizontal) resistance is much less well defined and is usually expressed as a polygenic trait. Single genes contributing to quantitative resistance usually act additively (Jørgensen 1994). However, quantitative resistance to *Bgh* might be partly mediated by *R*-like factors because QTL for powdery mildew resistance co-localise with chromosomal *R* clusters (Backes et al. 2003).

3. Publications: Chapters 1-14 with special introductions

Chapter 1

Hückelhoven R, Fodor J, Trujillo M and Kogel K-H (2000) Barley *Mla*- and *Rar*-mutants compromised in the hypersensitive cell death response against *Blumeria graminis* f.sp. *hordei* are modified in their ability to accumulate reactive oxygen intermediates at sites of fungal invasion. *Planta* 212: 16-24

Chapter 2

Hückelhoven R, Trujillo M, Kogel K-H (2000) Mutations in *Ror1* and *Ror2* genes cause modification of hydrogen peroxide accumulation in *mlo*-barley under attack from the powdery mildew fungus. *Mol Plant Pathol* 1: 287-292

Chapter 3

Trujillo M, Kogel K-H, Hückelhoven R (2004): Superoxide and hydrogen peroxide play different roles in non-host interactions of cereals and inappropriate formae speciales of *Blumeria graminis. Mol Plant-Microbe Interact* 17: 304-312

Chapter 4

Hückelhoven R, Kogel K-H (2003): Reactive oxygen intermediates in plant-microbe interactions: Who is who in powdery mildew resistance? *Planta* 216: 891–902

Chapter 5

Hückelhoven R, Dechert C, Trujillo M, Kogel K-H (2001) Differential expression of putative cell death regulator genes in near-isogenic, resistant and susceptible barley lines inoculated with the powdery mildew fungus. *Plant Mol Biol* 47: 739-748

Chapter 6

Schultheiss H, Dechert C, Király L, Fodor J, Michel K, Kogel K-H, Hückelhoven R (2003) Functional analysis of the barley *PR1b*-gene. *Plant Science* 165: 1275–1280

Chapter 7

Schultheiss H, Dechert C, Kogel K-H, Hückelhoven R (2002) A Small GTP-binding host protein is required for entry of powdery mildew fungus into epidermal cells of barley. *Plant Physiol* 128: 1447-1454

Chapter 8

Schultheiss H, Dechert C, Kogel K-H, Hückelhoven R (2003) Functional analysis of barley RAC/ROP G-protein family members in susceptibility to the powdery mildew fungus. *Plant J* 36: 589-601

Chapter 9

Hückelhoven R, Dechert C, Kogel K-H (2003) Overexpression of barley BAX inhibitor 1 induces breakdown of *mlo*-mediated penetration resistance to *Blumeria graminis*. *Proc Natl Acad Sci USA* 100: 5555-5560

Chapter 10

Eichmann R, Schultheiss H, Kogel K-H, Hückelhoven R (in press): The barley apoptosis suppressor homologue Bax Inhibitor-1 compromises nonhost penetration resistance of barley to the inappropriate pathogen *Blumeria graminis* f.sp. *tritici. Mol Plant-Microbe Interact* 17: 484-490

Chapter 11

Hückelhoven R (2004) BAX Inhibitor-1, an ancient cell death suppressor in animals and plants with prokaryotic relatives. *Apoptosis* 9: 299-307

Chapter 12

Hückelhoven R, Dechert C, Kogel K-H (2001) Non-host resistance of barley is associated with a hydrogen peroxide burst at sites of attempted penetration by wheat powdery mildew fungus. *Mol Plant Pathol* 2: 199-205

Chapter 13

Trujillo M, Troeger M, Niks R, Kogel K-H, Hückelhoven R (2004): Mechanistic and genetic overlap of barley host and non-host resistance to *Blumeria graminis*. *Mol Plant Pathol* 5: 389-396

Chapter 14

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu J-L, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P (2003) SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* 425:973-7

Special introduction to chapters 1-4:

The work of the following four chapters analysed the accumulation of reactive oxygen intermediates in the interaction of barley and wheat with inappropriate and appropriate *formae speciales* of *B. graminis.* The use of susceptible barley *rar* and *ror* mutants as well as the comparison of host with non-host resistance should provide an overview about the distinct roles of H_2O_2 and $O_2^{\bullet-}$ in these interactions. Since all susceptible mutants showed at least a partial lack of H_2O_2 accumulation in response to *Bgh* attack, one can assume that H_2O_2 is crucial for both effective *mlo*- and *Mla*-mediated resistance. Interestingly, the role of $O_2^{\bullet-}$ appears to be different from that of H_2O_2. This issue is discussed in detail in chapter 4 that aims to summarize the current knowledge of the role of reactive oxygen in powdery mildew resistance.

Chapter 1

Hückelhoven R, Fodor J, Trujillo M and Kogel K-H (2000) Barley *Mla*- and *Rar*-mutants compromised in the hypersensitive cell death response against *Blumeria graminis* f.sp. *hordei* are modified in their ability to accumulate reactive oxygen intermediates at sites of fungal invasion. *Planta* 212: 16-24

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Planta (2000) 212: 16–24

Planta

Barley *Mla* and *Rar* mutants compromised in the hypersensitive cell death response against *Blumeria graminis* f.sp. *hordei* are modified in their ability to accumulate reactive oxygen intermediates at sites of fungal invasion

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Abstract. The pathogenesis-related accumulation of superoxide radical anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) was comparatively analyzed in a barley line (*Hordeum vulgare* L. cv Sultan-5) carrying the powdery mildew (*Blumeria graminis* f.sp. *hordei*, *Speer*, *Bgh*) resistance gene *Mla12*, and in susceptible mutants defective in *Mla12* or in genes "required for *Mla12*-specified disease resistance" (*Rar1* and *Rar2*). In-situ localization of reactive oxygen intermediates was performed both by microscopic detection of azide-insensitive nitroblue tetrazolium (NBT) reduction or diaminobenzidine (DAB) polymerization, and by an NBT-DAB double-staining procedure. The *Mla12*-mediated hypersensitive cell death occurred either in attacked epidermal cells or adjacent mesophyll cells of wild-type plants. Whole-cell H_2O_2 accumulation was detected in dying cells, while $O_2^{\bullet-}$ emerged in adjacent cells. Importantly, all susceptible mutants lacked these reactions. An oxalate oxidase, which is known to generate H_2O_2 and has been implicated in barley resistance against the powdery mildew fungus, was not differentially expressed between the wild type and all mutants. The results demonstrate that the *Rar1* and *Rar2* gene products, which are control elements of *R*-gene-mediated programmed cell death, also control accumulation of reactive oxygen intermediates but not the pathogenesis-related expression of oxalate oxidase.

Key words: *Erysiphe* – *Hordeum* (cell death) – Oxidative burst – Programmed cell death

Abbreviations: *Bgh*A6 = *Blumeria* (*syn. Erysiphe*) *graminis* f.sp. *hordei* (Speer) race A6; DAB = 3,3-diaminobenzidine; ESH = elongated secondary hyphae; hai = hours after inoculation; HR = hypersensitive response; M22 = susceptible *Rar2*-mutant; M66 = susceptible *Mla12*-mutant; M100 = susceptible *Rar1*-mutant; NBT = nitroblue tetrazolium; ROI = reactive oxygen intermediate

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Introduction

Reactive oxygen intermediates (ROIs) are involved in programmed cell death triggered in plants in response to pathogen attack. However, whether ROIs are essential signal components of plant defense is still an open question. Although several lines of evidence suggest that ROI accumulation may be a signal for activation of resistance responses (Doke 1983; Levine et al. 1994; Jabs et al. 1997), it also seems to be a consequence of resistance (Heath 1998) or a hallmark of successful pathogenesis (von Gönner and Schlösser 1993; von Tiedemann 1997).

Recent studies have reinforced the view that the rapid accumulation of ROIs after pathogen recognition is not sufficient to elicit a hypersensitive cell death response (HR; Glazener et al. 1996; Hückelhoven and Kogel 1998; Dorey et al. 1999). Nitric oxide was suggested to be a complementing component for HR induction by hydrogen peroxide (Delledonne et al. 1998).

The role of ROIs in the interaction of barley with the powdery-mildew fungus (*Blumeria graminis* f.sp. *hordei*, *Bgh*) has been extensively studied. Subcellular, pathogenesis-related accumulation of $O_2^{\bullet-}$, detected as nitroblue tetrazolium (NBT) reduction to blue formazans, and of H_2O_2, observed as 3,3-diaminobenzidine (DAB) polymerization to brownish polymers, has been described. Barley seedlings bearing the *Mla12* resistance gene characteristically exhibit an $O_2^{\bullet-}$ burst in response to fungal penetration into attacked epidermal cells and in mesophyll cells neighboring the HR (Hückelhoven and Kogel 1998). Accumulation of H_2O_2 induced by *Bgh* attack has been detected in cell wall appositions formed as penetration barriers (papillae) and in cells expressing HR (Thordal-Christensen et al. 1997). The source of ROIs accumulating in barley after powdery mildew attack has not yet been identified. Accumulation of $O_2^{\bullet-}$ in attacked cells is barely sensitive to the NADPH oxidase-inhibiting agent diphenyleneiodonium chloride in-situ, and an NADPH oxidase like that acting in animal phagocytes has not yet been proven to exist in barley. Instead, peroxidases and oxalate oxidases

accumulate in barley after powdery-mildew attack and both may support the HR and cell-wall strengthening by the generation of H_2O_2, which is also a substrate for peroxidases during lignification (Kerby and Somerville 1992; Olson and Varner 1993; Zhou et al. 1998).

A comparative analysis of several barley lines carrying different genes for powdery-mildew resistance pointed to a complex role for $O_2^{\bullet-}$ in this pathosystem. Strikingly, *Mla12*-mediated HR, but not HR in plants bearing the resistance gene *Mlg*, was preceded by epidermal $O_2^{\bullet-}$ generation, while both genes mediate $O_2^{\bullet-}$ generation in the mesophyll and H_2O_2 accumulation (Hückelhoven and Kogel 1998; Hückelhoven et al. 1999).

The barley mutants M22, M66, and M100, derived from the double haploid *Mla12*-bearing resistant line Sultan-5 (Torp and Jørgensen 1986), are excellent tools for further elucidation of the role of ROIs. All mutants are disturbed in the *Mla12*-mediated HR, which is expressed in wild-type plants after penetration of epidermal cells by the avirulent *Bgh* race A6 (*Bgh*A6, Freialdenhoven et al. 1994). While M66 is affected in the *Mla12* gene itself, M22 and M100 are disturbed in genes required for *Mla*-specified resistance (*Rar2* and *Rar1*). The *Rar*-mutations also affect expression of other *Mlx*-mediated types of barley resistance against *Bgh* (Jørgensen 1996; Peterhänsel et al. 1997). Recently, the *Rar1* gene product was shown to be a small zinc-binding protein belonging to a novel class of proteins involved in cell death signaling in plants and development in animals (Shirasu et al. 1999). We show here that mutants defective in *Mla12*, *Rar1* or *Rar2* lack not only H_2O_2 accumulation in epidermal cells penetrated by *Bgh*A6 but also $O_2^{\bullet-}$ and H_2O_2 accumulation in subjacent mesophyll cells.

Material and methods

Plants, pathogens and inoculation

The barley (*Hordeum vulgare* L.) cultivar Sultan-5 and the mutants M22, M66 and M100 were obtained from J. Helms Jørgensen (Risø National Laboratory, Roskilde, Denmark). Their generation was described previously (Torp and Jørgensen 1986). Plants were grown in a growth chamber at 16 °C with 60% relative humidity and a photoperiod of 16 h (60 µmol photons m^{-2} s^{-1}). Inoculation of primary leaves was performed with 20 (for microscopy) or 50 conidia mm^{-2} (for gene transcript analysis) from *Blumeria graminis* f.sp. *hordei*, race A6, at the 7th day after germination. The fungus was cultivated on the barley cultivar Golden Promise under the same conditions.

Microscopic analysis, and staining of $O_2^{\bullet-}$ and H_2O_2

Whole-cell autofluorescence and discontinuity of cytoplasmic strings were taken as a reliable measure of cell death (Görg et al. 1993). Penetration of attacked cells was ascertained by detection of haustoria formation or development of elongated secondary hyphae (ESH). Bright-field and fluorescence microscopy was performed as described by Hückelhoven and Kogel (1998).

The solution for the in-situ detection of $O_2^{\bullet-}$ contained 1 mg mL^{-1} NBT in 10 mM NaN_3, 10 mM potassium phosphate buffer, pH 7.8 (Ádám et al. 1989). At 6, 12, 16, 19, 24, 30, 36, 40, 48 hours after inoculation (hai), the solution was injected (Hagborg 1970) into primary leaves of barley seedlings (Hückelhoven and Kogel 1998). Because the rate of fungal penetration into short and long epidermal cells is different, only short cells were evaluated (cell-type A and B covering parenchymatic mesophyll tissue near stomata; for leaf topography, see Koga et al. 1990).

Detection of H_2O_2 was performed using the DAB-uptake method as described previously (Thordal-Christensen et al. 1997; Hückelhoven et al. 1999).

In-situ double-detection of H_2O_2 and $O_2^{\bullet-}$ was performed by injection of 0.5 mg mL^{-1} DAB (pH 3.8, adjusted with HCl), followed 2 h later by in-situ detection of $O_2^{\bullet-}$ in the same leaves. In-situ detection of $O_2^{\bullet-}$ must follow after detection of H_2O_2, because DAB staining depends on endogenous peroxidases being inhibited by azide included in the NBT assay to make it specific for $O_2^{\bullet-}$ generation (compare also Auclair and Voisin 1985; Doke 1983; Heath 1998; Murphy et al. 1998).

Both NBT and DAB staining procedures led to weak background staining in mesophyll cells beneath short epidermal cells. Background staining was more intensive near vascular bundles where ROI accumulation was not evaluated in this study (see also Thordal-Christensen et al. 1997; Hückelhoven et al. 1999).

Extraction of RNA and northern blotting

Total RNA was extracted from 10 primary leaf segments (5 cm long) using an RNA extraction buffer (AGS, Heidelberg, Germany) according to the manufacturer's instructions. For barley mRNA detection (barley oxalate oxidase, clone *HvOxOa*, Gen Bank accession number Y14203, obtained from Hans Thordal-Christensen; Zhou et al. 1998; barley *PR1b*, clone *HvPR1b*, Gen Bank accession number X*74940*, obtained from David Collinge; Bryngelsson et al. 1994), 10 µg of total RNA from each sample was separated in agarose gels and blotted by capillary transfer to positively charged Nylon membranes. Detection of RNAs was performed according to the DIG System User's Guide after digoxygenin or fluorescein labeling of an RNA probe by in-vitro transcription (DIG-Luminescence detection Kit, Boehringer, Mannheim, Germany; Kogel et al. 1994). Prior to immunodetection of RNA-RNA hybrids, blots were washed stringently two times for 20 min in 0.1% (w/v) SDS, 0.1 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.0) at 68 °C.

Results

Loss of hypersensitivity in Mla12-, Rar1 and Rar2 mutants

In previous studies, we found that accumulation of ROIs preceded and accompanied the HR of attacked epidermal cells and/or the subjacent mesophyll tissue induced in *Mla12*-barley by the avirulent fungal race *Bgh*A6 (Hückelhoven and Kogel 1998; Hückelhoven et al. 1999). In the present study, we analyzed the mutants M22, M66 and M100, which are suppressed in the *Mla12*-mediated defense response, for their ability to drive an oxidative burst. The microscopic interaction phenotypes of M22 (genotype *Mla12*, *Rar1*, *rar2*), M66 (*mla12*, *Rar1*, *Rar2*) and M100 (*Mla12*, *rar1–2*, *Rar2*) with *Bgh*A6 have been determined earlier by Freialdenhoven et al. (1994) and Shirasu et al. (1999). In accordance with these studies, the frequency of cell wall penetration by the fungus was about 70% of interaction sites on the *Mla12*-resistant cultivar Sultan-5 and all the

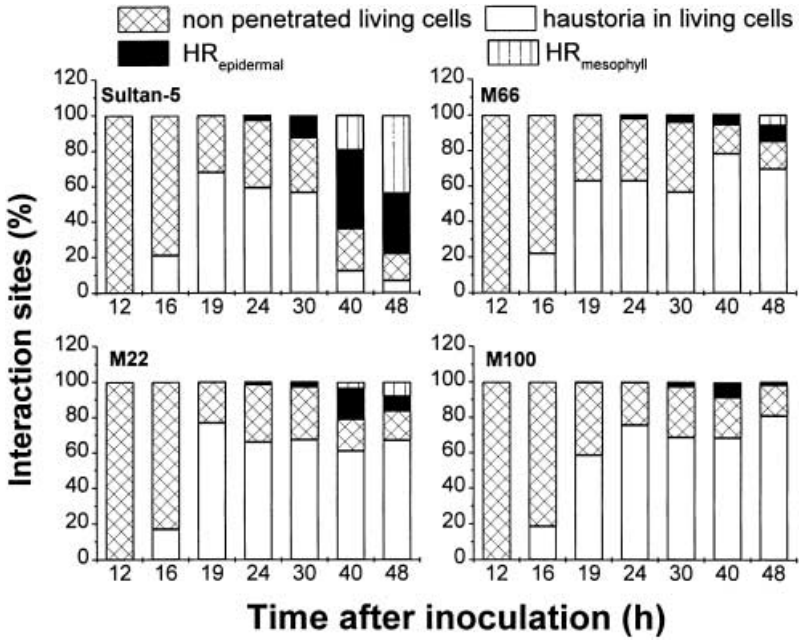


Fig. 1. Defense responses of the *Mla12*-resistant barley cv. Sultan-5 and susceptible loss-of-function mutants M22, M66 and M100 upon inoculation with *Blumeria graminis* f.sp. *hordei*, race A6 (*avrMla12*). Seven-day-old primary leaves of Sultan-5 and mutants defective in the genes *Mla12* (M66), *Rar2* (M22) and *Rar1* (M100) were inoculated with 10 conidia mm^{-2} of *Bgh*A6 and analyzed early in the interaction. *White columns*: frequency of established non-differentiated or differentiated haustoria in the living host cell first attacked by the pathogen (no defense). The low values observed from 30 hai onward in Sultan-5 result from increased frequencies of dead, penetrated cells and of dead mesophyll cells at late time points (see black and striped columns). *Checkered columns*: frequency of interaction sites restricted to a single living host cell in which the fungal penetration attempt was not successful. None of these infection sites exhibited a hypersensitive cell death. At these sites the fungus is arrested within an effective cell wall apposition (papilla). Before 19 hai it was not possible to distinguish finally between effective and non-effective papillae. *Black columns*: frequency of interaction sites in which the attacked cell shows a characteristic yellow whole-cell autofluorescence upon UV light excitation. Autofluorescence is a reliable measure of cell death (single-cell hypersensitive response, HR) in the barley-powdery mildew interaction (Koga et al. 1990; Görg et al. 1993). Autofluorescence of epidermal cells was not seen before 19 hai. *Striped columns*: frequency of interaction sites with mesophyll cells exhibiting yellow whole-cell autofluorescence (mesophyll-HR). Autofluorescent mesophyll cells were not seen before 30 hai. Mesophyll-HR was normally detected in the presence of branched ESH. Each column represents the average for triplicates of each 100 interaction sites per leaf. Repetition of the experiments led to results very similar to those shown in the figure

mutants at early interaction stages (19–24 hai, Fig. 1). At later time points, haustorium formation in mutants was followed by development of elongated secondary hyphae (ESH, 70–80% of interaction sites by 48 hai). The frequency of interaction sites showing an HR was less than 20% at each time of evaluation. In contrast, by 48 hai, Sultan-5 showed an HR of either the penetrated cell or subjacent mesophyll cells at nearly 80% of interaction sites (Fig. 1). If the epidermal HR failed to appear, the fungus was stopped after mesophyll-HR when it had already developed branched ESH.

Cellular localization of ROI

In barley, both H_2O_2 and $O_2^{\bullet-}$ were shown to accumulate after powdery-mildew attack. Their accumulation seems to be regulated differently after fungal attack (Hückelhoven and Kogel 1998; Hückelhoven et al. 1999). Therefore, we examined the accumulation of H_2O_2 and $O_2^{\bullet-}$ simultaneously and independently by in-situ DAB polymerisation and in-situ NBT reduction.

Certain patterns of ROI accumulation could be detected in all genotypes after fungal attack. In agreement with recent studies, the dark blue formazan dye indicative for NBT reducing activity of $O_2^{\bullet-}$ was confined to sites of cell wall penetration and the vicinity of haustorial initials at 16–24 hai. In the mesophyll tissue, formazans were detected in the apoplast and/or the cytoplasm surrounding chloroplasts at 12–30 hai. Polymerization of DAB driven by H_2O_2 was seen as a reddish-brown staining at interaction sites. The DAB polymers were detected 14–48 hai in non-penetrated cell wall appositions and 24 hai in anticlinal cell walls of living, penetrated cells (see Thordal-Christensen et al. 1997; Hückelhoven and Kogel 1998; Hückelhoven et al. 1999).

In Sultan-5, haustorial initials in HR cells showed bright autofluorescence under exposure to UV light, indicating accumulation and demobilization of phenolic compounds. At these sites, haustorial initials were also stained by DAB (Fig. 2A,B).

To address the question whether $O_2^{\bullet-}$ and H_2O_2 originate from the same source, we analyzed the temporal and spatial patterns of ROIs by a double-staining procedure (Fig. 2C–O). When attacked cells of Sultan-5 underwent HR, these cells were stained by DAB while adjacent cells were stained intensively by

formazan (Fig. 2C). In contrast, these staining responses were absent in all mutants (representative picture in Fig. 2D).

At sites with epidermal HR in Sultan-5, whole-cell DAB staining was associated with formazan staining in chloroplasts and DAB staining in the apoplast of subjacent mesophyll cells (Fig. 2E–G). Additionally, an intense brown coloration could be often detected around the nucleus in HR-cells (Fig. 2H). Membranes stained by DAB and/or DAB-stained cytoplasm permeating pits were observed between epidermal and adjacent cells in Sultan-5 (Fig. 2I,J,F). Mesophyll cells that had begun to collapse in the wild type accumulated DAB polymers in chloroplasts and cell walls, whereas $O_2^{\bullet-}$ was exclusively found in cells which appeared intact (Fig. 2K,L). The DAB staining in chloroplasts of cells mounting an HR was not detected by use of the DAB-uptake method in a former study (Hückelhoven et al. 1999). Lesions from dead DAB-stained mesophyll cells were characteristically surrounded by two rings of cells that were differently stained: the inner ring of cells showed DAB and formazan staining, while the outer ring of fully turgid cells was intensively stained with formazan, especially in chloroplasts (Fig. 2M–O). Simultaneous injection of 10 mM ascorbate and DAB into barley leaves completely prevented in-situ polymerization of DAB (data not shown).

Kinetic analysis of $O_2^{\bullet-}$ generation at interaction sites

In-situ accumulation of $O_2^{\bullet-}$ at interaction sites was analyzed in Sultan-5 and all mutants at 12, 16, 19, 24, 30, 36, 40 and 48 hai. Accumulation of formazans was seen in Sultan-5 with a time course similar to that found earlier in an *Mla12*-backcross line of cultivar Pallas (data not shown; Hückelhoven and Kogel 1998). Because the previous study had shown that temporal occurrence and frequency of formazan staining in attacked epidermal cells and underlying mesophyll cells after *Bgh* attack may differ (Hückelhoven and Kogel 1998), we evaluated these types of staining separately. In Sultan-5, the highest frequency of formazan-stained epidermal cells was detected at 24 hai (22% of sites). The frequency of sites with formazans in attacked cells was in all mutants similar to Sultan-5 at 24 hai (Fig. 3A). Strikingly, in the mutants this was not followed by cell death. Thus, $O_2^{\bullet-}$ generation in attacked epidermal cells did not correlate with the frequency of the HR (compare Fig. 1).

No genotype-specific differences in the mesophyll formazan staining were found during early interaction stages (12–24 hai, data not shown). However, when an HR occurred in Sultan-5, formazan staining in the mesophyll (Fig. 2C,G) increased strongly, resulting in a maximum level of 55% of all sites by 40 hai. All mutants showed this pattern of staining less frequently (Fig. 3B). Thus, in contrast to the situation in attacked cells (Fig. 3A), mesophyll staining with formazans positively correlated with HR.

Kinetic analysis of H_2O_2 accumulation at interaction sites

In an independent experiment the frequency of H_2O_2 accumulation at interaction sites was analyzed. Before the onset of HR in penetrated cells (24 hai), 60–70% of interaction sites were free of DAB staining, independent of the genotype. The DAB polymers were identified at 20–30% of sites, in non-penetrated papillae or anticlinal cell walls near penetration sites. However, at 36 hai the frequency of interaction sites with whole-cell H_2O_2 accumulation in attacked Sultan-5 cells rose to about 30%, while the corresponding value for all mutants was at most 10% (Fig. 4). Differences in DAB staining were detected even more strikingly in the mesophyll (48 hai). On Sultan-5, H_2O_2 was detected this time at more than 40% of interaction sites, whereas the mutants lacked this reaction almost completely (Fig. 4).

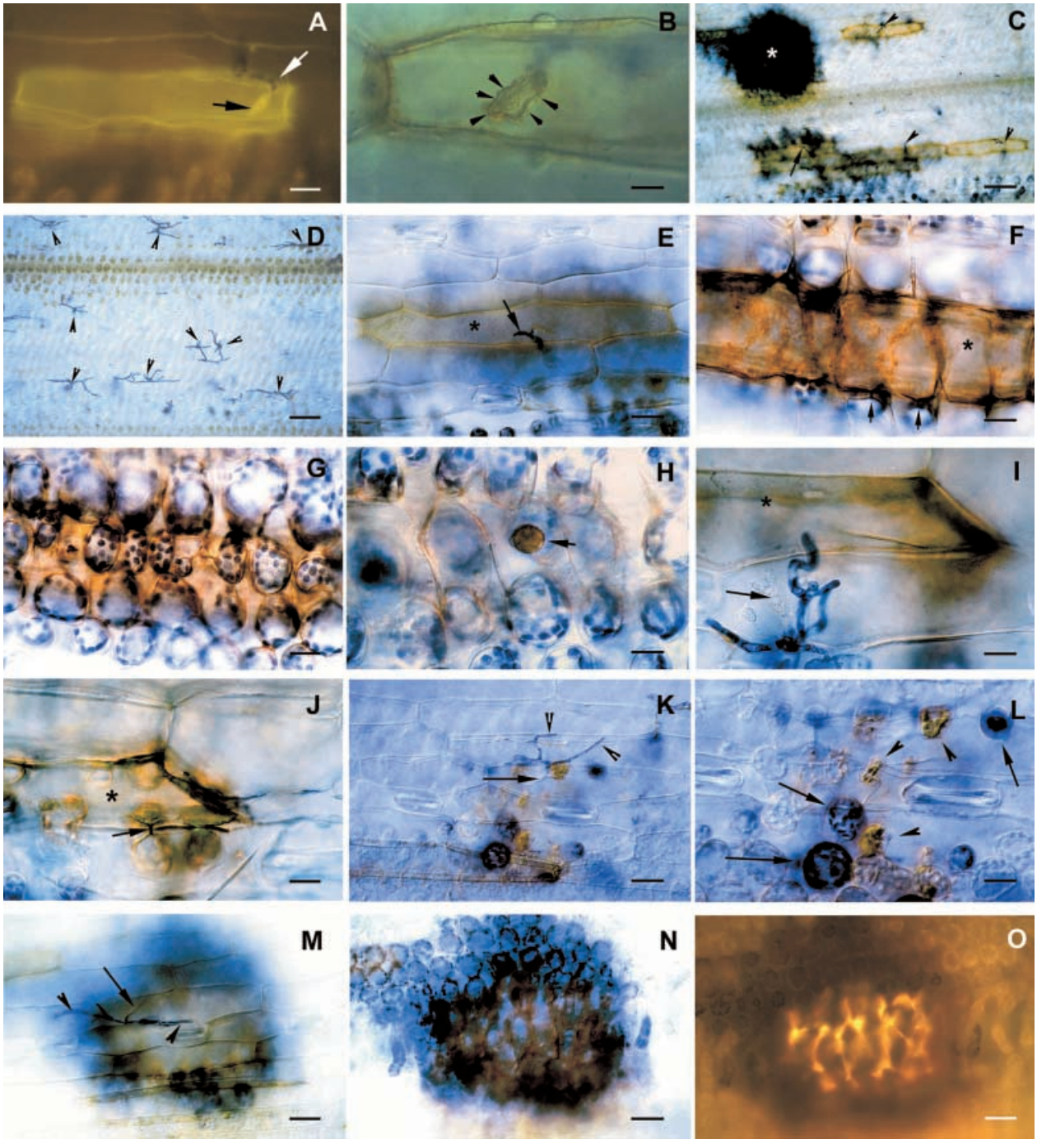
Expression of HvOxOa

Barley oxalate oxidase (*HvOxOa*) was previously shown to be expressed in near-isogenic barley lines of cultivar Pallas after inoculation with virulent and avirulent races of *Bgh* (Zhou et al. 1998). We examined whether any of the mutants were compromised in the accumulation of *HvOxOa* transcripts after inoculation with *Bgh*A6. We extracted total RNA from non-inoculated and inoculated plants at 3, 8, 15, 24, 36 and 48 hai for northern analysis. Expression of *Pr1b* was used as a marker for pathogenesis-related gene expression. As displayed in Fig. 5, the *HvOxOa* gene was constitutively expressed. A weak pathogenesis-related enhancement of the *HvOxOa* transcript level was observed from 8 hai onward. Compared to the wild type, none of the mutants showed a reduced amount of *HvOxOa* expression. In contrast, *PR1b* expression was lower in all mutants compared with Sultan-5.

Discussion

The present study demonstrates that accumulation of different ROIs in barley after *Bgh*A6 attack depends on the function of *Mla12*, *Rar1* and *Rar2* in a tissue-specific manner. While $O_2^{\bullet-}$ accumulates equally in attacked epidermal cells of each genotype, mesophyll $O_2^{\bullet-}$ generation as well as whole-cell H_2O_2 accumulation in attacked epidermal cells or subjacent mesophyll cells occur exclusively in plants bearing functional *Mla12*, *Rar1* and *Rar2* genes. Thus, in penetrated cells superoxide generation is an unspecific plant response whereas whole-cell H_2O_2 accumulation is dependent on a functional signal-transduction chain.

In attacked cells, $O_2^{\bullet-}$ was produced after the fungus succeeded in cell wall penetration (Hückelhoven and Kogel 1998). Since $O_2^{\bullet-}$ accumulated in attacked cells of all mutants (Fig. 3A), a functional *Mla12* pathway is not required for this reaction. On the other hand, the presence of H_2O_2 and phenolic compounds (Fig. 2A,B)



at haustorial initials in HR-cells indicates a role for ROIs in lignification and killing of the pathogen. Here, $O_2^{\bullet-}$ might have a role in defense whereas it is not a key signal for the epidermal HR.

Generation of $O_2^{\bullet-}$ was often detected in non-attacked cells in the neighborhood of dying or dead cells. Low-level, apoplastic $O_2^{\bullet-}$ concentrations were suggested to act as a signal for limitation of cell lesions (Jabs et al. 1996). On the one hand, at sites of spreading lesions in Sultan-5, $O_2^{\bullet-}$ generation preceded H_2O_2 accumulation and HR. On the other hand, at sites of single-cell HR, $O_2^{\bullet-}$ generation in neighboring cells did not result in an HR. Taking this into account, the balance of $O_2^{\bullet-}$ and H_2O_2 could be crucial for the discrimination between cell survival and cell death after powdery-mildew attack.

Mutations of *Mla12*, *Rar1 or Rar2* caused a lack of $O_2^{\bullet-}$ generation in the mesophyll at 40 hai. At the same time, $O_2^{\bullet-}$ was associated with chloroplasts. Allan and Fluhr (1997) showed that ROI accumulation after cryptogein elicitor treatment of tobacco epidermal tissue was partly associated with chloroplasts of stomatal guard cells. Whether chloroplastic $O_2^{\bullet-}$ generation is a direct consequence of pathogen recognition or a sec-

Fig. 2A–O. Microscopic detection of cell death and ROIs in epidermal cells during the *Mla12*-specified resistance response after inoculation with *Bgh*A6. Primary leaves of *Mla12*-resistant barley and the susceptible mutant M66 were treated with the ROI-indicative compounds NBT and DAB and analyzed microscopically for the accumulation of formazans and/or DAB polymers at 40 hai. **A** Epifluorescence photography of an interaction site where a penetrated host cell underwent HR. The whole cell and the haustorial initial (*black arrow*) show autofluorescence, indicating the accumulation and demobilization of phenolic compounds. *White arrow*, appressorial germ tube (not focused). Bar = 20 μm. **B** Interaction site where a single penetrated host cell underwent HR. Reddish-brown DAB polymers indicating H_2O_2 surround the haustorial initial (*black arrowheads*). Polymerization of DAB is also visible in association with cell walls. Bar = 8 μm. **C** Double-stained leaf of Sultan-5. Development of fungal germlings (*arrow and arrowheads*) was stopped by a single-cell HR. A multi-cell mesophyll HR at the interaction site (*white star*) is visible as a result of the single-cell-compatible status of a fungal germling with an epidermal cell (for a close-up see also Fig 2M–O). Brownish DAB polymers in dead cells and dark-blue formazan staining in adjacent mesophyll cells are visible at sites of HR. Bar = 80 μm. **D** Double-stained leaf of M66. Fungal germlings (*arrowheads*) developed branched ESH. Interaction sites show neither DAB polymers nor formazan staining. Bar = 80 μm. **E** A single-cell epidermal HR on Sultan-5. Fungal development was stopped after production of a short ESH (*arrow*). The penetrated cell (*star*) is stained entirely with DAB polymers while mesophyll cells show formazan staining. Bar = 25 μm. **F** Close-up of a single cell (*star*) showing HR and whole-cell DAB staining. The cytoplasm of the cell appears discontinuous. Additionally, sites of symplastic cell-cell contact (pits, *arrows*) show DAB staining. Bar = 12 μm. **G** Same interaction site as shown in **F**. Focused mesophyll cells show chloroplasts stained blue with formazan and brownish DAB polymers in the apoplast. Bar = 12 μm. **H** Same interaction site as shown in **E**. The nucleus (*arrow*) of the dead cell lies on the cell bottom and shows DAB staining. Bar = 8 μm. **I** Interaction site on Sultan-5. Whereas the attacked cell bearing a haustorium (*arrow*) is free of staining, DAB polymers are visible in the adjacent epidermal cell (*star*) and subjacent mesophyll cells (out of focus). Bar = 16 μm. **J** Same interaction site as in **I**, focused to the bottom of the cell marked with a star in **I**. The cell shows a symplastic cell-cell contact site (pit, *arrow*) with the subjacent mesophyll cell. The contact site is stained with DAB. Bar = 12 μm. **K** Leaf of Sultan-5. The fungus has penetrated into an epidermal cell and developed a mature haustorium (*arrow*) and branched ESH (*arrowheads*). Beginnings of cell death, along with DAB and formazan staining, are visible exclusively in the mesophyll (see **L**). Bar = 35 μm. **L** Close-up of the mesophyll subjacent to the layer focused in **K**. Some intact cells (*arrows*) are stained mainly with formazan in chloroplasts. Other cells that have started to shrink show chloroplasts stained with DAB (*arrowheads*). Bar = 20 μm. **M** Same interaction site as marked in **C** with a white star. The fungus has penetrated into an epidermal cell and developed a mature haustorium (*arrow*) and branched ESH (*arrowheads*). In the subjacent mesophyll, a spreading multi-cell HR is visible. Bar = 35 μm. **N** Same interaction site as in **M**. Mesophyll HR in the center of the picture is characterized by collapsed cells stained with DAB. These cells are surrounded by two rings of stained cells: one ring of cells directly neighbors dead cells showing DAB and formazan staining, and one ring of fully turgid cells exhibits intensive formazan staining in chloroplasts. Bar = 35 μm. **O** Epifluorescence photography of the interaction site in **M**. Mesophyll cells in the center of the picture are collapsed and show bright autofluorescence. Bar = 35 μm. **A** Non-stained leaf. **B** DAB-stained leaf. **C–O** DAB-NBT double-stained leaves

ondary effect of cell death is not yet clear. Chloroplastic $O_2^{\bullet-}$ generation is dependent on light (Hückelhoven and Kogel 1998) and might be caused by H_2O_2 from unidentified sources that inhibits the redox-sensitive Calvin cycle, thereby provoking $O_2^{\bullet-}$ generation by the Mehler reaction. Vice versa, chloroplastic $O_2^{\bullet-}$, its dismutation and/or overloading of H_2O_2-scavenging capacities in the chloroplasts may be involved in H_2O_2 accumulation during mesophyll-HR in barley under light conditions. This is supported by the finding that chloroplastic H_2O_2 accumulation was detected in dying mesophyll cells (Fig. 2K–N).

Reactive oxygen intermediates generated in the mesophyll may also be involved in the execution of epidermal HR. Support for this hypothesis comes from experiments with coleoptile epidermal monolayers which are unable to mount a *Mla12*-mediated HR (Schiffer et al. 1997). Cell-to-cell transport of ROIs from the mesophyll into the epidermal layer is indicated by the observation that H_2O_2 accumulates at cell structures permeating pits connecting epidermal cells with adjacent epidermal and mesophyll cells (Fig. 2F,J).

During the interaction of the mutants with *Bgh*A6, we found a compatible single-cell interaction in the penetrated epidermal cell as the predominant type of interaction. This reaction was also found, though to a lesser extent, in the resistant wild type. Importantly, it was only there that establishment of a compatible single-cell interaction and development of branched ESH were associated with an HR in the subjacent mesophyll tissue. This mesophyll reaction was strongly dependent on the function of *Mla12*, *Rar1* and *Rar2*, an observation that supports the assumption of the genetical control of mesophyll cell death.

The frequencies of whole-cell DAB staining in the resistant wild type and all mutants differed clearly. In the *Mla12* mutant M66 and the *Rar*-mutants M22 and M100, cell death occurred rarely and mostly in association with unsuccessful fungal penetration. Cell death and DAB staining of non-invaded cells occurred to some extent even in susceptible barley lines and may be a secondary effect after fungal arrest (Schiffer et al. 1997; Hückelhoven et al. 1999). When an HR occurred, patterns of formazan and DAB staining were similar in all genotypes (see also Hückelhoven and Kogel 1998; Hückelhoven et al. 1999). Importantly, in the mutants whole-cell H_2O_2 accumulation was never detected in cells penetrated by *Bgh*A6 or subjacent mesophyll cells. These results show that whole-cell H_2O_2 accumulation following haustorium formation by *Bgh*A6 needs race-specific recognition by the *Mla12*-product and the function of *Rar-1* (see also Shirasu et al. 1999) and *Rar-2*.

Expression of *PR1b* was linked to endogenous H_2O_2 generation because both were missing in the mutants (Figs. 4 and 5B; Freialdenhoven et al. 1994). In contrast, expression of the oxalate oxidase gene (*HvOxOa*) was constitutive and was only slightly enhanced in all genotypes after inoculation with *Bgh*A6. Because the mutants did not express an HR to the same extent as the wild type, *HvOxOa* expression is not sufficient for H_2O_2 generation and HR induction. However, the

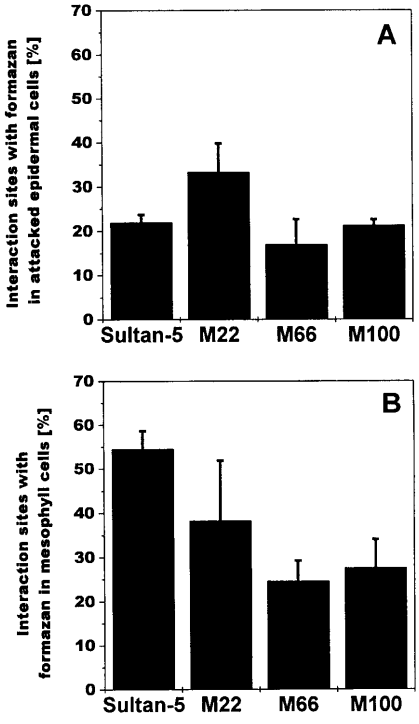


Fig. 3A,B. Generation of superoxide radical anions at interaction sites of the *Mla12*-resistant cv. Sultan-5 and susceptible mutants M22, M66 and M100 with *Blumeria graminis* f.sp. *hordei*, race A6. **A** Interaction sites with superoxide-indicative formazan staining in attacked epidermal cells. Maximal rates of staining were reached when leaves were injected with a NBT solution at 24 hai. The percentage of sites in which the attacked cell is stained with formazan is displayed. **B** Interaction sites with formazan in mesophyll cells. Maximal rates of mesophyll staining were reached when leaves were injected with an NBT solution at 40 hai. The percentage of sites with formazan in mesophyll cells directly beneath attacked epidermal cells is displayed. Each column represents the average (±SD) of triplicates of each 100 interaction sites per leaf. Repetition of the experiments led to results similar to those shown in the figure

possibility cannot be excluded that H_2O_2 generation is regulated at the protein level of oxalate oxidases which are active in the mesophyll of barley after powdery-mildew attack (Zhou et al. 1998). The different expression patterns of *PR1b* and *HvOxOa* in the mutants indicate a distinct regulation of these two genes during *Bgh* attack.

In incompatible interactions of dicotyledonous plants with microbial pathogens, a biphasic oxidative burst was often detected. While the first phase occurs also in compatible interactions, the second, prolonged phase usually depends on the presence of a corresponding pair of an *R*- and an *Avr*-gene (Baker and Orlandi 1995). In the barley powdery-mildew interaction, it is also possible to define distinct phases of the oxidative burst. However, it is notable that these phases appear to be temporally less restricted than in most dicotyledonous plants. Also, it is difficult to identify distinct phases clearly, because several different subcellular locations of ROI accumulation have been identified in this study. On the basis of a certain simplification of the cytological picture, three phases of ROI accumulation might be distinguished. The first H_2O_2 burst which occurs from 6 hai onward is elicited when the fungal primary germ tube attaches to the leaf surface and the plant reacts by formation of a cell wall apposition beneath this fungal structure (Thordal-Christensen et al. 1997). A second phase is associated with the fungal penetration attempt. In the case of successful penetration, ROI accumulation becomes visible around the haustorial initial and in anticlinal cell walls near the penetration site from 18 hai onward (Hückelhoven and Kogel 1998; Hückelhoven et al. 1999). In the case of an unsuccessful penetration, H_2O_2 accumulates beneath the appressorial germ tube in cytoplasmatic vesicles and cell wall appositions from 14 hai onward (Thordal-Christensen et al. 1997; Hückelhoven et al. 1999). These two phases of the

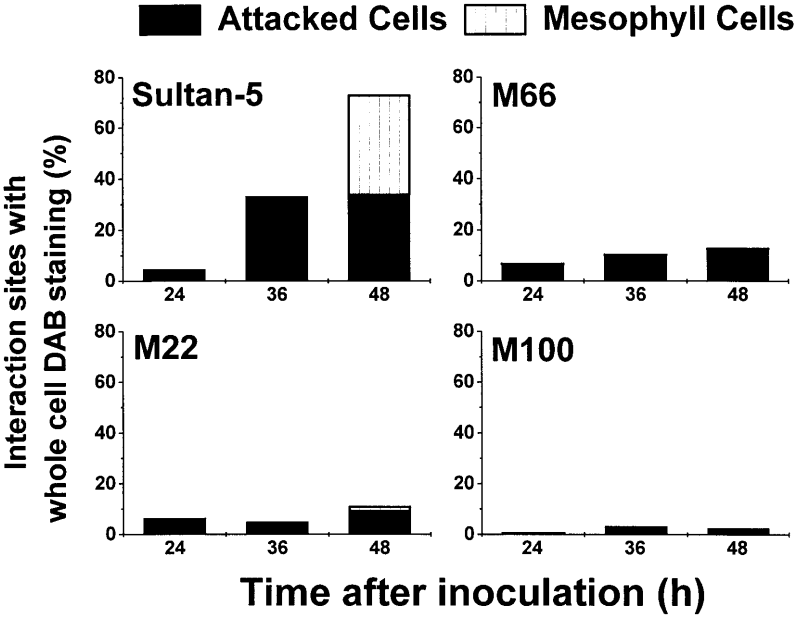


Fig. 4. Accumulation of hydrogen peroxide at interaction sites of the *Mla12*-resistant cv. Sultan-5 and susceptible mutants M22, M66 and M100 with *Blumeria graminis* f.sp. *hordei*, race A6. Sultan-5 and the mutants M66, M22 and M100 were inoculated with 10 conidia mm^{-2} of *Bgh*A6 (*avrMla12*). Primary leaves were excised at 18, 30 and 42 hai, placed in a solution of DAB and collected 6 h later for microscopic detection of DAB polymerization at interaction sites (indicated time points). Interaction sites with whole-cell DAB staining in attacked cells (*black columns*) or neighboring mesophyll cells (*striped columns*) were analyzed. Each column represents the average of triplicates of each 100 interaction sites per leaf. Repetition of the experiments led to results very similar to those shown in the figure

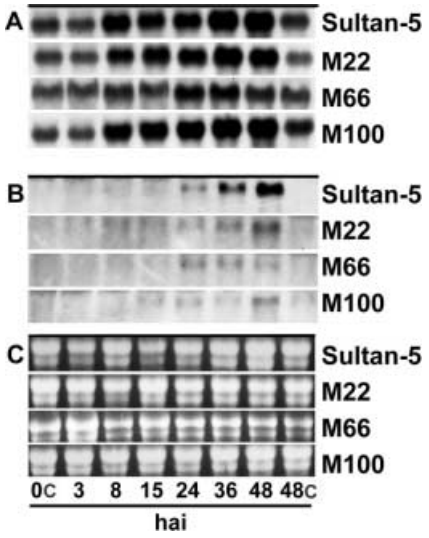


Fig. 5A–C. Gel blot analysis of barley oxalate oxidase (*HvOxOa*) and *HvPR1b* transcript accumulation after inoculation of resistant Sultan-5 and susceptible mutants M22, M66 and M100 with *Bgh*A6 (*avrMla12*). Seven-day-old primary leaves were inoculated with 50 conidia mm^{-2}. Sampling of eight leaves for total RNA extraction was carried out at 3, 8, 15, 24, 36 and 48 hai. Non-inoculated controls were taken at 0 and 48 hai (*0c and 48c*). Each gel slot was filled with 10 μg of total RNA. Transcripts of *HvOxOa* (**A**) and *HvPR1b* (**B**) were hybridized with labeled antisense RNA probes. **C** In-gel ethidium bromide staining of rRNA, performed before blotting to confirm equal sample loading. Repetition of the experiments led to results very similar to those shown in the figure

oxidative burst are independent of a race-cultivar specific recognition of the fungus by the plant. The third phase [previously designated phase II by Shirasu et al. (1999)] is exclusively confined to resistant barley bearing a functional *R*-gene-based signal transduction pathway coping with a corresponding avirulent race of *Bgh* (Fig. 4). In phase III, the burst takes place as whole-cell H_2O_2 accumulation in attacked epidermal cells or subjacent mesophyll from 24 hai onward and is closely linked to the onset of HR in these tissues. This phase seems to be analogous to phase II of bacterial-induced oxidative burst in cells of dicotyledonous plants (Levine et al. 1994; Baker and Orlandi 1995).

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Chapter 2

Hückelhoven R, Trujillo M, Kogel K-H (2000) Mutations in *Ror1* and *Ror2* genes cause modification of hydrogen peroxide accumulation in *mlo*-barley under attack from the powdery mildew fungus. *Mol Plant Pathol* 1: 287-292

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Mutations in *Ror1* and *Ror2* genes cause modification of hydrogen peroxide accumulation in *mlo*-barley under attack from the powdery mildew fungus

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SUMMARY

Race nonspecific resistance of barley against the barley powdery mildew fungus (*Blumeria Graminis* f.sp. *Hordei,* Speer, *Bgh*) is mediated by recessive *mlo* alleles and is controlled by at least two additional genes 'required for *mlo*-specified disease resistance' (*Ror1* and *Ror2*). The pathogenesis-related accumulation of hydrogen peroxide (H_2O_2) was comparatively analysed in a susceptible barley line (*Hordeum vulgare* L. Cv Ingrid, genotype *Mlo Ror1, Ror2*), a resistant Ingrid backcross line carrying the mutant allele *mlo5* (BCIngrid-*mlo5,* genotype *mlo5 Ror1 Ror2*), and in the moderately susceptible mutants A44 and A89 (genotypes *mlo5 Ror1 ror2* and *mlo5 ror1-2 Ror2,* respectively). *In situ* localization of H_2O_2 was performed by microscopic detection of 3,3-diaminobenzidine (DAB) polymerization. In BCIngrid-*mlo5*, penetration resistance against *Bgh* attack was closely correlated to H_2O_2 accumulation in cytoplasmic aggregates and cell wall appositions beneath the appressorium. In contrast, H_2O_2 accumulation was almost completely absent in susceptible Ingrid. Lines with mutations in *Ror* genes showed less H_2O_2 accumulation beneath appressoria, but more interaction sites with whole cell H_2O_2 accumulation and hypersensitive cell death response than resistant BCIngrid-*mlo5*. Thus, mutations in *Ror1* or *Ror2* genes influence the cellular pattern of H_2O_2 accumulation in *mlo* plants attacked by *Bgh*. The data support the hypothesis that H_2O_2 accumulation is involved in resistance to fungal penetration.

INTRODUCTION

The barley *Mlo* gene encodes a putative transmembrane receptor with seven membrane-spanning regions and a G-protein coupling site (Büschges *et al*., 1997; Devoto *et al*., 1999). Loss of *Mlo* function in barley mutants leads to resistance against a broad spectrum of barley powdery mildew isolates in young seedlings, along with spontaneous leaf cell death in late developmental stages. Resistance does not appear to be functionally linked to the spontaneous cell death response (Peterhänsel *et al*., 1997). Plants bearing *mlo* alleles express resistance against *Blumeria graminis* f.sp. *hordei* (*Bgh*) exclusively via penetration resistance, which is characterized by the formation of cell wall appositions (CWAs), accumulation of phytoalexins, pathogenesis-related gene transcripts and hydrogen peroxide (H_2O_2) (Hückelhoven *et al*., 1999; Peterhänsel *et al*., 1997; Stolzenburg *et al*., 1984; von Röpenack *et al*., 1998; Zeyen *et al*., 1993). All these characteristics are also expressed in susceptible plants, albeit to a lower extent, meaning that the *mlo* alleles confer a primed responsiveness for these defence reactions (Peterhänsel *et al*., 1997).

Some mutants of other plant species that execute cell death spontaneously show features of induced resistance and require endogenous salicylic acid for cell death and expression of pathogenesis-related genes (Dangl *et al*., 1996). In contrast, the resistance of *mlo5* plants does not rely on higher salicylic acid content than occurs in wild-type plants (Hückelhoven *et al*., 1999).

Penetration of epidermal cells by the powdery mildew fungus probably needs both turgor pressure and secreted hydrolases which disrupt the plant cell wall (Pryce-Jones *et al*., 1999). H_2O_2 is known to be necessary for lignification and protein cross-linking reactions (Brisson *et al*., 1994; Olson and Varner, 1993). Both processes participate in cell wall strengthening, which leads to a higher resistance of cell walls to mechanical pressure and hydrolytic activities of lytic enzymes.

Previous studies showed that most of the *mlo*-mediated reactions depend on the function of *Ror1* and *Ror2* (Freialdenhoven *et al*., 1996; Peterhänsel *et al*., 1997; von Röpenack *et al*., 1998). We show here that *Ror1* and *Ror2* are involved in the process governing H_2O_2 accumulation at sites of attempted fungal penetration in *mlo* barley.

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RESULTS

Loss of penetration resistance in Ror mutants

As expected from previous studies (Freialdenhoven *et al.*, 1996; Peterhänsel *et al.*, 1997), loss-of-function mutants A44 (*mlo5*, *Ror1*, *ror2*) and A89 (*mlo5*, *ror1-2*, *Ror2*) were compromised in penetration resistance.

Interaction phenotypes were compared from 12 to 36 h after inoculation (hai). At 12 hai, fungal appressoria adhered to the leaf surface, while no plant response was clearly detectable beneath the appressoria. At 16 hai, cytoplasmic aggregates were visible beneath the appressoria in most cells that were attacked but not penetrated by *Bgh*A6. This response appeared to be independent of the host genotype (Clark *et al.*, 1995). The first haustoria were observed at 16 hai. In susceptible Ingrid, the frequency of penetration rose from 16 hai onward, to a maximum level of 68% at 36 hai (Fig. 1). While no penetration was observed in BCIngrid-*mlo5*, the *ror1* mutant and the *ror2* mutant were penetrated at 22% and 13% of interaction sites, respectively (36 hai, Fig. 1).

A hypersensitive reaction (HR) occurred early, between 12 and 16 hai. Haustoria or elongated secondary hyphae were not observed at any HR sites. Interestingly, both *ror* mutants showed more HR than Ingrid and BCIngrid-*mlo5* in the time range 16–36 hai (Fig. 1; significant for $P = 0.05$, Student's *t*-test).

Accumulation of H_2O_2 at sites of attempted fungal penetration

To detect H_2O_2 accumulation during fungal penetration attempts, the (3,3-diaminobenzidine-) DAB-uptake method (Thordal-Christensen *et al.*, 1997) was applied at 8, 12, 16, 20, 26 and 32 hai. Four hours later, samples were taken for microscopic evaluation. Brownish staining indicating *in situ* DAB polymerization driven by H_2O_2 could not be observed in association with appressoria at 12 hai. Instead, staining was observed beneath primary germ tubes, which are unable to penetrate (Fig. 2A). For this observation, DAB was applied 8 hai. From 16 hai onward (DAB application at 12 hai), appressoria-associated staining was observed in CWAs, in cytoplasmic aggregates containing vesicles of different sizes, in halos around sites of attempted penetration, in anticlinal cell walls near sites of attempted penetration or as a whole cell accumulation when cells mounted a HR (Fig. 2B–F).

For frequency analysis of DAB staining types, we evaluated patterns of staining at 16, 20, 24 and 36 hai (Fig. 3). At 16 hai, H_2O_2 was frequently detected in cytoplasmic aggregates, vesicles and halos beneath appressoria. This pattern of DAB staining was often detected in all *mlo5* genotypes and to a

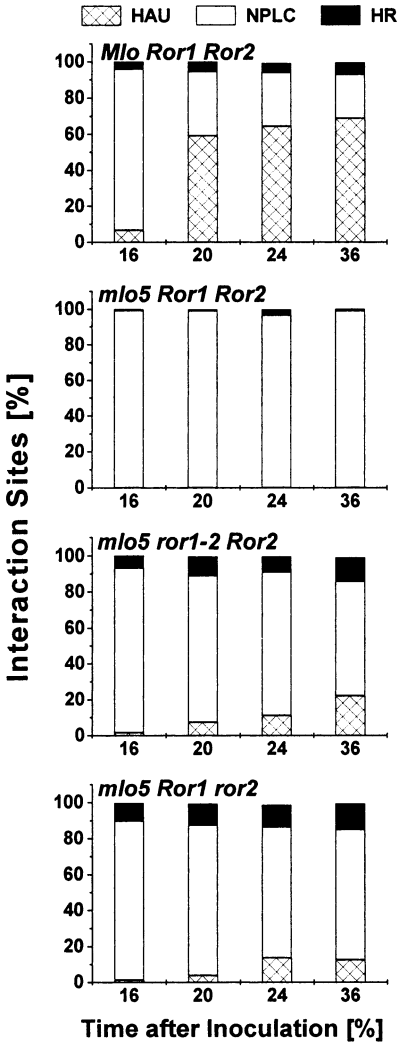

Fig. 1 Defence responses of the susceptible parent Ingrid (*Mlo*, *Ror1*, *Ror2*), a resistant barley line BCIngrid-*mlo5* (*mlo5*, *Ror1*, *Ror2*) and the susceptible mutants A89 (*mlo5*, *ror1-2*, *Ror2*) and A44 (*mlo5*, *Ror1*, *ror2*) following inoculation with *Blumeria graminis* f.sp. *hordei*, race A6. Checkered columns: Frequency of established nondifferentiated or differentiated haustoria (HAU) in the living host cell first attacked by the pathogen. No haustoria were detected at 12 hai. White columns: Frequency of interaction sites in which the fungal penetration attempt was unsuccessful (nonpenetrated living cells, NPLC). None of these infection sites exhibited a hypersensitive cell death. Black columns: Frequency of interaction sites restricted to a single host cell in which the attacked cell showed a characteristic yellow whole-cell autofluorescence upon UV light excitation. Whole-cell autofluorescence is a reliable measure of cell death (single-cell hypersensitive response, HR) in the barley–powdery mildew interaction (Görg *et al.*, 1993; Koga *et al.*, 1990). No HR was detected at 12 hai. Each column represents the average of 100 interactions scored on 3–5 leaves (overall 300–500 interaction sites per column). Repetition of the experiments led to results very similar to those shown in the figure.

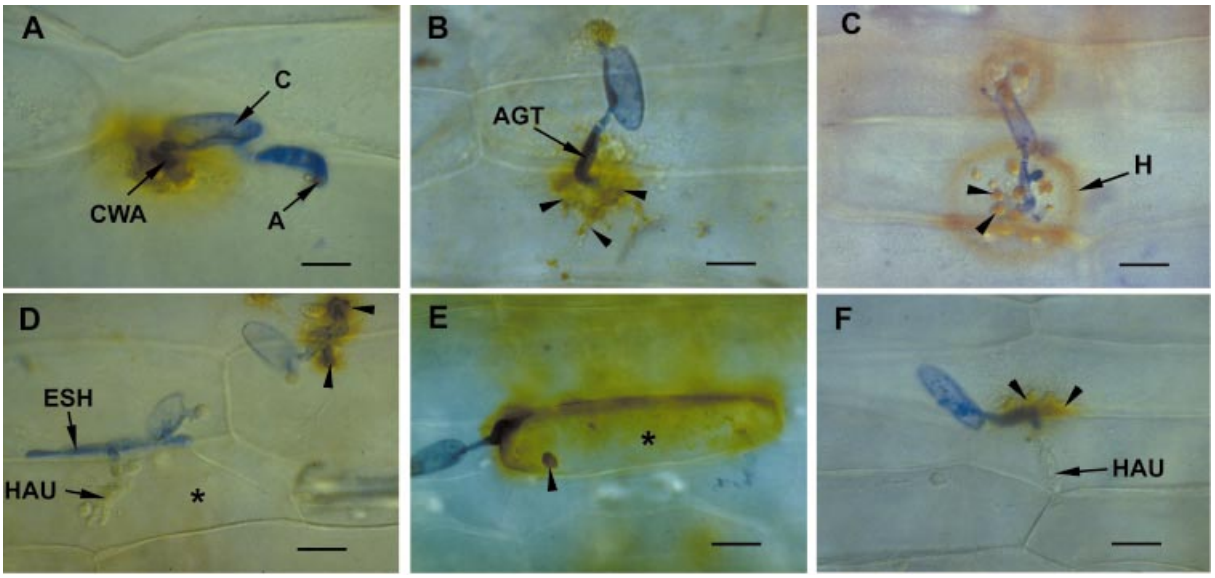

Fig. 2 Subcellular localization of H_2O_2 accumulation at interaction sites of barley and *Bgh*A6. At 8, 12, 16, 20, 26 and 32 hai, leaves were removed and placed in a solution of 1 mg/mL DAB. After 4 h leaf segments were analysed for DAB polymerization. (A) DAB staining of a cell wall apposition [CWA] beneath a primary germ tube at 12 hai (application of DAB at 8 hai) on BCIngrid-*mlo5*. Whereas a large CWA with DAB staining is visible beneath the primary germ tube, no reaction takes place beneath the appressorium [A]; [C], conidia; Bar = 8 μm. (B) Interaction site of Ingrid and *Bgh*A6 16 hai (application of DAB at 12 hai). Brownish DAB staining is visible in cytoplasmic aggregates (arrowheads) beneath the appresorial germ tube (AGT). Bar = 10 μm. (C) DAB staining in vesicles (arrowheads) and halos [H] at 30 hai in BCIngrid-*mlo5* (application of DAB at 26 hai). Bar = 12 μm. (D) Two neighbouring interaction sites on the same leaf of Ingrid at 36 hai (application of DAB at 32 hai). While DAB staining is visible in CWAs beneath two failed fungal penetration attempts (arrowheads), no DAB polymers can be seen at the site where the fungus successfully penetrated into the attacked cell [star], established a haustorium [HAU] and developed elongated secondary hyphae [ESH]. Bar = 10 μm. (E) Whole cell DAB staining of an attacked epidermal cell (star) of A89 20 hai (application of DAB 16 hai). The cell wall, the granular cytoplasm and the nucleus (arrowhead) are stained with DAB. Bar = 15 μm. (F) Interaction site on Ingrid at 20 hai (application of DAB at 16 hai). Whereas the site of haustorium formation [HAU] is fairly free of DAB polymers, staining is visible in the anticlinal cell wall and the neighbouring cell (arrowheads). Bar = 12 μm.

lesser extent also in the *Mlo* genotype Ingrid (Fig. 3A). Clear differences between *Mlo* and *mlo5* plants were found in the rate of stained CWAs. While 5% of all interaction sites in Ingrid were associated with DAB staining in papilla, nearly 30% of interaction sites in BCIngrid-*mlo5* were stained in CWAs at 16 hai (Fig. 3B). This difference was even more pronounced between 16 and 20 hai, the period in which most fungal penetration attempts succeeded in Ingrid and failed in BCIngrid-*mlo5*. At 20, 24 and 36 hai, *ror1* and *ror2* mutants showed 10–36% less interaction sites with stained CWAs than BCIngrid-*mlo5*. This corresponded roughly to the penetration frequencies in these genotypes. The rate of DAB stained CWA that could be penetrated by the fungus was less than 2% for each genotype and time of evaluation.

Along with HR of attacked cells, the rate of whole cell DAB staining was higher in both *ror* mutants than in Ingrid and BCIngrid-*mlo5* (Fig. 3C). No whole cell staining was found at 12 hai (data not shown).

The percentage of attacked cells without detectable DAB polymers increased during the time of observation in Ingrid to 80% (summarized in Fig. 3D). Staining occurring in anticlinal cell walls of penetrated cells appeared to originate from neighbouring cells and was therefore counted as uncoloured attacked cells (Fig. 2F, striped columns in Fig. 3D). Staining restricted to this subcellular location was seen in Ingrid at 20 and 24 hai (16 and 24% of interaction sites). Finally, at 36 hai, cells neighbouring penetrated cells were also free of DAB staining (Fig. 2D).

DISCUSSION

The present study demonstrates that the functions of the genes *Ror1* and *Ror2* are involved in accumulation of H_2O_2 in *mlo* barley during *Bgh* attack. The data suggest that H_2O_2 may be required for penetration resistance and, *vice versa*, suppression of H_2O_2 accumulation may be a strategy of *Bgh* to maintain compatibility after haustoria formation.

Because the pattern and frequency of H_2O_2 accumulation were altered in both *ror* mutants, we concluded that H_2O_2 accumulation in BCIngrid-*mlo5*, like penetration resistance, is influenced by the function of *Ror1* and *Ror2*. H_2O_2 shows some

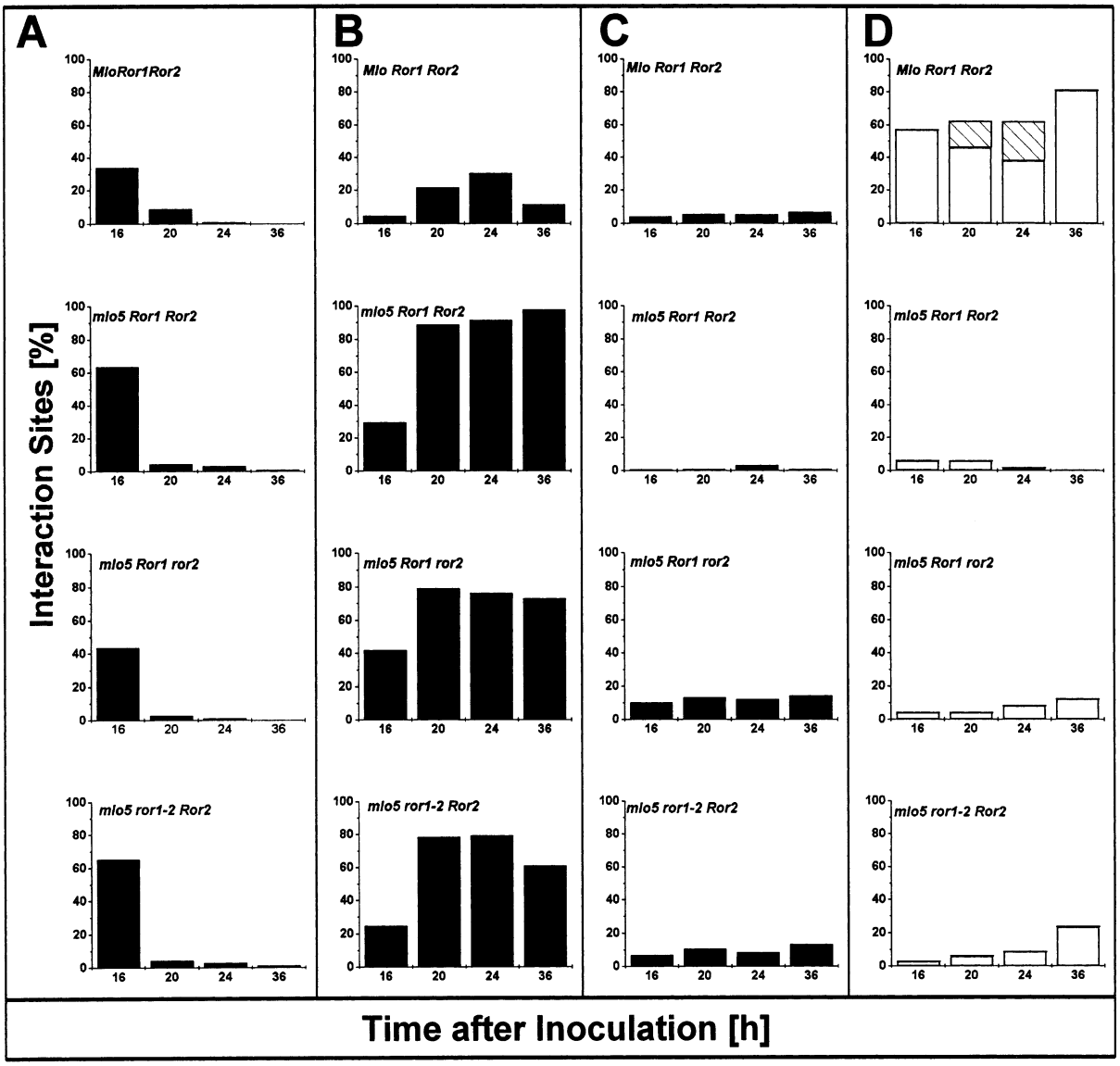

Fig. 3 Incidence of interaction sites with different patterns of H_2O_2 accumulation in attacked cells of Ingrid (*Mlo, Ror1, Ror2*), BCIngrid-*mlo5* (*mlo5, Ror1, Ror2*), A89 (*mlo5, ror1-2, Ror2*) and A44 (*mlo5, Ror1, ror2*) after inoculation with *Bgh*A6. At 12, 16, 20 and 32 hai, leaves were removed and placed in a solution of 1 mg/mL DAB. After 4 h (indicated time points in the figure) leaf segments were analysed for DAB polymerization in cells subjacent to appressorial germ tubes. (A) Frequency of interaction sites with diffuse DAB staining. Staining was visible in cytoplasmic aggregates, vesicles or halos. (B) Frequency of interaction sites with DAB staining in a cell wall apposition beneath an appressorium. (C) Frequency of interaction sites with whole cell DAB staining of the attacked cell. (D) Frequency of interaction sites where the attacked cell was free of DAB staining (white columns). Striped columns represent clear cells where a DAB staining at anticlinal cell walls occurred in the neighbouring cell (Fig. 2F). Each column represents the average of 3–5 times 100 interaction sites scored in parallel leaves. Repetition of the experiments led to results very similar to those shown in the figure.

antimicrobial activity (Peng and Kuc, 1992) and is a substrate for lignification and protein cross-linking (Bradley *et al.*, 1992; Thordal-Christensen *et al.*, 1997), which together may contribute to penetration resistance in *mlo*-barley.

Pathogenesis-related gene transcripts and antifungal p-coumaroyl-hydroxyagmatine accumulate to a high extent in BCIngrid-*mlo5*, whereas *Mlo* plants and *ror* mutants accumulate less of these defence compounds (Peterhänsel *et al.*, 1997; von Röpenack *et al.*, 1998). The degree of gained ability to accumulate defence compounds in BCIngrid-*mlo5* is reminiscent of that found here for H_2O_2 accumulation. Interestingly, in barley bearing the race-specific resistance gene *Mla12* or

mutants derived from it, endogenous accumulation of H_2O_2 and pathogenesis-related gene transcripts are temporally and quantitatively linked (Freialdenhoven *et al*., 1994; Hückelhoven *et al*., 2000; Shirasu *et al*., 1999). It is tempting to speculate that endogenous H_2O_2 acts in barley as a potent inducer of phytoalexin accumulation and pathogenesis-related gene expression.

Peterhänsel *et al*. (1997) showed that HR occurring in *Mlo* barley after an attack from the nonhost pathogen *Blumeria graminis* f.sp. *tritici*, isolate JIW28 is suppressed in BCIngrid-*mlo5*. In contrast, *ror* mutants show higher rates of cell death than BCIngrid-*mlo5* after JIW28 attack. Thus, *Ror* gene products are not only necessary for *mlo*-mediated resistance but also for suppression of host cell death in barley cells exhibiting mlo-type resistance against the powdery mildew fungus (Peterhänsel *et al*., 1997). In the current study, *ror* mutants exhibited higher rates of HR and whole cell H_2O_2 accumulation than BCIngrid-*mlo5* and Ingrid. Considered together, levels of H_2O_2 which are higher than in Ingrid might have been sufficient to trigger an overshoot reaction resulting in HR in *mlo ror* genotypes not exhibiting *mlo*-type suppression of epidermal cell death. Interestingly, in barley, HR of cells attacked but not penetrated by the powdery mildew fungus may also be a consequence of penetration resistance (Görg *et al*., 1993; Schiffer *et al*., 1997).

The *mlo5* allele confers a paradoxical deregulation of cell death in epidermis and mesophyll cells (Peterhänsel *et al*., 1997). The occurrence of HR in the *ror* mutants may indicate that the Mlo Ror pathway is also involved in regulation of cell death after *Bgh* attack in wild-type plants. This would suggest that the functional Mlo receptor perceives a cell death suppressor that may be released constitutively in early developmental stages by the plant or by the biotrophic fungus *Bgh*. This interpretation appears to contradict the fact that *mlo* plants do not show HR of attacked epidermal cells. On the other hand, this hypothesis is consistent with the observation that *mlo5* barley shows spontaneous cell death in late developmental stages (Peterhänsel *et al*., 1997) and accelerated mesophyll cell death after attack by *Magnaporte grisea* (Jarosch *et al*., 1999). In this context it seems questionable whether BCIngrid-*mlo5* is suppressed in epidermal cell death or whether the elicitor for HR is missing in these plants. This could be due to a higher resistance of the cell walls of BCIngrid-*mlo5* against fungal hydrolytic activity and therefore a lower level of endogenous cell death elicitors. Concordant with this hypothesis, *mlo ror* genotypes show more epidermal cell death because their cell walls are less stable and release more elicitor active fragments under fungal attack. In susceptible *Mlo* plants, race nonspecific HR-elicitors are probably also released, however, H_2O_2 accumulation and HR might be suppressed after a successful penetration of attacked cells. This is supported by the observation that, in susceptible *Mlo* and *mlo ror* genotypes, the total percentage of attacked cells showing H_2O_2 accumulation decreased after penetration and during haustorial maturation. Cells containing a haustorium showed only a weak accumulation of H_2O_2 at 20–24 hai and were essentially free of H_2O_2 at 36 hai. H_2O_2 accumulation at penetration sites was often restricted to anticlinal cell walls and appeared to originate not in the penetrated cell but rather in a neighbouring cell (Fig. 2F). Thus, the establishment of compatibility was accompanied by a nonoxidative status in the penetrated cell. Interestingly, a recent study by Lyngkjær and Carver (1999) discovered that even *mlo5* genotypes are more accessible to *Bgh* when preinoculated with the laboratory-selected '*mlo5*-virulent' isolate HL3/5. This induced accessibility was associated with a decreased ability of *mlo5*-plants to accumulate autofluorogens near fungal penetration attempts. In these processes, suppression of the H_2O_2 accumulation required to drive polymerization of phenolic compounds is likely to take place. A nonoxidative status may be a prerequisite to keep a penetrated cell alive during the period in which *Bgh* completes its nonsexual life cycle.

EXPERIMENTAL PROCEDURES

Plants, pathogens and inoculation

The barley (*Hordeum vulgare* L.) cv Ingrid (*Mlo Ror1 Ror2*), the backcross line BCIngrid-*mlo5* (*mlo5 Ror1 Ror2*) and the mutants A44 (*mlo5 Ror1 ror2*) and A89 (*mlo5 ror1-2 Ror2*) were obtained from P. Schulze-Lefert (Max-Plank-Institute for Plant Breeding, Cologne, Germany). Their generation has been described previously (Freialdenhoven *et al*., 1996). Plants were grown in a growth chamber at 16 °C with 60% relative humidity and a photoperiod of 16 h (60 µmol photons/m^2/s). Inoculation of primary leaves was performed with 10 conidia/mm^2 from *Blumeria graminis* f.sp. *hordei,* race A6, at the 7th day after germination. The fungus was cultivated on barley cv Golden Promise under the same conditions.

Microscopic analysis, staining of H_2O_2

Penetration of attacked cells was ascertained by detection of haustoria formation or development of ESH. Whole-cell autofluorescence and discontinuity of cytoplasmic strands were taken as a reliable measure of cell death (Görg *et al*., 1993; Koga *et al*., 1990). Bright-field and fluorescence microscopy was performed as described by Hückelhoven and Kogel (1998).

Because the rate of fungal penetration into short and long epidermal cells is different, only short cells directly adjacent to stomata (cell type A) and short cells not directly adjacent to stomata (type B) were evaluated, whereas long epidermal cells covering vascular tissue (type C) were excluded (for leaf topography see Koga *et al*., 1990). To avoid misinterpretation due to

the effects of induced accessibility or induced inaccessibility in cells where penetration was successful or unsuccessful, respectively (Lyngkjær and Carver, 1999), we evaluated exclusively interaction sites where only one fungus per cell attempted to penetrate.

Detection of H_2O_2 was performed using the DAB-uptake method as described previously (Hückelhoven *et al*., 1999; Thordal-Christensen *et al*., 1997).

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Chapter 3

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Chapter 4

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REVIEW

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Reactive oxygen intermediates in plant-microbe interactions: Who is who in powdery mildew resistance?

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Abstract Reactive oxygen intermediates (ROIs) such as hydrogen peroxide (H_2O_2) and the superoxide anion radical ($O_2^{\cdot-}$) accumulate in many plants during attack by microbial pathogens. Despite a huge number of studies, the complete picture of the role of ROIs in the host–pathogen interaction is not yet fully understood. This situation is reflected by the controversially discussed question as to whether ROIs are key factors in the establishment and maintenance of either host cell inaccessibility or accessibility for fungal pathogens. On the one hand, ROIs have been implicated in signal transduction as well as in the execution of defence reactions such as cell wall strengthening and a rapid host cell death (hypersensitive reaction). On the other hand, ROIs accumulate in compatible interactions, and there are reports suggesting a function of ROIs in restricting the spread of leaf lesions and thus in suppressing cell death. Moreover, in situ analyses have demonstrated that different ROIs may trigger opposite effects in plants depending on their spatiotemporal distribution and subcellular concentrations. This demonstrates the need to determine the particular role of individual ROIs in distinct stages of pathogen development. The well-studied interaction of cereals with fungi from the genus *Blumeria* is an excellent model system in which signal transduction and defence reactions can be further elucidated *in planta*. This review article gives a synopsis of the role of ROI accumulation, with particular emphasis on the pathosystem *Hordeum vulgare* L.–*Blumeria graminis*.

Keywords *Blumeria* · Cell wall strengthening · GTP-binding protein · *Hordeum* · Hypersensitive reaction · Oxidative burst

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Abbreviations *Avr*-gene: avirulence gene · CWA: cell wall apposition · DAB: 3,3-diaminobenzidine · HR: hypersensitive reaction · NBT: nitroblue tetrazolium · *R*-gene: resistance gene · *Rar:* gene required for *Mla12*-specified resistance · ROI: reactive oxygen intermediate · ROP: RHO (RAS—rat sarkome oncogene product—homologue) of plants · *Ror:* gene required for *mlo*-specified resistance · SA: salicylic acid

Introduction

Reactive oxygen intermediates (ROIs) derive from molecular oxygen by stepwise incomplete electron uptake, finally leading to complete oxygen reduction and production of H_2O. Narrowly interpreted, the family of ROIs consists of the superoxide radical anion $O_2^{\cdot-}$, the hydroperoxyl radical $HO_2^{\cdot}$, hydrogen peroxide H_2O_2, and the hydroxyl radical $HO^{\cdot}$. Superoxide, its protonated form $HO_2^{\cdot}$ and $HO^{\cdot}$ are relatively short-lived whereas H_2O_2 is comparatively stable and can cross membranes. In particular, the hydroxyl radicals among the ROIs are toxic due to their extraordinary ability to react spontaneously with organic molecules such as phenols, fatty acids, proteins and nucleic acids. In plants, the best-studied ROIs are $O_2^{\cdot-}$ and H_2O_2 whereas only little information is available on $HO^{\cdot}$ due to its extremely short half-life (Baker and Orlandi 1995; Hammond-Kosack and Jones 1996; Grant and Loake 2000).

ROI accumulation (in the sense of ROIs becoming detectable by biochemical or histochemical methods) is closely associated with the induction of plant defence reactions against viral, bacterial, and fungal pathogens, such as the hypersensitive reaction (HR), defence gene expression, and cell wall strengthening via cross-linking reactions of phenylpropanoids and proteins (Bradley et al. 1992; Levine et al. 1994; Jabs et al. 1997; Thordal-Christensen et al. 1997; reviewed by Lamb and Dixon 1997; Grant and Loake 2000). Likewise, the oxidative burst, triggered by a peptide elicitor from the non-host

pathogen *Phytophthora sojae* in parsley cells, is necessary and sufficient to induce phytoalexin production (Jabs et al. 1997). However, in this system, early defence gene expression is controlled ROI-independently by a mitogen-activated protein kinase (Kroj et al. 2002). Additionally, local and systemic H_2O_2 accumulation might be required for establishment of systemic acquired resistance in *Arabidopsis* and tobacco (Alvarez et al. 1998; Fodor et al. 2001). Evidence for the direct implication of ROIs in plant resistance to pathogens was provided by concomitant inhibition of ROI accumulation and plant defence by chemicals like diphenylene iodonium chloride that is supposed to block a ROI-producing NADPH oxidase (Levine et al. 1994; Jabs et al. 1997). Likewise, *in planta* ROI-producing systems triggered plant defence mechanisms (Wu et al. 1997; Chamnongpol et al. 1998). This relatively clear picture has been blurred by recent reports suggesting that a successful pathogenesis of some necrotrophic or hemibiotrophic fungal pathogens relies on or is at least supported by a high concentration of hydrogen peroxide (von Tiedemann 1997; Govrin and Levine 2000; Kumar et al. 2001).

Plant cells respond to bacterial challenge with a rapid and transient, biphasic accumulation of host cell-produced ROIs called the oxidative burst. While the first (unspecific) phase occurs in both compatible and incompatible interactions, the second prolonged phase usually precedes host cell death and depends on the presence of a corresponding pair of resistance (*R*) and avirulence (*Avr*) genes causing incompatibility (Baker and Orlandi 1995). However, there is evidence that H_2O_2 accumulation is not generally sufficient for host cell death: *hrmA* mutants of the bacteria *Pseudomonas syringae* pv. *syringae* and *P. fluorescens* elicited the second phase of the oxidative burst in tobacco suspension cells but not the HR (Glazener et al. 1996). Also, harpin or β-megaspermin elicitors derived from different pathovars of *Pseudomonas syringae* or *Phytophthora megasperma*, respectively, induced an HR in tobacco cell cultures, which could not be inhibited by blocking the accompanying H_2O_2 accumulation (Dorey et al. 1999; Ichinose et al. 2001). Recently, Delledonne et al. (2001) suggested that H_2O_2 needs the presence of nitric oxide ($NO^{\cdot}$) to provoke cell death whereas $O_2^{\cdot-}$ captures $NO^{\cdot}$ as $ONOO^-$, which might not trigger cell death in plants (see also Beligni and Lamattina 1999). Importantly, contrasting roles of $O_2^{\cdot-}$ and H_2O_2 in cell death regulation are also known from mammalian systems. For instance, H_2O_2 is a potent trigger of apoptosis in mammalians whereas $O_2^{\cdot-}$ is also involved in cell proliferation and cell survival (Irani et al. 1997; Clément et al. 1998).

For many years, it has been assumed that ROIs accumulate sequentially from $O_2^{\cdot-}$ as the primary origin. Today, however, we know that different ROIs can be produced independently by different sources, which seems reasonable because ROI accumulation must be under stringent control to avoid toxicity. Though there are various sources for infection-related ROI accumulation in the plant kingdom, the most prominent are cell-wall-bound peroxidases, membrane integral NADPH oxidases, amine oxidases and oxalate oxidases (Zhang et al. 1995; Allan and Fluhr 1997; Lamb and Dixon 1997; Bolwell et al. 2002; Torres et al. 2002). French bean cell wall peroxidases can oxidise unknown reductants to produce H_2O_2 in a pH-dependent manner. However, alkalisation of the apoplast to neutral pH values is thereby a prerequisite for peroxidase activity (Bolwell et al. 2002). In lettuce, apoplastic ROI accumulation in response to the nonhost pathogen *Pseudomonas syringae* pv. *phaseolicola* is sensitive to cyanide and azide, indicating a possible contribution of peroxidase to ROI generation (Bestwick et al. 1997).

The plasma-membrane NADPH oxidase is the major ROI-producing enzyme in mammalian phagocytes during internalisation of bacterial pathogens. The enzyme assembles in the phagocyte plasma membrane after phosphorylation of cytoplasmic subunits (for reviews, see Morel et al. 1991, Babior et al. 2002). The active complex consists of up to six subunits (Bokoch 1995; Lamb and Dixon 1997). In plants, GP91PHOX has been identified as a homologue of the mammalian NADPH oxidase large subunit of the heterodimeric membrane flavocytochrome b_{558} protein (Groom et al. 1996). Except for small GTP-binding proteins out of the RAC (ROP) family (Hassanain et al. 2000; Park et al. 2000; Ono et al. 2001), no other subunits, neither the flavocytochrome b_{558} subunit P22PHOX nor cytoplasmic interacting proteins (P40PHOX, P47PHOX, P67PHOX) of plant NADPH oxidases have been definitively identified, suggesting a different enzyme regulation in plant and mammalian cells. The presence of cytoplasmic Ca^{2+}-binding EF-hand motifs and oxidase stimulation by Ca^{2+} implies that plant GP91PHOX homologues produce ROIs in a Ca^{2+}-regulated manner (Keller et al. 1998; Sagi and Fluhr 2001).

This review aims at presenting an overview on the role of ROIs in the establishment and maintenance of inaccessibility and accessibility (resistance/susceptibility on the cellular level) during attack by a fungal plant pathogen. Our current knowledge predicts that the effects of ROIs in plant pathogenesis depend on many factors, of which the lifestyle (biotrophy or necrotrophy) of the pathogen is a major one. At present, therefore, it seems impossible to give a complete picture of ROI function in host–parasite interactions. We reply to this problem by focussing on a case study that examines the interaction of barley with the biotrophic powdery mildew fungus.

The interaction of barley with the barley powdery mildew fungus

The barley powdery mildew fungus *Blumeria graminis* (DC Speer) f.sp. *hordei* (Marchal) (*Bgh*) is a biotrophic pathogen that requires successful host cell wall pene-

tration, development of a functional haustorium and maintenance of host cell integrity to establish a stable compatible interaction with its host barley (*Hordeum vulgare* L.). Resistance to *Bgh* is expressed by prevention of penetration through localised cell wall strengthening, by hypersensitive host cell death, or a combination thereof depending on the host genotype (Jørgensen 1994; Thordal-Christensen et al. 1999; Schulze-Lefert and Vogel 2000).

Cell wall strengthening by wall appositions (CWAs syn. papillae) is typically observed in race-non-specific resistance responses such as the *mlo* resistance or quantitative background resistance (Stolzenburg et al. 1984; Zeyen et al. 1993; Carver et al. 1994). In contrast, the HR is the prevailing plant response in gene-for-gene resistance as exemplified by the barley *Mla* traits (Koga et al. 1990; Freialdenhoven et al. 1994). During *Bgh* attack, ROIs accumulate in epidermal and mesophyll tissue close to infection sites. In situ techniques have revealed the high spatiotemporal complexity of pathogen-elicited ROI accumulation patterns, and their association with the particular race–cultivar interaction. Detailed cytological analyses of the situation have allowed a comprehensive insight into the biology of this plant–microbe interaction and particularly into the hypothetical role of ROIs.

Subcellular patterns of *Bgh*-induced ROI accumulation

The accumulation of $O_2^{\cdot-}$ and H_2O_2 at interaction sites of barley with *Bgh* has been studied histochemically using the ROI-specific dyes nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB; Thordal-Christensen et al. 1997; Hückelhoven and Kogel 1998). $O_2^{\cdot-}$ and H_2O_2 show clearly distinguishable accumulation patterns during establishment of accessibility and inaccessibility. A definite temporal and spatial coincidence of the onset of defence reactions and DAB staining suggests an association of H_2O_2 with host cell inaccessibility (Thordal-Christensen et al. 1997; Hückelhoven et al. 1999, 2000a, 2000b; Vanacker et al. 2000). Pathogen-induced H_2O_2 accumulation occurs in three phases: Phase I (Fig. 1A) coincides with the attachment of the primary, non-in-

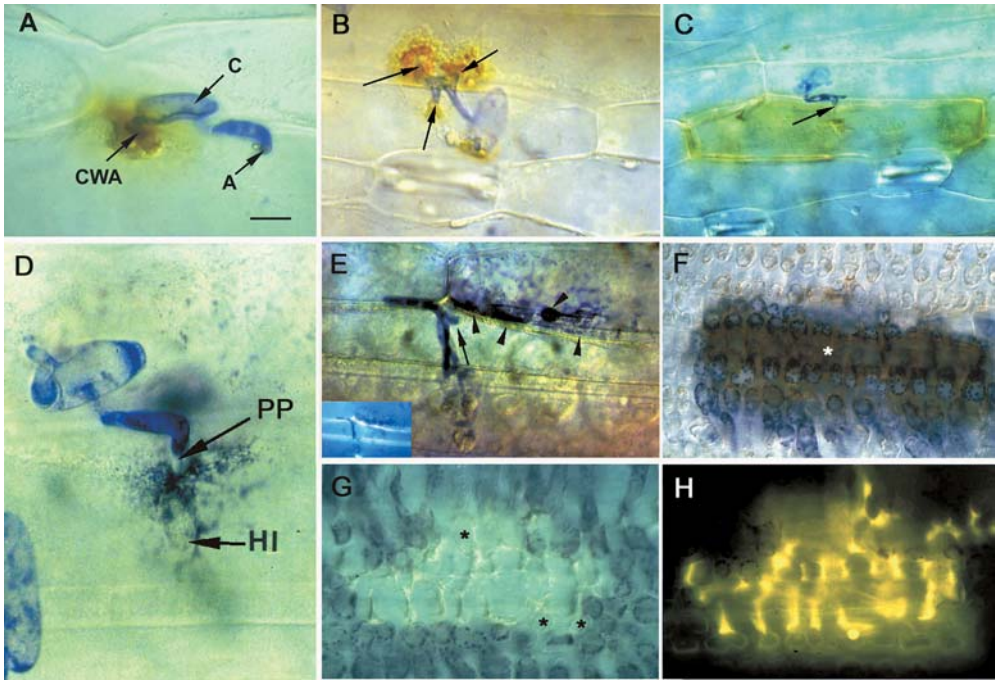

Fig. 1A–H Microscopic view of ROI accumulation patterns during attack of *Blumeria graminis* f.sp. *hordei* (*Bgh*) on barley (*Hordeum vulgare*). **A** H_2O_2 accumulation in late phase I (12 h after spore landing). Starting from its conidium (*C*) the fungus has built a primary germ tube and an appressorium (*A*). Barley has built a CWA beneath the primary germ tube where H_2O_2 accumulation is visible (*reddish-brown* DAB staining); *bar* 8 μm. **B** H_2O_2 accumulation phase II during formation of CWAs beneath three fungal penetration attempts (*arrows*). **C** H_2O_2 accumulation phase III during the HR of *Mla12* barley. The fungus penetrated successfully (*arrow*) and triggered whole-cell H_2O_2 accumulation (30 h after spore landing). **D** Superoxide accumulation phase I. Originating from the appressorium, *Bgh* formed a penetration peg (*PP*) and a haustorium initial (*HI*). Superoxide accumulation is indicated by *dark-blue* NBT staining around the penetration site. **E** Superoxide accumulation phase II. HR (UV-autofluorescence image in the *left corner*) of an attacked (*arrow*) cell is accompanied by $O_2^{\cdot-}$ accumulation in the neighbouring cell. $O_2^{\cdot-}$ is visible at the nucleus and along the anticlinal cell wall (*arrowheads*). **F** NBT–DAB double staining showing H_2O_2 accumulation phase III and superoxide accumulation phase II associated with an HR (*asterisk*). **G, H** Superoxide phase II during multi-cell mesophyll HR in *Mla12* barley. Dark-blue NBT staining indicates superoxide in tissues around dead cells that are free of stain. Cells immediately before collapse (*asterisks*) also do not show NBT staining. Blue-light excitation reveals yellow autofluorescence of dead cells. Panels **A** and **D** with permission from Hückelhoven et al. 2000b and Hückelhoven and Kogel 1998, respectively

fective germ tube onto the leaf surface. The germ tube tip is locally linked to the formation of a DAB-positive host CWA from 3 h after spore landing onward. The second phase proceeds from 14 h after inoculation onward when the pathogen attempts to penetrate from its secondary, appressorial germ tube. Phase II is subcellularly confined to the cytoplasm close to the site of attack, CWAs and anticlinal cell walls. The pattern and strength of H_2O_2 accumulation in phase II strictly depends on the outcome of the fungal penetration attempt. In successfully penetrated CWAs and adjacent anticlinal cell walls, DAB staining is weak, and H_2O_2 can be detected only occasionally around developing haustoria. In clear contrast, "effective" CWAs, which prevent penetration, stain strongly with DAB. Near such CWAs, DAB-positive vesicle-like structures can be commonly detected that most likely transport cell wall fortification material to the site of fungal attack (Fig. 1B; Hückelhoven et al. 1999).

The third phase of H_2O_2 accumulation spreads over the whole cell, meaning it is not restricted to subcellular sites (Fig. 1C). H_2O_2 starts to accumulate either at the mesophyll–epidermis interface or near penetration sites depending on the type of *R*-gene that mediates the defence response. In any case, onset of phase III is closely linked to subsequent cell death and arrest of the pathogen (Thordal-Christensen et al. 1997; Hückelhoven et al. 1999; Vanacker et al. 2000).

In clear contrast to H_2O_2, the superoxide radical anion ($O_2^{\cdot-}$, Fig. 1D) accumulates in attacked epidermal cells strictly in association with a successful penetration by *Bgh* (phase I of $O_2^{\cdot-}$ accumulation; Hückelhoven and Kogel 1998), which indicates that $O_2^{\cdot-}$ is related to cellular accessibility. Accordingly, $O_2^{\cdot-}$ was not detected in and near effective CWAs, indicating that H_2O_2 accumulation in CWAs might be independent of $O_2^{\cdot-}$ production. $O_2^{\cdot-}$ is also not detectable in attacked, non-penetrated epidermal cells that undergo an HR. A kinetic inspection of successfully penetrated cells of resistant barley demonstrated, that the number of interaction sites, where $O_2^{\cdot-}$ could be detected, declined concomitantly with the onset of HR (Hückelhoven and Kogel 1998). In this regard, it appears difficult to predict whether the rate of $O_2^{\cdot-}$ generation decreased or if enhanced superoxide dismutase (SOD) activity could have contributed to this effect. Significantly, living epidermal and mesophyll cells in direct contact with cells that underwent HR strongly accumulated $O_2^{\cdot-}$ ($O_2^{\cdot-}$ accumulation phase II) in chloroplasts, the cytoplasm and the apoplast (Fig. 1E–H), and these cells normally survive the oxidative stress exerted by the neighbouring HR-cells. Together, these data suggest that H_2O_2 but not $O_2^{\cdot-}$ is coupled with the death of *Bgh*-attacked barley cells whereas superoxide is involved in restriction of, rather than being a signal for, cell death (Hückelhoven et al. 2000a).

Although the powdery mildew fungus does not penetrate the mesophyll, this tissue executes a strong oxidative burst beneath the sites of attempted fungal infection. Typically, *R*-gene-mediated multi-cell mesophyll HR lesions are characterised by a central section in which cells accumulate H_2O_2 and undergo cell death, and an encircling layer of living, $O_2^{\cdot-}$-accumulating cells. Again, this observation is in accordance with the notion that $O_2^{\cdot-}$ is implicated in cell death restriction (Jabs et al. 1996; Hückelhoven and Kogel 1998).

Grading the ROI accumulation profile into different phases might imply interdependent processes. However, this may not necessarily be the case. For instance, penetration resistance due to effective CWAs is associated with strong H_2O_2 accumulation in phase I and particularly in phase II, whereas phase III and $O_2^{\cdot-}$ accumulation phases I and II are missing (Hückelhoven and Kogel 1998; Hückelhoven et al. 1999; Vanacker et al. 2000). Therefore, the phases of ROI accumulation likely represent independent processes, which are characterised by certain sources, elicitors and regulation. Figure 2 displays a schematic survey of ROI accumulation patterns and phases in the barley–barley powdery mildew fungus interaction.

Biochemical sources of *Bgh*-induced ROIs

The biochemical sources producing superoxide or hydrogen peroxide in barley under attack from *Bgh* have not yet been identified. In analogy to the mammalian phagocyte system, generation of ROIs might be driven by a plasma-membrane NADPH oxidase (Groom et al. 1996; Hückelhoven et al. 2001a), though the NADPH oxidase inhibitor diphenylene iodonium is only slightly effective in barley (Hückelhoven and Kogel 1998; and unpublished results). NBT solutions for histochemical in situ detection of $O_2^{\cdot-}$ contained NaN_3 to avoid unspecific NBT reduction. NaN_3 inhibits $O_2^{\cdot-}$ production by peroxidases and by the mitochondrial respiration chain. Azide-insensitive $O_2^{\cdot-}$ has been detected near plasma membranes as well as in cytoplasmic organelles and in chloroplasts (Hückelhoven and Kogel 1998). A putative NADPH oxidase gene and a gene encoding a possibly NADPH oxidase-regulating small G-protein are constitutively expressed in barley epidermis, and transcript levels do not strongly change during interaction with *Bgh* (Hückelhoven et al. 2001a; and unpublished results). However, constitutive expression might be sufficient to allow contribution of an azide-insensitive plasma-membrane NADPH oxidase to *Bgh*-induced $O_2^{\cdot-}$ production. Chloroplastic $O_2^{\cdot-}$ generation may stem from photosynthetic electron transport, which is known to be a source for oxygen reduction by ferredoxin oxidoreductase under stress conditions (Elstner and Osswald 1994). NBT-reduction in cytoplasmic organelles suggests other azide-insensitive sources of $O_2^{\cdot-}$ generation. Little is known about the contribution of $O_2^{\cdot-}$-generating peroxisomal membrane proteins (Corpas et al. 2001) as possible sources of $O_2^{\cdot-}$ in plant–microbe interactions. In general, the role of intracellular (Naton et al. 1996) or azide-sensitive ROI sources is not well

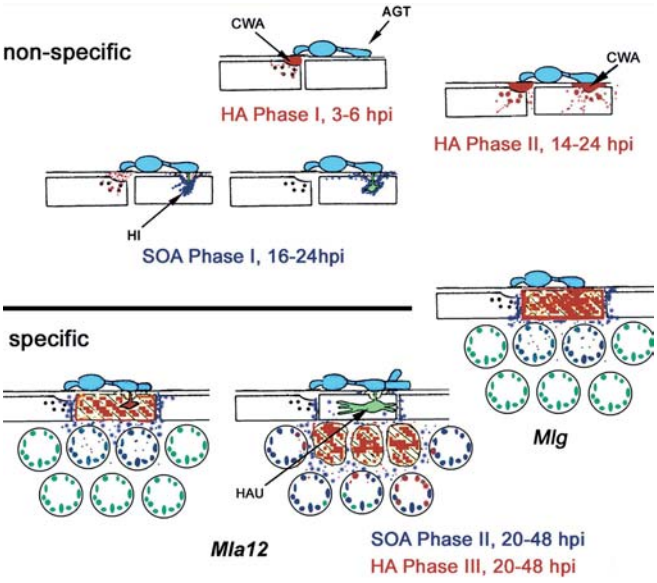

Fig. 2 Schematic drawing of *Bgh*-induced, localised ROI accumulation patterns. For illustration, patterns are simplified in the following way: H_2O_2 (*reddish-brown*) accumulates in three phases whereas $O_2^{\cdot-}$ (*dark-blue*) accumulates in two phases. First, H_2O_2 accumulates beneath the primary germ tube along with formation of a CWA in an epidermal cell (H_2O_2 accumulation phase I, HA phase I, *brown colouration* for H_2O_2). If *Bgh* is able to penetrate a host cell from its appressorial germ tube (*AGT*), H_2O_2 accumulation near CWAs is weak and $O_2^{\cdot-}$ accumulation is triggered at the penetration site (superoxide accumulation phase I, SOA phase I, *dark-blue colouration* for $O_2^{\cdot-}$). Where the plant prevents penetration effectively, H_2O_2 accumulates strongly in CWAs (HA phase II) whereas $O_2^{\cdot-}$ is hardly detectable. During onset and execution of HR (e.g. *Mlg*- or *Mla12*-mediated), H_2O_2 accumulates in the entire attacked cell (HA phase III) while $O_2^{\cdot-}$ accumulates in neighbouring mesophyll and epidermal cells that survive (SOA phase II). In the mesophyll HR (*Mla12*-mediated multi-cell death), dying mesophyll cells accumulate H_2O_2 and surrounding cells accumulate $O_2^{\cdot-}$ in the apoplast, cytoplasm and chloroplasts (*dark-green* if not ROI-accumulating). SOA phase I and HA phases I and II are triggered in race-non-specific plant responses. SOA phase II and HA phase III are associated with an HR that depends mostly on race-specific recognition. To a certain extent, early HR of apparently non-penetrated cells as typically mediated by *Mlg* is also triggered non-specifically. The time course of events differs depending on the active *R*-gene or on environmental influences. Drawing was inspired by Kita et al. 1981. *HI* Haustorial initial, *HAU* haustorium, *hpi* onset of the particular ROI accumulation phase, hours post inoculation

understood. Lipoxygenases, cytochrome p450 and mitochondrial respiration might play a role, although clear evidence is lacking. Recently, Asthir and co-workers (Bavita Asthir, Scottish Agricultural College, Edinburgh, UK, personal communication) discovered that diamine oxidase but not polyamine oxidase activity is strongly enhanced in barley under *Bgh* attack. This effect was stronger when comparing a resistant to a fully susceptible cultivar, indicating a possible involvement of diamine oxidase in *Bgh*-induced H_2O_2 accumulation. Hydrogen peroxide originates partly from $O_2^{\cdot-}$. However, since the spatiotemporal accumulation patterns of $O_2^{\cdot-}$ and H_2O_2 during *Bgh* attack are different, H_2O_2 might at least partly be generated independently of $O_2^{\cdot-}$. Alternative sources for H_2O_2 are peroxidases and oxalate oxidases, which have been shown to accumulate and to be active in attacked barley (Kerby and Somerville 1992; Freialdenhoven et al. 1994; Zhang et al. 1995; Zhou et al. 1998). Peroxidases are present in CWAs where H_2O_2 can be localised (Scott-Craig et al. 1995; Bestwick et al. 1998; Brown et al. 1998; McLusky et al. 1999). Peroxidases may contribute to H_2O_2 accumulation and utilise H_2O_2 as a substrate in lignification-like processes. Apoplastic alkalisation is involved in activation of a French bean peroxidase that produces H_2O_2 (Bolwell et al. 2002). However, it has been suggested that infection by *Bgh* induces apoplastic acidification rather than alkalisation, and that this change in pH results in activation of oxalate oxidases contributing to H_2O_2 accumulation by oxalate oxidation (Zhou et al. 2000). Thus more data about infection-related pH shifts are necessary to understand the role of milieu-dependent H_2O_2 sources at infection sites. Since oxalate oxidase-like proteins, which accumulate in barley epidermis upon *Bgh* attack (Wei et al. 1998), exhibit SOD activity (Woo et al. 2000; A. Christensen and H. Thordal-Christensen, Risø National Laboratory, Roskilde, Denmark, personal communication), they could contribute to H_2O_2 accumulation by acceleration of $O_2^{\cdot-}$ disproportionation, given that $O_2^{\cdot-}$ is produced and H_2O_2 degradation remains unchanged.

Oxidative defence

The expression "oxidative defence" has been used to characterise ROI-dependent plant defence reactions. DAB-positive staining of CWA is a reliable histochemical marker that distinguishes non-effective from effective CWAs. *Bgh*-induced, highly localized cell wall fortification is characterised by cross-linking reactions of phenolic compounds and proteins leading to lignin-like bioinert material and detergent-insoluble protein networks, respectively. Lignification processes are detectable by formation of yellow autofluorogens in CWAs (e.g. Lyngkjaer and Carver 1999; Hückelhoven et al. 1999). In resistance conferred by the *mlo* gene, the extremely effective CWAs contain autofluorogens, which are less sensitive to saponification at early infection stages. This indicates a possible oxidative bonding of phenolics to the cell wall (von Röpenack et al. 1998). Strikingly, barley *mlo* genotypes exhibit a much higher rate of CWAs with strong H_2O_2 accumulation than the respective *Mlo* wild type (Hückelhoven et al. 1999, 2000b). Protein immobilisation in effective CWAs has been demonstrated by Coomassie staining, and a reduced protein solubility by SDS buffers (Thordal-Christensen et al. 1997). Cross-linking and immobilisation of structural cell wall compounds may also be involved in heat-induced penetration resistance of barley to *Bgh* because this reaction is associated with an oxidative burst throughout the leaf tissue (Vallélian-Bindschedler et al. 1998a). *Bgh* apparently penetrates a

barley leaf by both hydrolytic activity and mechanical force (Pryce-Jones et al 1999). H_2O_2-driven formation of inert cell wall materials limits ingress of fungal hydrolases to plant cell walls and thus penetration. Additionally, lignin-like substances should hamper mechanical penetration by *Bgh*. Altogether, the evidence indicates that H_2O_2 is probably essential for apoplastic defence against the powdery mildew fungus.

Salicylic acid (SA) has been shown to support oxidative defence, likely by enhancement of NADPH oxidase activity, as demonstrated in soybean suspension cells (Shirasu et al. 1997). In barley, basal levels of free and conjugated SA do not change after *Bgh* attack (VallélianBindschedler et al. 1998b). A thorough kinetic analysis of free and total SA in several incompatible powdery mildew interactions including single-cell and multi-cell HR defence phenotypes confirmed this earlier finding, clearly indicating that hypersensitive cell death neither requires nor provokes SA accumulation in barley (Hückelhoven et al. 1999). Although plants were grown under identical conditions, total SA concentrations of different barley leaves varied from 150 to 1,000 ng SA/g fresh weight. Interestingly, this variation did not reflect the resistance status of the plants (Hückelhoven et al. 1999). Tissue-specific or subcellular SA distribution in barley could be important for understanding these findings.

ROI and signal transduction

$O_2^{\cdot-}$ and compatibility

In barley, ROI accumulate in both compatible and incompatible interactions (Hückelhoven and Kogel 1998; Hückelhoven et al. 1999). During fungal penetration and early haustorium development, a strong $O_2^{\cdot-}$ accumulation and a very faint H_2O_2 accumulation can be detected around the penetration site and adjacent anticlinal cell walls. The trigger of $O_2^{\cdot-}$ accumulation is non-specific in the sense that it is not affected by *R*-gene mutation (Hückelhoven et al. 2000a). *Bgh*-derived molecules that induce $O_2^{\cdot-}$ have not yet been identified. Functionally, such molecules might act as non-specific elicitors or pathogenicity factors (Hückelhoven and Kogel 1998; Kogel and Hückelhoven 1999).

H_2O_2 in race-unspecific resistance mechanisms

It is noteworthy that the implication of H_2O_2 in background resistance can be monitored in compatible interactions that lack *R*-gene-mediated responses. Thereby, H_2O_2 accumulates in CWAs and HR cells. CWAs in long epidermal cells (>400 μm), known to exhibit high potential for background resistance (Koga et al. 1990), show H_2O_2 at high frequencies (our unpublished observations). These observations support the notion that histochemically detectable H_2O_2 is linked to execution of certain defence responses rather than to race-specific signal transduction. Moreover, H_2O_2 patterns in non-host resistance of barley to wheat powdery mildew fungus (*Blumeria graminis* f.sp. *tritici*) qualitatively resemble those of background and race-specific resistance (Hückelhoven et al. 2001b; M. Trujillo: unpublished results from our laboratory).

Chemically induced resistance after treatment of barley with 2,6-dichloroisonicotinic acid results in enhanced penetration resistance and a higher rate of epidermal HR in response to *Bgh* (Kogel et al. 1994). Since induced plants show less $O_2^{\cdot-}$ accumulation (phase I) and more H_2O_2 accumulation (phases II and III) in attacked cells (Kogel and Hückelhoven 1999; Hückelhoven et al. 1999), an ambivalent role for ROIs seems likely, and enhanced H_2O_2 accumulation in induced plants shows that race-specific recognition of the attacking pathogen is not a general prerequisite for H_2O_2 accumulation. Although constitutive resistance does not rely on SA accumulation in barley, exogenously applied SA analogues are able to enhance background resistance, and both types of resistance share common post-inoculation features such as H_2O_2 accumulation. Therefore, one may assume possible cross-talk between the pathways of constitutive and induced resistance. However, it is not clear whether such cross-talk includes common signal transduction elements, as shown for NPR1 (non-expressor of PR-1; Cao et al. 1997; Ryals et al. 1997) in dicotyledonous plants, or late downstream events during execution of defence.

ROIs in race-specific resistance responses: *Mlg*-mediated resistance

In barley bearing the semi-dominant resistance gene *Mlg*, ROI accumulation patterns are qualitatively indistinguishable from those in background resistance and chemically induced resistance. However, in clear contrast to susceptible barley, *Mlg* barley accumulates H_2O_2 at nearly all interaction sites in phase III (Hückelhoven et al. 1999). Meanwhile, $O_2^{\cdot-}$ accumulation in attacked cells can hardly be observed (Hückelhoven and Kogel 1998). Because *Mlg* barley shows an HR at up to 80% of all interaction sites (Görg et al. 1993; Hückelhoven et al. 1999), this demonstrates that $O_2^{\cdot-}$ accumulation is not necessary for the HR. Interestingly, cells that undergo an HR are not penetrated in *Mlg* barley, which possibly explains the lack of $O_2^{\cdot-}$ accumulation.

Mla_x-mediated resistance

The barley MLA-proteins are members of the presumably cytoplasmic coiled-coil, NBS-LRR class of *R*-gene products (Wei et al. 1999; Halterman et al. 2001). Barley lines bearing *Mla12* show a late HR after penetration by *Bgh*. Accordingly, $O_2^{\cdot-}$ accumulates in attacked *Mla12* cells in advance of the subsequent cell death reaction.

Importantly, the $O_2^{\cdot-}$ burst coincides temporally and locally with fungal penetration and declines before H_2O_2 accumulation and the HR. However, on the basis of the present cytological and histochemical data it is not possible to distinguish as to whether $O_2^{\cdot-}$ is necessary as a source for H_2O_2 or whether $O_2^{\cdot-}$ is responsible for an extended cell survival indicated by the typically delayed cell death reaction in *Mla12* genotypes. In the latter case, cell death may only occur after removal of superoxide, as convincingly shown in animals and plants (Irani et al. 1997; Delledonne et al. 2001). Mutants of *Mla12* and *Rar1*, a gene required for *Mla*-specified resistance, are susceptible to *avrMla12 Bgh* isolates (Torp and Jørgensen 1986; Freialdenhoven et al. 1994). RAR1 is likely part of the ubiquitination/proteasome machinery (Azevedo et al. 2002). Both types of mutant, *mla12* and *rar*, lack phase III of H_2O_2 accumulation (Shirasu et al. 1999; Hückelhoven et al. 2000a). While *mla12* mutants are presumably impaired in initiation of H_2O_2 accumulation phase III, *rar1* mutants might be unable to remove a negative regulator of H_2O_2 accumulation via targeted proteolysis. In contrast, unspecifically triggered phase I $O_2^{\cdot-}$ accumulation was not affected in *mla12* and *rar* mutants (Hückelhoven et al. 2000a).

In *Mla12* barley, phase-III H_2O_2 accumulation, and cell death, alternatively takes place either in the epidermis or in the mesophyll. The fact that penetrated *Mla* cells occasionally survive fungal attacks whereas underlying mesophyll cells die points to both local cell death suppression by *Bgh* and signal transport out of the attacked cell into the mesophyll tissue (Hückelhoven et al. 1999).

Changes in the redox status of *Mla1* barley attacked by avirulent *Bgh* have been shown by analysis of the antioxidant system (Vanacker et al. 2000): contents of glutathione and the ratio of reduced to oxidized glutathione are greatly affected during H_2O_2 accumulation preceding the HR.

Redox regulation via MLO?

The functional MLO protein has been proposed to be a central negative regulator of defence mechanisms and cell death in barley. Loss of MLO function leads to unspecific *Bgh* resistance and provokes early senescence-like phenomena such as spontaneous cell death and chlorophyll degradation (Schulze-Lefert and Vogel 2000; Piffanelli et al. 2002). Powdery mildew-resistant *Mlo* mutants (*mlo5*) accumulate H_2O_2 at sites of *Bgh* attack (phase II) more frequently, earlier and apparently to higher concentrations (Hückelhoven et al. 1999, 2000b; Piffanelli et al. 2002). Additionally, *Mlo* expression is triggered by pathogen attack and by oxidative stress, suggesting that MLO is both a putative sensor and an effector of the cellular redox status (Piffanelli et al. 2002; Kim et al. 2002b). Since *mlo*-mediated resistance is race-unspecific, subcellular H_2O_2 accumulation (phase II) in CWAs should be triggered unspecifically and under negative control of MLO. Phase II of H_2O_2 accumulation depends partly on the function of *Ror1* and *Ror2* (Hückelhoven et al. 2000b; Piffanelli et al. 2002), two genes that are required for *mlo*-specified resistance (Freialdenhoven et al. 1996). This indicates that *Ror1* and *Ror2* gene products are involved in subcellular H_2O_2 accumulation. Additionally, penetrated *ror* mutants show a decreasing rate of H_2O_2 accumulation after fungal establishment whereas non-penetrated *mlo*/*Ror* genotypes show longer-lasting H_2O_2 accumulation. Fungal antioxidants or suppressors of H_2O_2 production may play a role in post-penetration defence suppression in susceptible barley and wheat (see also Wäspi et al. 2001). *mlo*-mediated penetration resistance runs without detectable $O_2^{\cdot-}$ accumulation (Hückelhoven and Kogel 1998). In contrast, susceptible *Mlo* barley accumulates $O_2^{\cdot-}$ at penetration sites, raising the question of whether MLO and $O_2^{\cdot-}$ accumulation are functionally linked. MLO represents a putative transmembrane receptor with seven membrane-spanning domains reminiscent of an animal G-protein-coupled receptor (Büschges et al. 1997; Devoto et al. 1999). However, instead of being dependent on the function of heterotrimeric G-proteins, MLO interacts Ca^{2+}-dependently with calmodulin to completely fulfil its role in barley susceptibility to *Bgh* (Kim et al. 2002a; Stein and Somerville 2002). Both Ca^{2+} and small G-proteins of the ROP, Rho (RAC) of plants, 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). At least one barley small G-protein, RACB, appears to be required for susceptibility because RNA interference by double-stranded RNA of *HvRacB* induced *Ror1*-dependent resistance to *Bgh*. *Ror1*-dependency of this effect suggests a link between MLO and small-G-proteins (Schultheiss et al. 2002; Stein and Somerville 2002). Together, small G-proteins and Ca^{2+} are possibly involved in both $O_2^{\cdot-}$ production and susceptibility to *Bgh*. This appears to be in clear contrast to other plant–pathogen interactions, where Ca^{2+} and G-proteins have been associated with plant defence (Blume et al. 2000; Romeis et al. 2000; Ono et al. 2001). However, it is imaginable that MLO, monitoring Ca^{2+} and ROI activities, antagonizes non-specific *Bgh* defence. ROP GTPases are involved in localized Ca^{2+} influx, actin remodelling and membrane transport during polar growth (Yang 2002). Actin remodelling and membrane transport are involved in many cellular processes such as NADPH oxidase activation in phagocytes (e.g. el Benna et al. 1994), CWA formation in barley (Kobayashi et al. 1997) and certainly also plasma-membrane invagination by fungal haustoria. Thus, ROP proteins and the cytoskeleton are possibly involved in processes leading to both accessibility and inaccessibility of barley cells.

Interestingly, both functional RACB and functional MLO play negative roles in resistance to *Bgh*, whereas losses of RAC1 or MLO function lead to hypersusceptibility to the fungal pathogen *Magnaporthe grisea* in

rice and barley, respectively (Jarosch et al. 1999; Ono et al. 2001; Stein and Somerville 2002). It is not clear yet whether barley RACB and rice RAC1 have analogous functions in disease resistance. However, MLO and RAC G-proteins are signal transduction elements that play ambivalent roles in resistance to biotrophic *Bgh* and hemibiotrophic *M. grisea*. Additionally, *mlo*-mutant genotypes are more sensitive to fungal toxins from culture filtrates of the hemibiotroph ascomycete *Bipolaris sorokiniana*. These toxins thereby induce accumulation of H_2O_2 (Kumar et al. 2001). However, the role of H_2O_2 in hemibiotrophy is unclear. It occurs early and is strictly associated with CWA formation and the HR in the epidermal layer when *B. sorokiniana* attempts to penetrate (biotrophic phase) and subsequently in the mesophyll during fungal spreading (necrotrophic phase, Kumar et al. 2001, 2002). Thus, it is possible that H_2O_2 contributes to penetration resistance to hemibiotrophs but is additionally involved in mesophyll cell collapse facilitating fungal growth during tissue disintegration.

Causalities

There has been a rise in the amount of data supporting the notion that ROIs are crucially involved in both cellular accessibility and inaccessibility to the powdery mildew fungus. Recently, Mellersh et al. (2002) have shown that non-host resistance of cowpea to plantain powdery mildew fungus (*Erysiphe cichoracearum*) could be partially broken by exogenous application of catalase. In comparable experiments, superoxide dismutase was inefficient. Overexpression of peroxidases, oxalate oxidase and germin-like proteins (oxalate oxidase-like proteins) enhanced background resistance to penetration by *Blumeria graminis* f.sp. *tritici* in transiently transformed wheat cells (Schweizer et al. 1999a, 1999b). Interestingly, constructs encoding mutant proteins without oxalate oxidase activity partly retained their resistance-enhancing effect. Thus, the effect of overexpression apparently did not completely rely on oxalate oxidase activity. Instead a structural role for oxalate oxidase proteins was suggested because they were partly immobilised at sites of attempted penetration (Schweizer et al. 1999b). An HR-supporting role of oxalate oxidase activity, which is dependent on apoplastic acidification, was indicated by proton extrusion after application of sublethal doses of the fungal toxin fusicoccin. The same treatment resulted in a higher frequency of epidermal HR upon attack by *Bgh* (Zhou et al. 2000).

As already mentioned, cell-autonomous silencing of the barley *RacB* gene encoding a possibly NADPH oxidase-activating small G-protein (Hückelhoven et al. 2001a; Schultheiss et al. 2002) led to enhanced penetration resistance to *Bgh*. Accordingly, overexpression of a constitutively active $RACB^{V15}$ mutant enhanced susceptibility to *Bgh* and therefore elucidated RACB as a susceptibility factor (H. Schultheiss: unpublished results from our laboratory). In accordance with the finding that successful penetration by *Bgh* is associated with $O_2^{\cdot-}$ accumulation, one can suppose that $O_2^{\cdot-}$ plays a negative role in barley penetration resistance to *Bgh*. However, a direct link between RACB and $O_2^{\cdot-}$ accumulation has not been shown yet. In many plant–microbe interactions, a phase of the oxidative burst that is independent of the *R–Avr*-gene has been observed (Baker and Orlandi 1995). To our knowledge this unspecific burst has never been ascribed as being provoked by the pathogen for its own benefit or to play a role in cellular accessibility. Recently, Torres et al. (2002) have shown that the *Arabidopsis atrboh* mutants lacking one or two different NADPH oxidase core subunits (*gp91phox* homologues) show, when compared to wild type Col-0 plants, less H_2O_2 accumulation but enhanced resistance and HR in response to *Peronospora parasitica*. On this basis, it is possible to question whether NADPH oxidase-dependent ROI production is favourable for resistance to invading fungi and oomycetes. The *atrboh* mutants additionally show smaller size and spontaneous cell death in mature leaves. This underscores a role of GP91PHOX in developmental processes and survival of leaf cells. However, although spontaneous cell death occurs in late stages whereas resistance to *Peronospora* is measurable in young leaves, it is difficult to distinguish direct effects from induced resistance in these mutants. It will be interesting to see whether *atrboh* mutants exhibit expression of acquired-resistance marker genes in young leaves. Interestingly, transient silencing of *pNAox* (barley *gp91phox*) expression in barley epidermal cells by RNA interference with *pNAox* double-stranded RNA enhanced resistance to *Bgh* penetration (M. Trujillo: unpublished results from our laboratory). Recently, superoxide was shown to interfere negatively with cell death via protecting cells from nitric oxide by formation of $ONOO^-$ (peroxinitrite), which is apparently not highly toxic for plants. $NO^{\cdot}$ together with H_2O_2 triggered cell death, indicating that the balance of $O_2^{\cdot-}$ and H_2O_2 is crucial for cell death induction (Delledonne et al. 2001). A cell death-restricting role of $O_2^{\cdot-}$ in *Arabidopsis* is demonstrated by the fact that $O_2^{\cdot-}$ triggered spreading lesions in *lsd1* mutants but not in wild-type plants (Jabs et al. 1996). In this system, the wild-type LSD1 zinc-finger protein seems to act as an $O_2^{\cdot-}$ sensor that controls CuZn-SOD upregulation as an initial stage of cell death protection (Kliebenstein et al. 1999).

Future work should include the search for downstream events of ROI accumulation in barley, which are not yet identified. ROIs were shown to induce Ca^{2+}-channel opening in stomatal guard cells (Murata et al. 2001). If ROIs were to play a similar role in *Bgh*-attacked cells, ROIs could operate upstream of the Ca^{2+} influx required for full calmodulin-dependent MLO activity in cellular accessibility to *Bgh*.

Because accumulation of *PR*-gene transcripts and of an antifungal *p*-coumaroyl-hydroxyagmatine correlates with H_2O_2 accumulation after *Bgh* attack (Freialdenhoven et al. 1994; Peterhänsel et al. 1997; von Röpenack et al. 1998; Shirasu et al. 1999; Hückelhoven

et al. 1999, 2000a), it seems likely that H_2O_2 is a messenger for other defence compounds (Levine et al. 1994; Chamnongpol et al. 1998). In our hands, infiltration of barley leaves with sublethal mixtures of glucose (2 mM) and glucose oxidase (25 units/ml) producing H_2O_2 *in planta* induced accumulation of pathogenesis-related PR-1 protein transcripts (József Fodor, Hungarian Academy of Sciences, Budapest, Hungary, personal communication. Other downstream events of ROI might result in local or systemic induced resistance to *Bgh* (Ouchi et al. 1974; Thordal-Christensen et al. 1988; Vallélian-Bindschedler et al. 1998a; Lyngkjaer and Carver 2000). Both types of induced resistance are associated with penetration resistance, for which cell wall cross-linking reactions driven by H_2O_2 might be a prerequisite (Bradley et al. 1992; Brisson et al. 1994; Olson and Varner 1993). Vice versa, one may speculate that induced accessibility to *Bgh*, which takes place after inoculation of barley with virulent *Bgh*, might be associated with an enhanced H_2O_2-scavenging capacity of cells penetrated by *Bgh* (Lyngkjaer and Carver 1999; Hückelhoven et al. 1999). This is also supported by the finding that susceptible barley infected by *Bgh* shows partly enhanced antioxidative capacities on the level of enzyme activities (El-Zahaby et al. 1995; Vanacker et al. 1998; Burhenne and Gregersen 2000). Considered together, one can speculate that the successful fungus suppresses H_2O_2-dependent plant defence or triggers plant endogenous survival pathways possibly via MLO to support its biotrophic life style.

Conclusions

The role of H_2O_2 and $O_2^{\cdot-}$ in host–parasite interactions is complex. We still do not have unequivocal evidence for the role of diverse ROIs in resistance and susceptibility of barley to *Bgh*. The sources of ROIs are not fully elucidated and although detailed data about the spatiotemporal distribution of ROIs exist, it is not clear whether we miss certain aspects due to so far limited technical access to, for example, hydroxyl radicals or the interplay of ROIs with each other and with other signal transduction compounds. Identification of genes encoding candidates for ROI-generating proteins and antioxidants provides a basis for reversed genetic approaches (e.g. Schweizer et al. 1999a, 2000) in different barley backgrounds and should shed brighter light into the role of ROIs in mechanisms of plant disease resistance and susceptibility.

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Special introduction to chapters 5-8:

In chapters 5-8 the isolation and characterisation of candidate genes with a potentially essential role in the interaction of barley with *Bgh* is described. The approach followed a reason-based strategy starting with published mRNA sequences or expressed sequence tag sequence information in public databases. This led to the isolation of a set of copy DNA sequences. The corresponding genes were partly activated in response to inoculation with *Bgh*. Differential expression of a few of these genes in differentially resistant barley lines gave the first hint to a potential function in determining resistance or susceptibility to *Bgh*. Functional assessment based on microprojectile mediated single-cell transformation with subsequent challenge of transformed cells by *Bgh* allowed characterisation of some genes as putative resistance or susceptibility factors.

The barley family of RAC/ROP-type small GTPases was characterised in detail. This provided, in excess of their role in the barley-*Bgh* interaction, basic sequence information, a phylogenetic analysis and information about subcellular localisation of the proteins.

The role of the gene products is discussed with respect to penetration resistance and in particular with respect to their role in production of reactive oxygen intermediates and in cytoskeleton rearrangement.

Chapter 5

Hückelhoven R, Dechert C, Trujillo M, Kogel K-H (2001) Differential expression of putative cell death regulator genes in near-isogenic, resistant and susceptible barley lines inoculated with the powdery mildew fungus. *Plant Mol Biol* 47: 739-748

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Differential expression of putative cell death regulator genes in near-isogenic, resistant and susceptible barley lines during interaction with the powdery mildew fungus

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Abstract

We analysed pathogenesis-related expression of genes, that are assumed to be involved in ubiquitous plant defence mechanisms like the oxidative burst, the hypersensitive cell death reaction (HR) and formation of localized cell wall appositions (papillae). We carried out comparative northern blot and RT-PCR studies with near-isogenic barley (*Hordeum vulgare*L. cv. Pallas) lines (NILs) resistant or susceptible to the powdery mildew fungus race A6 (*Blumeria graminis* f.sp. *hordei, Bgh*A6). The NILs carrying one of the R-genes *Mla12*, *Mlg* or the *mlo* mutant allele *mlo5* arrest fungal development by cell wall appositions (*mlo5*) or a HR (*Mla12*) or both (*Mlg*). Expression of an aspartate protease gene, an ascorbate peroxidase gene and a newly identified cysteine protease gene was up-regulated after inoculation with *Bgh*A6, whereas the constitutive expression-level of a *BAS* gene, that encodes an alkyl hydroperoxide reductase, was reduced. Expression of a newly identified barley homologue of a mammalian cell death regulator, Bax inhibitor 1, was enhanced after powdery mildew inoculation. An oxalate oxidase-like protein was stronger expressed in NILS expressing penetration resistance. A so far unknown gene that putatively encodes the large subunit of a superoxide generating NADPH oxidases was constitutively expressed in barley leaves and its expression pattern did not change after inoculation. A newly identified barley *Rac1* homologue was expressed constitutively, such as the functionally linked NADPH oxidase gene. Gene expression patterns are discussed with regard to defence mechanisms and signal transduction.

Abbreviations: aa, amino acids; BCP*Mlx*, *Mlx* backcross line in cv. Pallas; *Bgh*A6, *Blumeria graminis* f.sp. *hordei* race A6; EtBr, ethidium bromide; hai, hours after inoculation; HR, hypersensitive cell death reaction; NIL, near-isogenic backcross line; ORF, open reading frame; PCD, programmed cell death

Introduction

The barley-powdery mildew pathosystem has been widely studied in the past decades to support our understanding of the interaction of an economically important crop plant with a harmful biotrophic fungal pathogen. R-genes (dominant or semidominant resistance genes) or mutant alleles (*mlo*) were identified that mediate resistance against *Blumeria graminis* f.sp. *hordei* (*Bgh*) either in a race-specific or in a race-non-specific manner (Jørgensen, 1994). Resistance against *Bgh* is expressed as penetration resistance or under execution of a hypersensitive cell death reaction (HR). HR is widely accepted as a measure plants use to restrict the development of pathogens including *Bgh* (Freialdenhoven *et al*., 1994). However, it is still a question whether cell death is a cause or a consequence of effective defence (Király *et al*., 1972; Schiffer *et al.*, 1997). To study the functional role of HR, it is necessary to identify elements which are required for plant cell death. In barley, two genes *Rar1* and *Rar2* were identified which are required for many but not all R-gene-specified resistance types to *Bgh* (Freialdenhoven *et al.*, 1994; Jørgensen 1996;

Peterhänsel *et al*., 1997). While the product of *Rar2* is still unknown, RAR1 is a small zinc-binding protein (Shirasu *et al.*, 1999). These genes also control the accumulation of reactive oxygen intermediates (ROI) which is closely linked to HR in barley and is absent in susceptible *Rar1* and *Rar2* mutants (Thordal-Christensen *et al*., 1997; Hückelhoven and Kogel 1998; Hückelhoven *et al*., 1999; Shirasu *et al*., 1999; Hückelhoven *et al*., 2000b). Despite these evidences, the plant oxidative burst cannot be judged as causal for the expression of HR in general (Glazener *et al*., 1996; Hückelhoven and Kogel, 1998; Dorey *et al*., 1999; Heath, 2000). As a basis for future gene function studies, it is necessary to isolate plant genes which are hypothetically involved in the oxidative burst and programmed cell death (PCD).

In the present work, we used barley genes which might be involved in PCD and studied their role in barley defence against *Bgh*. We studied gene expression in resistant and susceptible, near-isogenic barley lines (NILs) bearing different traits (*Mla12*, *Mlg* or *mlo5*) that mediate resistance in a race-specific (*Mla12*, *Mlg*) or race-non-specific manner (*mlo5*). These genes govern fungal arrest at different stages of the interaction: (1) at the penetration stage while the attacked cell remains alive (*mlo*); (2) at the penetration stage on cells that subsequently undergo a HR (*Mlg*); or (3) after penetration by a subsequent single-cell or multi-cell HR (*Mla12*). The NILs are excellent tools to elucidate the role of gene expression after powdery mildew attack. Because the temporal occurrence of distinct defence responses is well known (Hückelhoven and Kogel, 1998; Hückelhoven *et al.*, 1999), kinetic gene expression analysis are able to provide correlative evidence for gene function in plant defence.

Material and methods

Plants, pathogens and inoculation

The barley (*Hordeum vulgare* L.) cv. Pallas and the *mlo5*-, *Mlg*- and *Mla12*-backcross lines in cv. Pallas (BCP*Mlx*) were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Their generation was described previously (Kølster *et al*., 1986). Plants were grown in a growth chamber at 16 °C, 60% RH and a photoperiod of 16 h (60 μmol m^{-2} s^{-1} photon flux density). The barley powdery mildew fungus *Blumeria* (syn. *Erysiphe*) *graminis* DC: Fr. f.sp. *hordei*, race A6 (*Bgh*A6) (Wiberg, 1974) was inoculated onto 7-day old barley primary leaves to give a density of 50 conidia per mm^2.

Cloning, sequencing and probe generation

cDNA fragments were isolated by RT-PCR with one-step RT-PCR kits (Life Technologies, Karlsruhe, Germany or Qiagen, Hilden, Germany). A complex RNA pool out of barley seedlings was used as a template. RNA was isolated from Pallas at 3, 5 and 7 days after germination. Additionally, RNA was isolated from the NILs at 1, 2 and 5 days after inoculation with *Bgh*A6 at 7 days after germination. RNA was also extracted from Pallas treated with the plant resistance activator 2,6-dichloroisonicotinic acid at 1, 2, and 5 days after treatment at 5 days after germination (Kogel and Hückelhoven, 1999). All isolated RNAs were diluted to a concentration of 1 μg/μl and pooled. Primers were designed with GenBank or EST data base information for specific barley sequences or rice and *Arabidopsis* sequences. To amplify a putative barley NADPH oxidase cDNA we designed primers from conserved regions in *gp91phox* homologue sequences from rice and *Arabidopsis* (GenBank accession numbers X93301 and AB008111). Primers 5′-garcaaggctcttttgattg-3′ and 5′-gaaatgctccttatggaattc-3′ were useful to generate a 378 bp RT-PCR product including 337 bp barley-specific sequence (*pNAox*). Further primers were 5′-gcagcccctcctcagcggcgc-3′ (5′ primer) and 5′-ctttttgggctgtgtctctgc-3′ (3′ primer) to obtain a 862 bp *Asprot* cDNA fragment (GenBank accession number X56136, Runeberg-Roos and Saarma, 1998); 5′cgcgccgcagccgagtacgac-3′ (5′ primer) and 5′-gtcacaaaaacacatgtaacc-3′ (3′ primer) to obtain a 674 bp barley *BAS* cDNA fragment (GenBank accession Z34917; Baier and Dietz, 1996); 5′-ggccgacatgcattcaccag-3′ (5′primer) and 5′-catctgatattgctgggtctg-3′ (3′ primer) to obtain a 506 bp *OXLP* cDNA fragment gene (GenBank accession number X93171; Wei *et al*., 1998); 5′-gccatggcgaagagctacccc-3′ (5′ primer) and 5′-agagatgattacttagcagtc-3′ (3′ primer) to obtain a 860 bp barley *APX* cDNA fragment (GenBank accession number AJ006358; Hess and Boerner, 1998); 5′-cgcagtaagcacagtagaaag-3′ (5′ primer) and 5′-gacgatccagtagtctttgcc-3′ (3′ primer) derived from Genbank accession number AF134153 to obtain another 454 bp barley *Cysprot* cDNA; 5′-ggattcaacgcgagcgcaggacaagc-3′ (5′ primer) and 5′-gtcgacgcggtgacggtatctacatg-3′ (3′ primer) to obtain

a 871 bp barley BaxI cDNA including an ORF of 741 bp; 5′-gttcatcaagtgcgtcaccgtg-3′ (5′ primer) and 5′-ttagcttcctcagttcttccctg-3′ (3′ primer) derived from a rice *Rac1* homologue (GenBank accession number AB029508) to obtain a 387 bp barley *Rac1* cDNA fragment; 5′-ctgtaggaaatggctgacgg-3′ (5′ primer) and 5′-tcggatcacctgacccat-3′ (3′ primer) to obtain a 758 bp barley *Actin* cDNA fragment (GenBank accession number AJ234400).

We isolated cDNAs from gels and cloned them into pGEM-T-Vektor (Promega, Mannheim, Germany). cDNAs were sequenced from plasmids by use of the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham, Freiburg, Germany) and analysed for similarities in the GenBank database using the BLAST algorithm (Altschul *et al*., 1997). For probe generation, plasmids were amplified in *Escherichia coli,* isolated and used for in *vitro* transcription with T7 or SP6 RNA polymerases and digoxygenin or fluorescein-labelled nucleotides (DIG-Luminescence detection Kit, Boehringer, Mannheim, Germany).

RNA extraction and northern blotting

Total RNA was extracted from 8–10 primary leaf segments (5 cm long) using RNA extraction buffer (AGS, Heidelberg, Germany) according to the manufacturer's instructions. For barley mRNA detection, 10 μg of total RNA from each sample were separated in agarose gels and blotted by capillary transfer onto positively charged nylon membranes. Detection of RNAs was performed according to the DIG system user's guide. Prior to immuno-detection of RNA-RNA hybrids, blots were washed stringently twice for 20 min in 0.1% w/v SDS, 0.1 (or 0.2) × SSC at 66–68 °C.

Reverse transcription-polymerase chain reaction

The OneStep RT-PCR kit (Qiagen, Hilden, Germany) was used for quantitative reverse transcription polymerase chain reaction (RT-PCR) following the manufacturer's instructions. To quantify template amounts the RT-PCR reaction was stopped during the exponential phase of amplification maintaining initial differences in target transcript amounts. PCR cycle number was deduced empirically for each first strand cDNA taking care that PCR cycle number was chosen so low that the PCR products could hardly be detected by ethidium bromide (EtBr) staining in agarose gels. Besides EtBr staining, PCR products were separated in agarose gels, denatured, blotted onto nylon membranes and detected with specific non-radioactively labelled RNA probes with standard protocols under stringent conditions. Hybridization, washing and immuno-detection were performed as described for northern blotting.

Results

For the present study, certain gene candidates were chosen because all of them are involved in different types of PCD in plants or animals. Candidates were chosen because of different reasons: A barley aspartate protease, phytepsin, was highly expressed during autolysis of developing tracheary elements and sieve cells (Runeberg-Roos and Saarma, 1998) and, therefore, phytepsin could also be involved in pathogen-induced cell death. Cysteine proteases are involved in soybean cell death triggered either by an avirulent strain of *Pseudomonas syringae* pv. *glycinea* or by oxidative stress (Solomon *et al*., 1999). Down-regulation of ascorbate peroxidases was involved in pathogen-induced PCD in tobacco (Mittler *et al*., 1998). H_2O_2 accumulates in chloroplastsof cells that undergo HR in response to barley powdery mildew inoculation (Hückelhoven *et al.*, 2000b). Therefore, down-regulation of chloroplast antioxidants such as thioredoxin dependent peroxidase BAS1 (Baier and Dietz, 1996) could be involved in HR. RAC1 and NADPH oxidase are believed to play key roles in the oxidative burst and PCD in plants (Groom *et al.*, 1996; Kawasaki *et al*., 1999). BAX inhibitor 1 is a protein that can suppress BAX-induced PCD in animals and yeast. This PCD is associated with BAX-mediated formation of pores in outer mitochondrial membranes, cytochrome c release from mitochondria and cysteine protease activity (Xu and Reed, 1998).

An oxalate oxidase like protein gene and an actin gene were chosen as controls for *Bgh*-induced and for constitutive gene expression, respectively (Wei *et al*., 1998).

Isolation of cDNAs

Five of the candidates were present in common databases. cDNA fragments of the corresponding genes were amplified with specific primers by RT-PCR using a RNA pool of cv. Pallas as a template (see Materials and methods).

Barley genes for the other candidates were not available and, therefore, these genes were isolated via

Table 1. Characteristics of newly identified barley cDNAs with putative functions in plant cell death.

cDNA	Length of cDNA (bp)	Putative protein function	Database entry with highest similarity, accession number[b] (organism) [a](identities/similarities of *x* aa)	Homologue from animals, accession number[b] (organism) [a](identities/similarities of *x* aa)
pNAox	336	large membrane-spanning subunit of NADPH oxidase	cytochrome b245 β chain homologue *rbohA,* T02024 (*Oryza sativa*) (100/110 of 112)	NADPH thyroid oxidase 1, AAF73921 (*Homo sapiens*) (31/55 of 110)
pRac1	387	small GTP-binding protein regulating NADPH oxidase	small GTP-binding protein RACBP, AAF91343 (*Oryza sativa*) (129/129 of 129)	rac GTPase, AAD50299 (*Xenopus laevis*) (96/110 of 139)
pBI-1	741	Bax inhibitor-1protein	Bax inhibitor-1, BAA89540 (*Oryza sativa*) (192/208 of 247)	testis enhanced gene transcript protein, AAB87479 (*Homo sapiens*) (78/120 of 228)
pCysprot	412	cysteine protease	cysteine protease, AAD55363 (*Hordeum vulgare*) (121/131of 137)	probable cysteine proteinase, T24387 (*Caenorhabditis elegans*) (69/89 of 135)

[a]Altschuhl *et al*., 1997.
[b]Genepept database.

a candidate gene approach (NADPH oxidase, Rac1, cysteine protease, Bax inhibitor 1). DNA sequences of known genes from animals or other plant species were taken to screen EST and other databases. If no barley ESTs could be found, sequences from rice were preferred to derive primer sequences for RT-PCR (see Materials and methods). In all cases RT-PCR generated cDNA fragments of the expected sizes. Four barley cDNAs were isolated and cloned. cDNAs were sequenced for homology searches in common databases (Altschuhl *et al*., 1997). Plant and animal homologues could be identified for all isolated cDNAs (Table 1). A barley putative NADPH oxidase cDNA fragment (*pNAox*, GenBank accession number AJ251717) spanning an open reading frame (ORF) of 112 amino acids (aa) showed 98% similarity to a rice oxidative burst oxidase cytochrome b245 β chain and 50% similarity to human NADPH thyroid oxidase. A *Rac1* cDNA fragment (*pRac1*, GenBank AJ290420) coded for a 129 aa ORF 100% identical to a rice small GTP-binding protein and 80% similar to another RAC-protein from *Xenopus leavis*. Both homologues are putative regulators of NADPH oxidases. A barley putative Bax inhibitor 1 gene (*BaxI*) was isolated probably as full-length clone (*pBI*-1, GenBank AJ290421). At the 3′ end two following stop codons were identified and at the 5′ end a start codon was identified in a mRNA region were all homologues share the translation initiation point. The ORF identified covered 247 aa. This was very similar to plant and animal Bax inhibitor 1 homologues encoding putative membrane proteins. High similarities of the *BaxI* ORF were found to a rice homologue (84% similarity) and to a human Bax inhibitor protein which was identified originally as a testis enhanced gene product (52% similarity). A cysteine protease cDNA fragment (clone *pCysprot*, GenBank AJ278817) encoded an ORF of 137 aa which was very similar to another barley cysteine protease (95% similarity) but also to a cysteine protease from *Caenorhabditis elegans* (66% similarity, Table 1). The *Cysprot* ORF encodes a protein domain typical of papain cysteine proteases.

Expression patterns of candidate genes in barley leaves after inoculation with Bgh

To elucidate the role of the candidate genes in the plant-microbe interaction of barley and *Bgh*, we inoculated four near-isogenic barley lines (NILs), that were derived from the susceptible cv. Pallas with 50 conidia/mm^2 of *Bgh* race A6. This race is avirulent to *Mla12* and *Mlg* barley (NILs: backcross Pallas *Mla12* (BCP*Mla12*) or backcross Pallas *Mlg* (BCP*Mlg*)) and, as all natural European barley powdery mildew isolates, also to *mlo*-barley (NIL: BCP*mlo5*). From these lines we took leaf samples for gene expression analy-

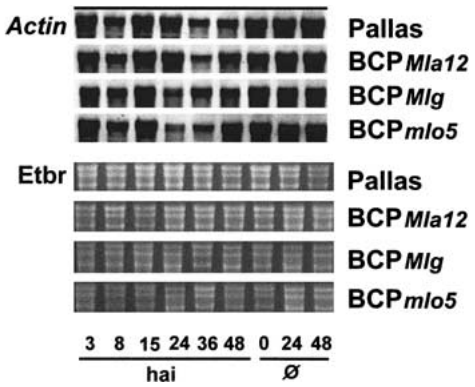

Figure 1. Gel blot analysis of *Actin* gene transcripts after inoculation of susceptible Pallas and resistant near-isogenic barley lines (NILs) with *Bgh*A6. Monogenic-acting R-genes *Mla12* and *Mlg* as well as recessive *mlo5* alleles mediate complete resistance to *Bgh*A6. Transcript accumulation was analysed at 3, 8, 15, 24, 36 and 48 hai in NILs bearing the different genes. Leaves from mock-inoculated controls (Ø) were analysed for mRNA accumulation at 0, 24 and 48 hai. The RNA amount loaded (10 μg) on the blotted gel was checked by EtBr staining of rRNAs.

sis early after inoculation, when the establishment of compatibility or successful defence takes place (Hückelhoven *et al*., 1999). To check disease development of the fungus and plant reactions, we additionally kept 5–10 inoculated plants from the same batch and judged the interaction type macroscopically 5–7 days after inoculation. Susceptible Pallas was covered completely with powdery mildew pustules 7 days after inoculation. Sporulation was not found on BCP*Mlg* and BCP*Mla12* whereas on BCP*mlo5* 1–5 small pustules per leaf were observed. This should be due to susceptibility of stomata guard cells of *mlo*-barley (Jørgensen, 1994). On BCP*Mla12,* multi-cell mesophyll HR beneath weakly developed mycelia was visible with the naked eye from the 5th day after inoculation onward.

Actin was expected to be expressed constitutively and used as a marker for equal loading. However, the gene used was expressed constitutively only in control plants, whereas in inoculated plants *Actin* expression decreased relatively to rRNAs transiently at 8 and 36 h after inoculation (hai) (Figure 1). Therefore, we used EtBr staining of rRNAs as a check for RNA loading. As a positive control for pathogenesis-related gene expression and an interesting candidate for differential expression in the NILs we analysed mRNA accumulation of the *OXLP* gene, which encodes an oxalate oxidase-like protein. As shown before by Wei *et al.* (1998), *OXLP* expression was induced strongly after powdery mildew inoculation. Interestingly, expression of *OXLP* was different in the NILs (Figure 2). Highest and earliest *OXLP* expression was found in BCP*Mlg* and BCP*mlo5*, that both build effective cell wall appositions at sites of attempted penetration.

We further analysed the expression of an aspartate protease gene (*Asprot*) that is involved in developmentally regulated barley cell death (Runeberg-Roos and Saarma, 1998). We found that *Asprot* expression was up-regulated under pathogen attack. This was most pronounced 22–36 hai in BCP*mlo5* coinciding with penetration resistance and 48 hai in BCP*Mla12* coinciding with mesophyll cell death (Figure 3 and Hückelhoven *et al*., 1999).

To study the role of selected antioxidants in this system, we analysed accumulation of mRNAs encoding cytosolic ascorbate peroxidase (*APX*) and alkyl hydroperoxide reductase (*BAS*). *APX* expression was not detected in northern analysis (see below). BAS expression was down-regulated within the time course of the experiment in mock-inoculated plants as well as in inoculated plants. Compared to mock controls, down-regulation occurred in a more pronounced way after inoculation with *Bgh.* This observation was made irrespective of the barley genotype (Figure 4). We further analysed the expression of *BaxI*. As shown in Figure 3, this gene is also responsive to *Bgh* inoculation. *BaxI* expression was up-regulated 24 and 48 hai in all lines. This effect was more distinct in BCP*Mla12* and BCP*Mlg,* that underwent HR upon inoculation with *Bgh*A6 (Figure 5).

Rac1, Cysprot, APX and *pNAox* were expressed too weakly to detect transcripts on standard northern blots with fluorescently or radioactively labelled probes. Gene expression of these genes was analysed by RT-PCR (Figures 6 and 7). *Rac1* was expressed constitutively. *Cysprot* and *APX* showed enhanced expression at 24 and 48 hai in both susceptible Pallas and resistant BCP*Mla12* (Figure 6). Expression of *pNAox* was studied in more detail. This gene was expressed to a higher level in young seedlings by 3 days after germination. At this time, a second RT-PCR product could be detected (Figure 7a). The second PCR product was larger and might stem from an alternatively spliced or immature transcript because the genomic sequence of the *pNAox* gene fragment includes two introns (data not shown). The non-spliced transcript of 850 bp could not be detected by RT-PCR. In older seedlings, *pNAox* was expressed constitutively after both mock and *Bgh*A6 inoculation (Figure 7b).

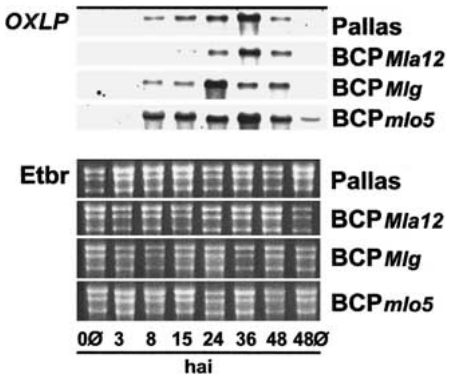

Figure 2. Gel blot analysis of oxalate oxidase-like protein gene (*OXLP*) transcripts after inoculation of susceptible Pallas and resistant near-isogenic barley lines (NILs) with *Bgh*A6. Monogenic-acting R-genes *Mla12* and *Mlg* as well as recessive *mlo5* alleles mediate complete resistance to *Bgh*A6. Transcript accumulation was analysed at 3, 8, 15, 24, 36 and 48 hai in NILs bearing the different genes. Leaves from mock-inoculated controls (Ø) were analysed for mRNA accumulation at 0 and 48 hai. Differences in the RNA amount loaded (10 μg) on the blotted gel were checked by EtBr staining of rRNAs. Repetition of the experiments led to very similar results.

Discussion

Numerous differential gene expression and random EST sequencing approaches started in recent years and revealed a mass of cDNA sequence information. Nowadays ten thousands of EST sequences are available for barley and the close relative rice. We used this information to select candidate genes for analysis of their role in the interaction of barley with powdery mildew. Interestingly, most of the selected candidates showed pathogenesis-related gene expression. Therefore, it seems that the candidate approach is a suitable tool to identify genes that are involved in plant defence mechanisms. Compared to differential display approaches and subtractive cDNA methods the candidate approach is less expensive and less time-consuming but, of course, also less comprehensive. All of these approaches are able to identify genes which are functionally redundant. A random mutagenesis approach followed by a screen for susceptible mutants would probably skip such genes as well as genes which are lethal as mutant alleles.

Expression of *OXLP* was up-regulated early and strongest in NILs forming effective cell wall appositions after *Bgh* attack (Figure 2). The oxalate oxidase like protein is probably involved in penetration resistance against *Bgh* because *OXLP* is expressed in barley epidermis during papilla formation (Wei *et al*., 1998). Additionally, over-expression of *TaGLP2a,* a wheat

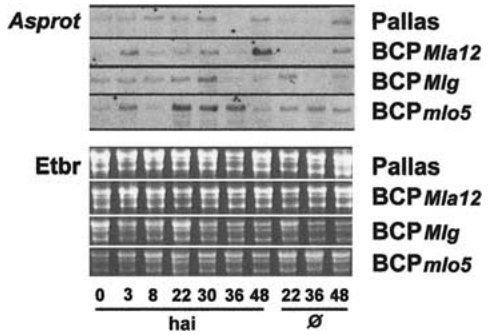

Figure 3. Gel blot analysis of a barley aspartate protease gene (*Asprot*) transcript after inoculation of NILs with *Bgh*A6. Transcript accumulation was analysed at 0, 3, 8, 22, 30, 36 and 48 hai in the NILs. Leaves from mock-inoculated controls (Ø) were analysed for mRNA accumulation at 22, 36 and 48 hai. Loading of the blotted gel (10 μg RNA) was checked by EtBr staining of rRNAs. Repetition of the experiments led to very similar results.

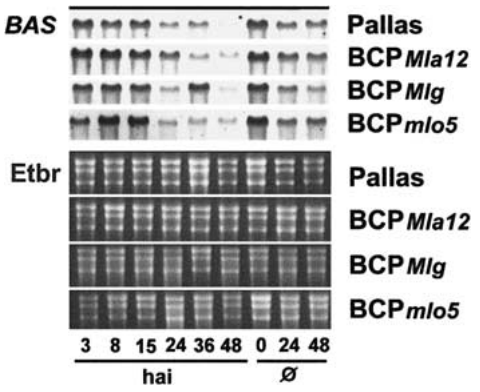

Figure 4. Gel blot analysis of BAS transcripts after inoculation of NILs with *Bgh*A6. Transcript accumulation was analysed at 3, 8, 15, 24, 36 and 48 hai in the NILs. Leaves from mock-inoculated controls (Ø) were analysed at 0, 24 and 48 hai. Loading of the blotted gel (10 μg RNA) was checked by EtBr staining of rRNAs. Repetition of the experiments led to very similar results.

homologue of *OXLP,* enhances penetration resistance (Schweizer *et al*., 1999b). The biochemical role of OXLP has not been elucidated yet but it may either take part in H_2O_2 accumulation or it may be a structural cell wall protein. This hypothesis is consistent with our recent finding that *mlo*-barley accumulates higher rates of H_2O_2 at sites of attempted penetration than wild-type barley (Hückelhoven *et al*., 2000a). Together, in *mlo*-barley high concentrations of H_2O_2, cross-linkable proteins and phenolics (von Röpenack *et al*., 1998) in cell wall appositions might contribute to penetration resistance.

The regulation of the antioxidative capacity of attacked barley leaves appears to be hard to interpret from gene expression data. While up-regulation of

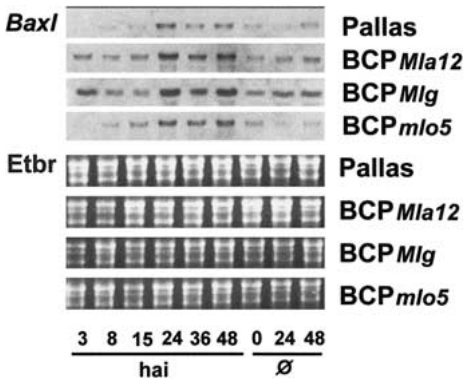

Figure 5. Gel blot analysis of and Bax inhibitor 1 gene (*BaxI*) transcripts after inoculation of NILs with *Bgh*A6. Transcript accumulation was analysed at 3, 8, 15, 24, 36 and 48 hai in the NILs. Leaves from mock-inoculated controls (Ø) were analysed at 0, 24 and 48 hai. Loading of the blotted gel (10 μg RNA) was checked by EtBr staining of rRNAs. Repetition of the experiments led to very similar results.

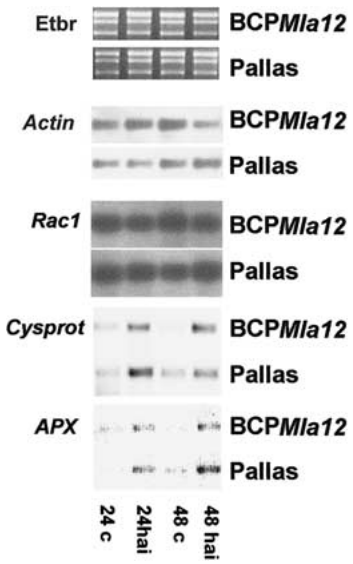

Figure 6. RT-PCR analysis of actin, Rac1, cysteine protease and ascorbate peroxidase gene transcripts after inoculation of NILs with *Bgh*A6. Transcript accumulation was analysed at 24 and 48 hai in Pallas and BCP*Mla12*. At the same time, leaves from mock-inoculated controls were analysed (24c and 48c). RT-PCR products were detected in agarose gels after separation and staining with EtBr staining (inverted positives shown; *APX*, 30 PCR cycles; *Cysprot*, 30 PCR cycles). Alternatively, PCR products were denatured in gels, blotted onto nylon membranes and detected with specific antisense RNA probes (*Actin* and *Rac1,* 27 PCR cycles). Equal loading of RT-PCR tubes (1 μg RNA) was confirmed by EtBr staining of rRNAs in separated check gels. Repetition of the experiments led to very similar results.

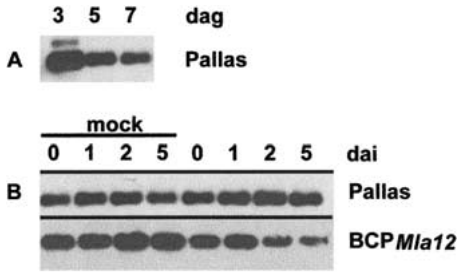

Figure 7. RT-PCR analysis of NADPH oxidase gene transcripts after inoculation of NILs with *Bgh*A6. A. Transcript accumulation was analysed in Pallas at 3, 5 and 7 days after germination (dag). B. Transcript accumulation was analysed in Pallas and BCP*Mla12* at 1, 2 and 5 days after inoculation at the seventh day after germination. During the same period leaves from mock-inoculated controls were analysed. PCR products (27 cycles, 1 μg RNA per tube) were denatured in gels, blotted onto nylon membranes and detected with specific antisense RNA probes (*pNAox*). Repetition of the experiments led to very similar results.

APX expression under powdery mildew development (Burhenne and Gregersen, 2000) was confirmed in this study, *BAS1* expression was down-regulated. *BAS1* encodes a haem-free peroxidase which reduces alkyl hydroperoxides and H_2O_2 in chloroplasts (Baier and Dietz, 1999). BAS1-protein protects the photosynthesis apparatus from fatty acid oxidation products and probably depends on photosynthetic reduction potential via thioredoxins. Down-regulation of *BAS1* expression might be caused by an acceleration of leaf ageing (Baier and Dietz, 1996) under pathogen attack or may be a prerequisite to drive an oxidative response to attack from *Bgh*. It is tempting to speculate that down-regulation of the chloroplast BAS1-protein is involved in the chloroplastic oxidative burst which can be detected in barley mesophyll cells after *Bgh* attack (Hückelhoven and Kogel, 1998; Hückelhoven *et al*., 2000b).

Several studies indicate an up-regulation of antioxidant capacities in barley during establishment of a compatible interaction with *Bgh* (El-Zahaby *et al*., 1995; Vanacker *et al*., 1998; Burhenne and Gregersen, 2000). Data from these studies fit well in with the fact that in compatible interactions successfully penetrated cells are free of H_2O_2 whereas in resistant lines H_2O_2 accumulates in cells that are attacked but not penetrated and in cells that undergo HR (Thordal-Christensen *et al*., 1997; Hückelhoven *et al*., 1999, 2000a, b).

As putative elements of a defence-related signal transduction chain (Dangl *et al*., 1996; Groom *et al*., 1996; Kawasaki *et al*., 1999), NADPH oxidase and

RAC1 are expected to be regulated on the activity level rather than on gene expression level. Therefore, it may not surprise that the *pNAox* gene and the *Rac1* gene whose product may regulate superoxide generation by NADPH oxidase were constitutively expressed during *Bgh* attack. Both, O_2^- generation and H_2O_2 accumulation occur in barley at sites of fungal attack. However, a causal relationship between activity of a barley NADPH oxidase, the oxidative burst and effective defence has not been demonstrated yet.

Similar to the role of caspases in animal apoptosis, plant cysteine and aspartate proteases are thought to represent proteins, that can disintegrate plant cells (Runeberg-Ros and Saarma, 1998; Solomon *et al*., 1999; Lam and del Pozo, 2000). For *Asprot* and *Cysprot* we found responsiveness to inoculation with *Bgh*. Thus, it seems possible that they contribute to HR in barley. *Cysprot* encodes a papain type protease. Papain proteases were also shown to be involved in apoptosis of human cells, probably *via* cleavage and activation of the pro-apoptotic factor Bid. Truncated Bid is thought to activate Bax which then forms pores in the outer mitochondrial membrane provoking cytochrome *c* release and subsequent caspase activation (Stoka *et al*., 2001). It was also shown for soybean that 'ectopic expression of cystatin, an endogenous cysteine protease inhibitor gene, inhibited cysteine protease activity and blocked cell death triggered either by an avirulent strain of *Pseudomonas syringae* pv. *glycinea* or by oxidative stress' (Solomon *et al*., 1999). Because up-regulated *Cysprot* expression was found in susceptible and resistant barley, we speculate that regulation of cysteine protease on the activity level may be crucial for HR. The pivotal event for protease activation may be the oxidative burst which is differentially triggered in resistant and susceptible barley (Hückelhoven *et al*., 1999, 2000b; Solomon *et al*., 1999).

Bax plays a central role in cytochrome *c* release from mitochondria which initiates a caspase cascade. In contrast, Bax inhibitor 1 abolishes the effect of Bax over-expression in animals and yeast (Kawai *et al*., 1999; Xu and Reed, 1998; Lam and del Pozo, 2000; Sanchez *et al*., 2000). As shown here, a gene encoding a barley Bax inhibitor 1 homologue was expressed in barley leaves upon attack by *Bgh*. This reaction was unexpectedly most pronounced in NILs undergoing HR. Because Bax inhibitors are expected to suppress cell death, the barley Bax inhibitor 1 may play a role in restriction of the spread of cell death in tissues mounting HR after fungal attack. Surprisingly, no Bax, Bid or other members of the Bcl-2 family have been identified in plants so far. We also failed to clone *Bcl-2* homologues via RT-PCR of barley mRNA with degenerated primers derived from conserved regions of several animal sequences (not shown). However, the presence of Bax inhibitors in plants suggests the conservation of a Bax/Bcl-2 analogue cell survival/cell death pathway in animals and plants. This is further supported by the finding that anti-apoptotic members of the Bcl-2 family, the animal cell death suppressors Bcl-x(L) and Ced-9, inhibit cell death in tobacco plants (Mitsuhara *et al*., 1999). Furthermore, in tobacco Bax can induce cell death with some features of the hypersensitive reaction (Lacomme *et al*., 1999).

Further gene function analysis of the candidates is required to confirm their role in barley resistance or susceptibility to *Bgh*. At present, tissue-specific expression analysis, isolation of full-length clones, transient gene expression and post-transcriptional gene silencing in barley cells challenged by *Bgh* is carried out (Nelson and Bushnell, 1997; Nielsen *et al*., 1999; Schweizer *et al*., 1999a, 2000) to provide a deeper insight into the role of the genes described here.

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Functional assessment of the pathogenesis-related protein PR-1b in barley

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Abstract

The pathogenesis-related protein 1 (PR-1b) of barley (*Hordeum vulgare* L.) is a marker for the attack by the powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*, *Bgh*) and other pathogens. PR-1b consists of 164 amino acids and has a potential signal peptide for export into the cell wall. Here, we show that *PR-1b* is differentially expressed in near-isogenic barley lines exhibiting various forms of defence phenotypes including papilla formation and the hypersensitive cell death. To elucidate PR-1b function, we transiently silenced *PR-1* expression by double stranded RNA (dsRNA) interference in the moderately susceptible barley double mutant line A89 (genotype: *mlo5–ror1*), which shows a papillae-based defence phenotype. Upon bombardment of leaf segments with *PR-1b* dsRNA and a GFP marker gene construct, *Bgh* slightly more frequently penetrated the plant cell wall of transformed epidermal cells relative to cells bombarded with human control dsRNA. We conclude that PR-1b contributes to penetration resistance to the powdery mildew fungus in barley. We also observed that *PR-1b* expression correlates with the production of H_2O_2 in responses to *Bgh* and *Bipolaris sorokiniana* and was induced upon infiltration of the H_2O_2 producing mixture of glucose and glucose oxidase.

Keywords: Gene silencing; MLO; Pathogen; Reactive oxygen intermediates; Resistance; Suppression

1. Introduction

The interaction between barley (*Hordeum vulgare* L.) and the powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*, *Bgh*) has been intensively studied in terms of plant defence reactions [1,2]. The plant response to pathogen attack includes alterations of the cell wall, production of phenolic metabolites and reactive oxygen intermediates, a hypersensitive cell death reaction (HR) as well as accumulation of pathogenesis-related (PR) proteins. In barley, early penetration resistance and physical reinforcement of the cell wall by appositions (CWAs, syn. papillae) are typically observed in race-non-specific resistance responses such as those governed by the *mlo* alleles or quantitative background resistance. HR is the predominant plant response in gene-for-gene resistance mediated by major restistance (*R*) genes such as *Mlg* and *Mla* [1]. Both race-non-specific formation of effective CWAs and *R* gene-mediated execution of HR correlate spatially and temporally with H_2O_2 accumulation [3].

PR proteins were initially identified as pathogen inducible proteins in leaves of tobacco after tobacco mosaic virus infection. Despite years of intensive research the biological function of the pathogenesis-related protein PR-1 remained unclear. It was reported that purified PR-1 proteins exhibit antimicrobial activities against *Phytophthora infestans* and that tobacco overexpressing PR-1 shows enhanced resistance to oomycetes. This provided first evidence for a causal role of PR-1 in plant defence. Expression of the gene increased after pathogen attack as well as after a number of abiotic stresses, including UV irradiation and wounding (for reviews see [4,5]). In dicotyledonous but not in monocotyledonous plants, PR-1 expression correlates with the onset of resistance induced biologically or by chemical resistance inducers like 2,6-dichloroisonicotinic acid and benzothiadiazoles [4,6].

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In barley, Mouradov et al. [7], Bryngelsson et al. [8] and Stevens et al. [9] identified altogether four different *Bgh*-responsive basic forms of PR-1. Southern analyses indicated a total number of 8–10 PR-1 family members in the genome [8]. Evidence for an involvement of PR-1 in the defence of barley against the powdery mildew fungus came from the observation that *PR-1* accumulated in response to inoculation with *Bgh* in resistant barley lines much stronger than in susceptible lines [10–12].

In this study we underline the involvement of the basic PR-1b [8] protein in the defence mechanism of barley against the powdery mildew fungus. We demonstrate differential *PR-1b* transcript accumulation during execution of different resistance mechanisms and show a clear association of *PR-1b* expression and *Bgh*-resistance. By *PR-1b* double stranded RNA (dsRNA) interference, we uncover contribution of PR-1b to penetration resistance to *Bgh*.

2. Materials and methods

2.1. Plant materials, pathogens and inoculation

The barley (*Hordeum vulgare* L.) lines Ingrid, Pallas and the backcross (BC) lines BCIngrid*mlo5*, BCPallas*Mla12* and BCPallas*Mlg* were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Their generation was described previously [13]. The mutant A89 was provided by Paul Schulze-Lefert (Max-Planck-Institute for Plant Breeding Research, Cologne, Germany [14]). Plants were grown in a growth chamber at 18 °C with 60% relative humidity and a photoperiod of 16 h (60 μmol m^{-2} s^{-1} photon flux density). The barley powdery mildew fungus, *Blumeria graminis* (DC) Speer f.sp. *hordei* Em. Marchal, race A6 was inoculated onto barley primary leaves to give a density of 50 conidia mm^{-2}. *Bgh* was maintained on barley cultivar Siri under the conditions described above.

Bipolaris sorokiniana had been isolated from the boot leaf of the wheat cultivar Sonalika growing under natural conditions at Banaras, eastern India, during April 1999. Single spore isolation and maintenance of the isolate has been described by Kumar et al. [15]. For inoculation, primary leaf segments of 6 cm length harvested from 7-day-old plants were laid flat by fixing their ends on surface of a steel sheet. A suspension containing approximately 20,000 spores/ml water plus 0.02% Tween-20 was sprayed onto the segments. Inoculated segments were immediately placed onto a 2 mm thick layer of benzimidazole 0.5% water agar (0.6 ppm benzimidazole) in a plastic dish closed tightly with a lid. Segments were incubated for 72 h at 25 °C with a photoperiod of 16 h.

2.2. RNA extraction and Northern blotting

Total RNA was extracted from 5 to 10 primary leaf segments (5 cm long) using a RNA extraction buffer (AGS, Heidelberg, Germany) according to the manufacturer's instructions. For barley mRNA detection, 2–4 μg of total RNA from each sample was separated in agarose gels and blotted by capillary transfer to positively charged Nylon membranes. Detection of RNAs was performed with antisense RNA probes (*HvPR-1*b, *HvOxOa* [2]) according to the DIG-System User's Guide (Roche, Mannheim, Germany). Prior to immunodetection of RNA–RNA hybrids, blots were washed stringently two times for 20 min in 0.1% (w/v) SDS, 0.1 × SSC at 68 °C. For probe generation, plasmids were amplified in *E. coli*, isolated and used for in vitro transcription using T7 RNA polymerases and digoxygenin or fluorescein labelled nucleotides (DIG-Luminescence Detection Kit, Roche, Mannheim, Germany).

2.3. Construction of pGY1-PR-1b and pGFP:PR-1b

For overexpression of the PR-1b protein the cDNA of *PR-1b* was cut out of the plasmid pHvPR-1b [8] with restriction enzymes *Sma*I and *Eco*RV and cloned into the *Sma*I site of pGY1 [16]. For expression of a GFP:PR-1b fusion protein the cDNA of the green fluorescent protein (GFP) was amplified from the plasmid pGFP under elimination of the GFP stop codon [16] by PCR (primers: 5′-GGATCCATGGTGAGCAAGGGCGAG-3′ and 5′-GGATCCTTGTACAGCTCGTCCAT-3′). PCR products were cloned directly into pGY1-PR-1b (cut with *Sma*I). The strategy was designed in a way, which allowed cloning of GFP upstream of the *PR-1b* 5′ end. Orientation of the insert was checked by PCR.

2.4. Transient transformation, RNAi, and evaluation of fungal development

To determine the function of PR-1b, we used a transient transformation assay as described previously [17,18]. Plants were grown in a growth chamber at 24 °C (20 °C in the dark) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). For dsRNA interference experiments, 312 μg of 1.1 μm tungsten particles (BioRad, Munich, Germany) were coated with dsRNA (2 μg) together with pGFP (1 μg; GFP under control of the Cauliflower Mosaic Virus (CaMV) 35S promoter [16]) as a transformation marker for each shot. As an unspecific RNA control, we used human thyroid receptor (hTR) dsRNA. Double stranded RNA was obtained by annealing of sense and antisense RNA synthesized in vitro [17]. For overexpression of PR-1b, 312 μg of 1.1 μm tungsten particles were coated with 0.5 μg pGFP as a marker and 0.8 μg pGY1-PR-1b or pGY1 (control), respectively.

Leaf segments were bombarded with coated particles 4 h before inoculation with *Bgh* race A6 (100 conidia mm^{-2}). Interaction outcome was evaluated subsequently by fluorescence and light microscopy. For each individual experiment, at least 100 interaction sites were counted as described in

[18]. PE was calculated for each experiment as number of penetrated cells divided by total number of attacked cells.

2.5. In planta production of H_2O_2

To produce H_2O_2 in planta we pressure-infiltrated barley first leaves with mixtures of glucose and glucose oxidase. Barley seedlings were infiltrated with 2 mM glucose (Gluc) in 10 mM $K_xH_xPO_4$ buffer pH 7.5 either alone or together with 25, 50, or 100 U/ml glucose oxidase (GOX EC 1.1.3.4 from *Aspergillus niger*, Sigma, Hannover, Germany). The starch/iodide assay was performed according to Wu et al. [19].

3. Results

3.1. Expression of PR-1b in response to biotrophic Blumeria graminis f.sp. hordei

To further elucidate a role of PR-1b in the barley defence against *Bgh*, we carried out gel blot analyses with total RNA from different susceptible and resistant near-isogenic barley lines after inoculation with 50 conidia of *Bgh*A6 per mm^2: (a) susceptible cultivars Ingrid and Pallas; (b) resistant backcross line BCIngrid*mlo5*; (c) moderately susceptible *ror*-mutant A89 (genotype *mlo5*, *ror1*); (d) resistant backcross lines BCPallas*Mlg* and BCPallas*Mla12*. The susceptible lines Pallas and Ingrid exhibited a late and transient *PR-1b* expression that decreased rapidly after successful haustoria formation (22–48 hai). The earliest detectable increase of *PR-1b* transcripts took place 3 hai in resistant BCIngrid*mlo5* (Fig. 1A). The mutant A89 showed an intermediate *PR-1b* transcript accumulation coinciding with an intermediate resistance phenotype between Ingrid and BCIngrid*mlo5*. In the *Mlg* and *Mla12* backcross lines strong *PR-1b* expression started between 8 and 22 hai and remained on a high level during expression of effective plant defence (Fig. 1B). The intensity of *PR-1b* signals correlated closely with the timing of different resistance responses in *mlo5*, *Mlg*, and *Mla12* genotypes.

3.2. PR-1b expression in response to the hemibiotrophic fungus Bipolaris sorokiniana

It was previously shown that *Bgh*-resistant *mlo*-barley is highly susceptible to certain hemibiotrophic fungi such as *Magnaporthe grisea* and *B. sorokiniana* [15,20]. We inoculated *Mlo* and *mlo5* plants by spraying with 20,000 spores/ml of *B. sorokiniana* and recorded *PR-1b* expression during the first 3 days post-inoculation. The *mlo5* genotype showed more severe spot blotch symptoms than the wild type parent *Mlo* line (not shown). *B. sorokiniana*-induced *PR-1b* transcript accumulation started from 16 hai onward and was stronger and earlier in highly susceptible *mlo5*-barley relative to the *Mlo* parent (Fig. 2).

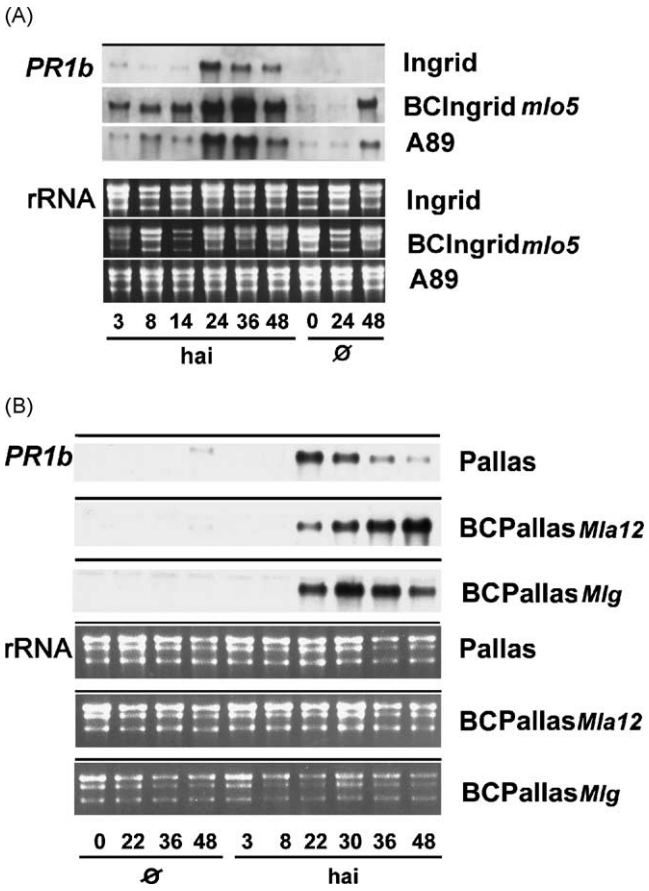

Fig. 1. Barley *PR-1b* expression in response to inoculation with *Blumeria graminis* f.sp. *hordei*. Northern blots of 2 μg total RNA were probed with antisense *PR-1b* RNA probes. Gene expression was analysed after mock (control, Ø) or conidial inoculation, respectively. Gel loading was documented after ethidium bromide staining of rRNAs. Total RNA was extracted at 0–48 h after inoculation (hai). (A) Transcript accumulation in near-isogenic susceptible cultivar Ingrid (genotype *Mlo*, *Ror1*), race-non-specific resistant BCIngrid*mlo5* (*mlo5*, *Ror1*) and moderately susceptible A89 (*mlo5*, *ror1*) after dense inoculation with *Bgh*. Resistant *mlo5*-barley builds effective cell wall appositions preventing penetration 14–24 h after inoculation. (B) Transcript accumulation in near-isogenic susceptible cultivar Pallas and race-specific resistant near-isogenic backcross lines BCPallas*Mla12* or BCPallas*Mlg* after dense inoculation with *Bgh* race A6 (*AvrMla12*, *AvrMlg*). Pallas is penetrated by *Bgh* 14–24 hai resulting in unrestricted fungal growth. BCPallas*Mla12* shows late post-penetration HR in epidermal cells and subjacent mesophyll cells 24–72 hai. BCPallas*Mlg* is resistant to fungal penetration and expresses early single-cell HR 18–30 hai.

3.3. PR-1b expression in response to H_2O_2

We tested whether H_2O_2 is a trigger for *PR-1b* expression. Barley first leaves were infiltrated with mixtures of glucose and different concentrations of glucose oxidase (Gluc/GOX) that oxidizes glucose accompanied by the production of H_2O_2. H_2O_2 accumulation in the intercellular washing fluid was confirmed by the starch/iodide assay (not shown). Glucose alone or in mixture with low concentration of GOX (25 U ml^{-1}) did not induce visible symptoms after infiltration into c.v. Pallas, whereas both 50 and 100 U GOX ml^{-1}

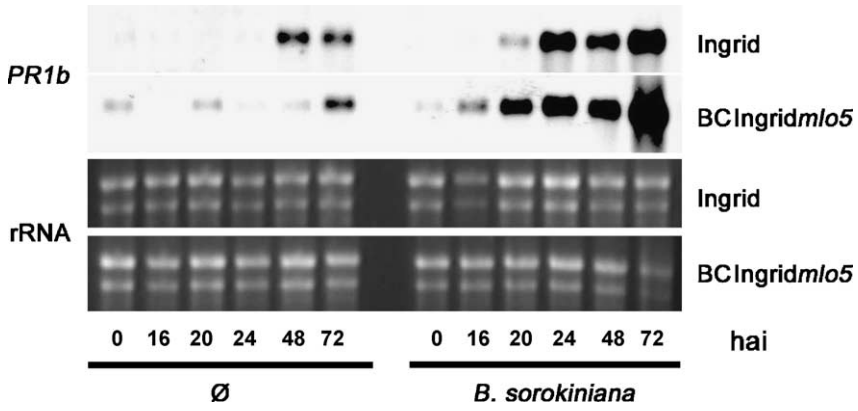

Fig. 2. Barley *PR-1b* expression in response to infection with *Bipolaris sorokiniana*. Northern blots of 3 μg total RNA were probed with antisense *PR-1b* RNA probes to analyse gene expression during fungal penetration (biotrophic phase) and tissue colonisation (necrotrophic phase) by the hemibiotrophic fungus *B. sorokiniana*. *Mlo*-wild type barley c.v. Ingrid and *Mlo*-mutant backcrossline BCIngrid*mlo5* were tested after spraying water (control, Ø) or conidial suspensions, respectively. Gel loading was documented after ethidium bromide staining of rRNAs. Total RNA was extracted at 0–72 h post-inoculation.

caused a mild leaf necrosis. We analysed *PR-1b* expression at 5 h and 24 h post-infiltration of Gluc/GOX and found increased gene expression at both time points even with 25 U GOX ml^{-1} while expression of *oxalate oxidase A* remained unchanged. Infiltration of Glucose solution did not induce detectable *PR-1b* expression (Fig. 3).

3.4. Assessment of PR-1b function

To determine whether PR-1b is crucial for penetration resistance, we performed epidermal single-cell *PR-1b* gene silencing experiments by sequence-specific dsRNA interference (RNAi) in a transient transformation assay [17,18].

Leaf segments of the moderately susceptible barley mutant A89 in which *PR-1b* is expressed at a high level (see Fig. 1A) were bombarded with *PR-1b* dsRNA together with a vector for expression of the green fluorescing protein (pGFP). Subsequently, the segments were inoculated with *Bgh*, and outcome of the interaction was evaluated 40 h later. After five independent experiments and evaluation of 772 interaction sites, we concluded that the transient knock down of PR-1b provoked a weak but significant increase in penetration efficiency (PE) of *Bgh* relative to controls that were bombarded with human control dsRNA (Fig. 4). Thereby, the relative PE on *PR-1b* dsRNA bombarded cells was enhanced from 20% in controls to approximately 25% (Fig. 4).

In order to demonstrate effectiveness and specificity of the RNA interference mechanism, we bombarded leaves with pGFP:PR-1b together with dsRNA of *PR-1b* or, alternatively, with a heterologous dsRNA of the human thyroid

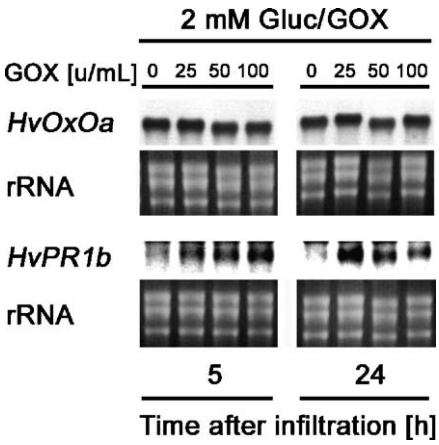

Fig. 3. Barley *PR-1b* expression in response to infiltration of mixtures of glucose and glucose oxidase provoking H_2O_2 accumulation. Barley cultivar Pallas seedlings were pressure-infiltrated with 2 mM glucose (Gluc) either alone (0) or together with 25, 50, or 100 U ml^{-1} glucose oxidase (GOX). Barley oxalate oxidase A (*HvOxOa*) and *PR-1b* expression was analysed in Northern blots at 5 and 24 h after infiltration. Gel loading was documented after ethidium bromide staining of rRNAs.

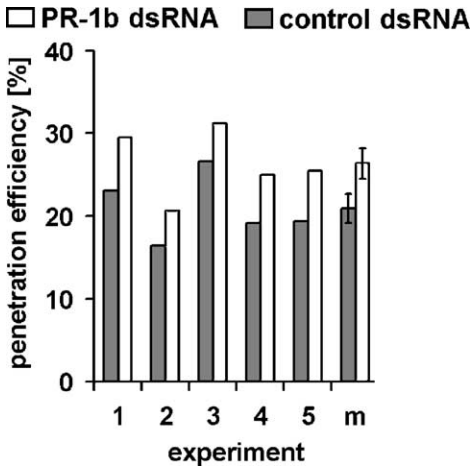

Fig. 4. Increase of penetration of barley mutant A89 (*mlo5*, *ror1*) by *Bgh* upon *PR-1b* dsRNA induced dsRNA interference. Penetration efficiencies were evaluated in five independent experiments with race *Bgh*A6. Penetration efficiency is enhanced by *PR-1b* dsRNA (white columns) relatively to controls (*TR* dsRNA; grey columns). Individual results (experiments 1–5) and means (*m*, columns with standard error bars) are displayed. Influence of *PR-1b* dsRNA on penetration efficiency in A89 is significantly different from the control at $p < 0.001$ (Student's *t*-test).

hormone receptor (TR). Expression of the GFP:PR-1b fusion protein in the presence of *TR* dsRNA resulted in 14.2 ± 0.5 GFP-fluorescent cells per segment (mean ± S.E.). In contrast, transformation of cells with GFP:PR-1b in the presence of *PR-1b* dsRNA reduced the number of green fluorescent cells to 3.9 ± 0.4 (mean ± S.E.; 72% reduction) per segment indicating sequence-specific, induced degradation of *GFP:PR-1b* mRNA.

Overexpression of PR-1b in the susceptible line Ingrid that exhibits a relatively late and transient expression of *PR-1b* (see Fig. 1) led to a small decrease in fungal PE from 51.9 ± 4.8 to 47.7 ± 2.7% (mean ± S.E.). However, this effect was not significant, although it was detectable in each of three independent experiments.

4. Discussion

The analysis of PR-1 conducted in this study characterised PR-1 as a stress response protein involved in several plant defence mechanisms. We have demonstrated that expression of PR-1b is apparently not sufficient to restrict the growth of fungal pathogens but is one factor that limits penetration of barley by the powdery mildew fungus.

In vitro studies revealed that recombinant barley PR-1b is not sufficient for the inhibition of *Bgh* germ tube development [21], whereas PR-1 isolated from beans inhibits differentiation of rust fungi [22]. Silencing the expression of PR-1b in A89 enhanced fungal penetration success although overexpression in the susceptible cultivar Ingrid enhanced resistance only weakly. Since overexpression was not very effective, our results support the notion that PR-1b does not exert a direct antimicrobial effect on *Bgh*. We speculate that PR-1b contributes to build up a physical barrier against plant cell wall penetration by *Bgh* together with other apoplastic host proteins. The removal of PR-1b from the resistance machinery resulted in a partial breakdown of penetration resistance. For gene silencing analysis by RNAi it was critical to choose the *mlo5–ror1* double mutant line A89 that shows strong *PR-1b* expression though the interaction phenotype with powdery mildew is moderately susceptible [14].

Many studies have demonstrated the responsiveness of the barley *PR-1b* gene to attack from *Bgh* (Fig. 1) [8,23]. *PR-1b* expression in susceptible barley might be a result of non-specific single-cell background resistance, which is responsible for the fact that development of *Bgh* is arrested at up to 50% of interaction sites even in the absence of an *R* gene. After successful fungal establishment, *PR-1b* expression attenuated at 36–48 hai possibly because the powdery mildew fungus actively suppresses defence reactions as a prerequisite to maintain biotrophy. Interestingly, transient defence gene expression in wheat upon inoculation with *B. graminis* reappeared after curative application of the resistance activating compound syringolin A, possibly counteracting defence suppression by the fungus [24]. In *mlo5*-barley, *PR-1b* expression in response to *Bgh*, *B. sorokiniana* and *M. grisea* was very high (Figs. 1 and 2) [6,11]. *Bgh*-resistant *mlo5*-barley is highly susceptible to the hemibiotrophic *B. sorokiniana*. Apparently, development of this fungus is not affected by strong expression of *PR-1b*.

Physiological changes induced by *Bgh* in barley are associated with effects on the host redox status [3]. For instance, *PR-1b* expression correlates temporally with accumulation of H_2O_2 during both effective defence reactions (papillae, HR) against *Bgh* and tissue colonisation by *B. sorokiniana* [3,15], which indicates that *PR-1b* expression could be affected by the plant redox status. Accordingly, *PR-1b* expression was induced by Gluc/GOX producing H_2O_2 in planta (Fig. 3). Though our results only hint to redox dependent PR-1b expression, this idea is further supported by the fact that the barley *PR-1b* promoter [23] contains several W-boxes that are supposed to be DNA sequences for binding of WRKY transcription factors that contain a potentially redox-sensitive zinc finger as DNA binding domain [25].

Acknowledgements

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A Small GTP-Binding Host Protein Is Required for Entry of Powdery Mildew Fungus into Epidermal Cells of Barley[1]

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Small GTP-binding proteins such as those from the RAC family are cytosolic signal transduction proteins that often are involved in processing of extracellular stimuli. Plant RAC proteins are implicated in regulation of plant cell architecture, secondary wall formation, meristem signaling, and defense against pathogens. We isolated a *RacB* homolog from barley (*Hordeum vulgare*) to study its role in resistance to the barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*). *RacB* was constitutively expressed in the barley epidermis and its expression level was not strongly influenced by inoculation with *B. graminis*. However, after biolistic bombardment of barley leaf segments with *RacB*-double-stranded RNA, sequence-specific RNA interference with *RacB* function inhibited fungal haustorium establishment in a cell-autonomous and genotype-specific manner. Mutants compromised in function of the *Mlo* wild-type gene and the *Ror1* gene (genotype *mlo5 ror1*) that are moderately susceptible to *B. graminis* showed no alteration in powdery mildew resistance upon *RacB*-specific RNA interference. Thus, the phenotype, induced by *RacB*-specific RNA interference, was apparently dependent on the same processes as *mlo5*-mediated broad resistance, which is suppressed by *ror1*. We conclude that an RAC small GTP-binding protein is required for successful fungal haustorium establishment and that this function may be linked to MLO-associated functions.

Complete resistance of barley (*Hordeum vulgare*) to the biotrophic, fungal pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*) is mediated by major resistance genes such as the *Mla* genes or by loss of MLO function in *Mlo*-mutant genotypes such as *mlo5*-barley (Jørgensen, 1994; Schulze-Lefert and Vogel, 2000). The latter is expressed exclusively via penetration resistance, which is characterized by formation of cell wall appositions and accumulation of phytoalexins, pathogenesis-related gene transcripts, and hydrogen peroxide (Stolzenburg et al., 1984; Zeyen et al., 1993; Peterhänsel et al., 1997; von Röpenack et al., 1998; Hückelhoven et al., 1999, 2000b). All of these characteristics are also found in susceptible barley, albeit to a lower extent, meaning that the *mlo* alleles confer a primed responsiveness for these defense reactions or the functional MLO is a control element of these fundamental resistance mechanisms (Büschges et al., 1997; Peterhänsel et al., 1997).

It is intriguing that *Bgh*-resistant *mlo* genotypes show hypersusceptibility to *Magnaporthe grisea* and to toxic culture filtrates of *Cochliobolus sativus* (Jarosch et al., 1999; Kumar et al., 2001). Thus, *Mlo* exerts an ambivalent role in controlling resistance to the biotroph *Bgh* and susceptibility to the hemibiotroph *M. grisea*. The MLO protein is a membrane-spanning protein reminiscent of a G-protein coupled receptor (Devoto et al., 1999). In animals, such proteins interact with heterotrimeric G-proteins and/or small GTP-binding proteins via different cytoplasmic domains (Naor et al., 2000). Small GTP-binding proteins such as those of the RAC family are cytosolic signal transduction proteins that often are involved in processing of extracellular stimuli. Plant RAC proteins are involved in regulation of plant cell architecture, secondary wall formation, meristem signaling, and defense against pathogens (Valster et al., 2000). Mammalian RAC1, in its GTP-binding form, is essential for stable assembly of an active NADPH oxidase complex in the plasma membrane of phagocytic and nonphagocytic cells. This complex is responsible for generation of superoxide radical anion ($O_2^{\cdot-}$) that is a signal molecule for cell proliferation in low concentrations, whereas it causes host cell death and pathogen killing in higher concentrations (Irani et al., 1997; Burstein et al., 1998; Irani and Goldschmidt-Clermont, 1998; Subauste et al., 2000).

Interaction of plant RAC homologs with the NADPH oxidase complex appears to regulate activity of NADPH oxidase that produces $O_2^{\cdot-}$ in response to pathogen attack (Hassanain et al., 2000; Ono et al., 2001). Rice (*Oryza sativa*) *Rac1*, when overexpressed in rice in its constitutive active form, leads to hypersensitive reaction (HR) at sites of attack by *M. grisea* and, therefore, to pathogen resistance. Expression of

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dominant negative forms of *Rac1* consistently results in enhanced susceptibility to *M. grisea* (Kawasaki et al., 1999; Ono et al., 2001).

Reactive oxygen intermediates (ROI) play multiple roles in plant pathogen interactions. $O_2^{\cdot-}$ or H_2O_2 induce defense mechanisms including pathogenesis-related gene expression and the HR. On the other hand, ROI are also signals that restrict cell death and lead to production of antioxidants. Spatial and quantitative differences in the occurrence of ROI are crucial for their mode of action (Levine et al., 1994; Tenhaken et al., 1995; Jabs et al., 1996). In barley, $O_2^{\cdot-}$ production takes place during attack by *Bgh* at sites of successful penetration of epidermal cells, but not at sites where fungal penetration is prevented (Hückelhoven and Kogel, 1998). In contrast, H_2O_2 accumulates subcellularly in barley at sites were penetration by *Bgh* is successfully prevented as well as in entire cells that undergo HR. Together, accumulation patterns of $O_2^{\cdot-}$ and H_2O_2 differ temporally and spatially in barley during attack by *Bgh* (Thordal-Christensen et al., 1997; Hückelhoven and Kogel, 1998; Kogel and Hückelhoven, 1999; Hückelhoven et al., 1999, 2000a, 2000b).

We show here that a barley RAC homolog is required for parasitic entry of the biotrophic powdery mildew fungus into epidermal host cells and, therefore, that this protein has a negative function in disease resistance of barley to *Bgh*.

RESULTS

Isolation of a Barley RACB Open Reading Frame

We recently isolated a partial coding sequence of a barley putative *Rac1* homolog (GenBank accession no. AJ290420; Hückelhoven et al., 2001). In this study, we isolated a complete open reading frame of the barley *Rac* homolog (see "Materials and Methods") that encodes a protein with more than 98% identity to RACB from rice and maize (*Zea mays*) and more than 55% identity to human RAC1 or RAC2. Therefore, the cDNA clone now is designated as barley *RacB* (GenBank accession no. AJ344223). The barley RACB homolog contains several conserved motifs that are essential for RAC function in animal systems. The CXXL motif is conserved at the C terminus. The Cys residue of this motif is the site of post-translational isoprenylation that directs active RAC proteins into the plasma membrane. The so-called effector loop of RAC protein can also be found in barley RACB (amino acids 28–48). This motif is responsible for interaction with target protein of RAC homologs such as NADPH oxidase. Barley RAC residues 127 to 140 resemble a specific effector loop that might be required for induction of $O_2^{\cdot-}$ generation via RAC (Hassanain et al., 2000). Motifs typically responsible for GTP binding and GTP hydrolysis, respectively, are also present in barley RACB. Together, the isolated barley cDNA encodes a protein that contains all typical motifs of small RAC GTP-binding proteins.

RacB Is Expressed in Epidermal Tissue

In our previous study, we described constitutive expression of the barley *RacB* homolog (designated as *Rac1*) in barley primary leaves. *RacB* expression was unaffected by inoculation with the powdery mildew fungus (*Bgh*; Hückelhoven et al., 2001). In this study, we wanted to know whether *RacB* is expressed in the epidermis of barley that is the only tissue attacked by *Bgh*. We analyzed tissue-specific expression of *RacB* in peeled abaxial epidermal strips and the residual part of primary leaves. Susceptible barley cultivars Pallas and resistant P10 were inoculated densely on the abaxial sides with *Bgh* race A6 by 24 h before sampling. As a positive control for epidermis-specific gene expression, an oxalate-oxidase like-protein gene (*OXLP*) was selected (Wei et al., 1998). Ubiquitin 1 (*Ubi*) was used as a marker for tissue-unspecific expression, and chloroplast-directed *BAS* (thioredoxin-dependent peroxide reductase) was selected as a marker for mesophyll expression. As shown in Figure 1, expression of *RacB* was stronger in peeled epidermal strips than in the rest of the leaves. Tissue specificity of *RacB* expression was similar to that of *OXLP* and different from that of *Ubi* and *BAS*.

We compared early expression of *RacB* in a highly resistant barley *mlo* line BCIngrid-*mlo5*, the respective susceptible near-isogenic parent Ingrid, and a susceptible mutant A89 (*mlo5 ror1*) between 0 and 24 h after inoculation (HAI). In Ingrid, about 50% to 60% of fungal penetration attempts lead to haustoria

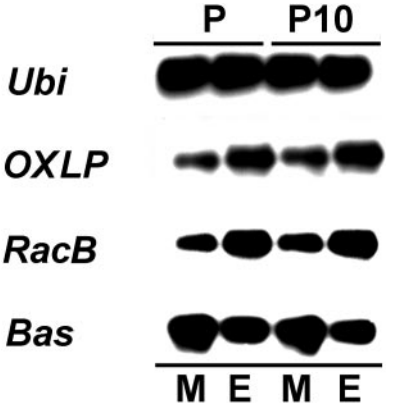

Figure 1. *RacB* is expressed in epidermal tissue. Reverse transcriptase (RT)-PCR with RNA from cv Pallas and cv BCP*Mla12* (P10) 24 HAI with *Bgh*A6. For extraction of total RNA, abaxial epidermal strips (E, inoculated site of the leaves) were separated from the mesophyll and adaxial epidermis (M). *Ubi* was selected as a marker for tissue-unspecific gene expression. *OXLP* was selected as a positive control for gene expression in the epidermal layer. *Bas* was selected as a positive control for gene expression in mesophyll cells. RT-PCR was carried out with 25 cycles under specific conditions. RT-PCR-products were denatured in gel, blotted, and detected by antisense RNA probes under stringent conditions.

formation between 12 and 24 HAI, whereas penetration rate in BCIngrid-*mlo5* was close to 0%. Cultivar A89, a *Mlo-Ror1* double-mutant line derived from BCIngrid-*mlo5*, is penetrated at 20% to 35% of the interaction sites by the *Bgh* isolate used (Hückelhoven et al., 2000b). *RacB* gene expression was slightly enhanced in response to *Bgh* inoculation as compared with *Ubi* expression that was taken as a constitutive marker. In the same RNA batch, the expression of *OXLP* as a positive control for *Bgh*-induced gene expression was enhanced from 8 HAI onward. At 14 HAI, when the first immature haustoria can be found in epidermal cells, *OXLP* expression was somewhat stronger in cv A89 and resistant BCIngrid-*mlo5* than in Ingrid (Fig. 2).

Sequence-Specific RNA Interference (RNAi) by *RacB*-double-stranded (ds) RNA Enhances Penetration Resistance

We addressed the question of whether RACB is involved in cellular accessibility or maintenance of basal resistance of barley to powdery mildew fungus. Host cell wall penetration and haustorium formation are the key steps in establishing host-pathogen compatibility. However, even susceptible barley cultivars such as Pallas or Ingrid prevent penetration at up to 50% of interaction sites, indicating a significant level of basal resistance. We used sequence-specific RNAi to induce gene silencing of *RacB*. RNAi produces phenotypes in plants that are very similar to those of knockout mutants (Waterhouse et al., 1998). It recently was shown that RNAi also functions transiently in barley if dsRNA is delivered into epidermal cells by biolistic bombardment (Schweizer et al., 2000). To test the efficiency of RNAi in induction of post-transcriptional gene silencing of RACB, we bombarded barley epidermal cells with *p*-green fluorescent protein (GFP):RACB that had been constructed for expression of a GFP:RACB fusion protein under control of the cauliflower mosaic virus 35 S promoter, together with *RacB*-dsRNA or heterologous control dsRNA (human thyroid hormone receptor dsRNA, *TR*), respectively. In four independent experiments, sequence-specific silencing of GFP:RACB led to a significant reduction of green fluorescing cells by 75% (Table I). This shows that dsRNA of *RacB* is suitable for inducing silencing of RACB in bombarded cells.

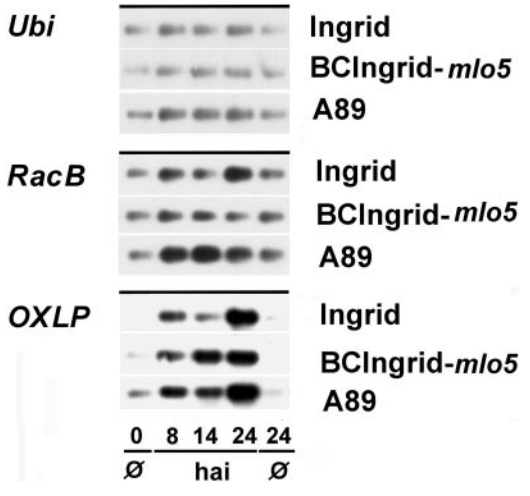

Figure 2. *RacB* expression in resistant and susceptible barley lines. RNA was isolated from cv Ingrid (*Mlo*, *Ror1*, susceptible), cv BCIngrid-*mlo5* (*mlo5*, *Ror1*, resistant), and cv A89 (*mlo5*, *ror1*, moderately susceptible) immediately before (0 Ø) inoculation at 8, 14, and 24 HAI with *Bgh* and 24 HAI from noninoculated control plants (24 Ø). *Ubi* was selected as a marker for constitutive gene expression. *OXLP* was selected as a positive control for *Bgh*-induced gene expression in the epidermal layer. RT-PCRs were carried out with 20 to 25 cycles under specific conditions. PCR products were denatured in gel, blotted, and detected by antisense RNA probes under stringent conditions.

Table I. *Effect of RacB-dsRNA on transient expression of a RACB:GFP fusion protein*

	No. of Green Fluorescing Cells per Leaf[a]
Control-dsRNA	11.3 ± 2.0
RacB-dsRNA	2.9 ± 1.8

[a] Mean ± SE of four independent experiments.

To elucidate the role of small GTP-binding proteins in basal resistance or cellular accessibility, we bombarded Pallas leaf segments with *RacB*-dsRNA together with a GFP expression vector (pGFP; Schweizer et al., 1999). Leaves were subsequently inoculated with *Bgh*, and the outcome of the interaction was evaluated 48 h later by in vivo light and fluorescence microscopy (Nielsen et al., 1999). Penetration into GFP-expressing cells was confirmed by detection of haustoria in living cells and by judgment of fungal development on these cells by fluorescence and light microscopy (see "Materials and Methods").

In each of six independent experiments, bombardment of cv Pallas with *RacB*-dsRNA led to a reduced number of cells that were successfully invaded by *Bgh* as compared with leaf segments bombarded with heterologous *TR*-dsRNA. The resistance-inducing effect of *RacB*-dsRNA resulted in an average reduction of penetration efficiency (PE) of *Bgh* by 44% (Fig. 3).

Broad prehaustorial resistance in barley against *Bgh* is controlled negatively by the wild-type MLO protein. Barley *mlo5* genotypes without a functional MLO protein are race nonspecifically resistant to penetration by *Bgh* (Büschges et al., 1997; Jørgensen, 1994). Because *RacB*-dsRNA inhibited haustorium formation in cv Pallas that bears no functional resistance gene against *Bgh*A6, we speculated that *RacB* and *Mlo* might be functionally linked. To test this hypothesis, we selected a *mlo5* genotype (cv A89, *mlo5 ror1*, background Ingrid) that is moderately susceptible to *Bgh* due to the mutation in *Ror1* (Freialdenhoven et al., 1996). In this double-mutant genotype, we tested the impact of *RacB*-dsRNA in

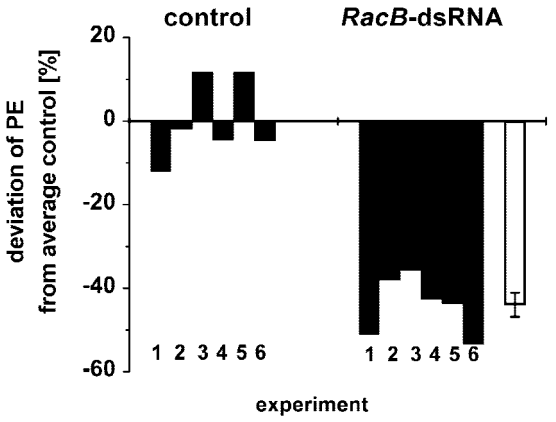

Figure 3. *RacB*-dsRNA interferes with the PE of *Bgh* in barley. Relative PE was evaluated in six independent experiments with *Bgh* on barley cv Pallas. PE of *Bgh* was reduced in cells that were bombarded with *RacB*-dsRNA compared with cells that were bombarded with control dsRNA (*TR*, human thyroid receptor-dsRNA). Negative and positive deviation of PE indicate reduced or enhanced PE, respectively, compared with average penetration frequency in six control experiments (adjusted to zero). Black columns, Relative PE at minimum 100 interaction sites in an independent experiment. White column, Average of the independent experiments with *RacB*-dsRNA. Error bar shows SE (relative PE of control and *RacB*-dsRNA are significantly different at $P = 0.000001$ level, Student's *t* test).

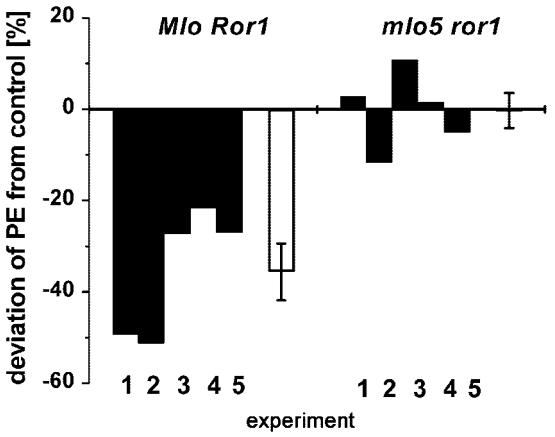

Figure 4. The influence of *RacB*-dsRNA on the PE of *Bgh* is dependent on the barley genotype. Relative PE was evaluated in five independent experiments with *Bgh* on barley lines Pallas, Ingrid, or A89. The PE of *Bgh* is reduced in cv Pallas (*Mlo Ror1*, experiments 1 and 2) or cv Ingrid (*Mlo Ror1*, experiments 3–5) cells that were bombarded with *RacB*-dsRNA compared with cells bombarded with control dsRNA (not shown). Penetration of susceptible double-mutant A89 (*mlo5 ror1*, experiments 1–5) was not affected by *RacB*-dsRNA. Black columns, Relative PE in an independent experiment. White columns, Average of five independent experiments with *RacB*-dsRNA. Error bars show SEs (influence of *RacB*-dsRNA on PE in *Mlo Ror1* and *mlo5 ror1* genotypes, respectively, is significantly different at $P < 0.002$, Student's *t* test).

comparison with wild-type *Mlo* genotypes. In five independent experiments, *RacB*-dsRNA did not prevent haustoria establishment in cv A89, whereas in the same experiments, PE was reduced by *RacB*-dsRNA in cv Pallas and cv Ingrid (*Mlo Ror1* genotypes; Fig. 4). Thus, resistance induced by *RacB*-dsRNA such as *mlo*-mediated resistance does not work in cv A89. It is remarkable that the *RacB*-dsRNA effect was stronger in cv Pallas than in cv Ingrid (Fig. 4, experiments 1 and 2 or 3–5, respectively). Absolute PEs are shown in Table II.

To rule out the possibility that *RacB*-dsRNA influences the transformation rate or the survival rate of attacked cells, we compared the number of GFP-expressing cells on control and *RacB*-dsRNA bombarded leaves (Table III). Microscopic evaluation showed that *RacB*-dsRNA did not influence the number of total or attacked GFP-expressing cells in any genotypes used. This demonstrates that RNAi by *RacB*-dsRNA strongly affects processes linked to successful establishment of the fungus but not cell death of host cells.

DISCUSSION

We have shown that *RacB*-dsRNA specifically interferes in barley epidermal cells with haustorium establishment by the plant parasitic, biotrophic powdery mildew fungus. Delivery of *RacB*-dsRNA into epidermal cells induced resistance with a similar efficiency as *Mlo*-dsRNA (Schweizer et al., 2000). Therefore, our results tag an RAC small GTP-binding protein as a host element that is required for successful invasion by *Bgh*.

Several lines of evidence could exclude nonspecific effects of *RacB*-dsRNA. First, in all experiments, the effect of *RacB*-dsRNA was compared with that of nonspecific *TR*-dsRNA, which has no plant homologs. An effect of *TR*-dsRNA was excluded in several experiments (data not shown). Second, the effect of *RacB*-dsRNA was genotype specific (Fig. 4). Third, *RacB*-dsRNA did not influence the number of nonattacked or attacked GFP-expressing cells (Table III). Fourth, when we bombarded barley with pGFP:RACB for expression of a GFP:RACB fusion protein together with *RacB*-dsRNA, the number of cells showing GFP fluorescence was reduced by 75% compared with experiments with heterologous *TR*-dsRNA. This shows that *RacB*-dsRNA induced gene silencing of the *RacB:GFP*-transgene. Thus, the biological effects of *RacB*-dsRNA are most likely a result

Table II. *Penetration frequencies of Bgh on barley leaves bombarded with dsRNA*

Line	Penetration Frequency[a]	
	Control-dsRNA	*RacB*-dsRNA
	%	
Pallas (*Mlo Ror1*)	57.0 ± 2.3	31.8 ± 1.6
Ingrid (*Mlo Ror1*)	53.8 ± 6.5	39.0 ± 4.0
A89 (*mlo5 ror1*)	27.4 ± 0.6	27.5 ± 1.6

[a] No. of penetrated cells divided by no. of attacked cells multiplied by 100 (mean ± SE).

Table III. *Transformation rates on barley leaves bombarded with dsRNA*

Line	No. of GFP-Expressing Cells per Shot[a,b]				n[c]
	Control-dsRNA		*RacB*-dsRNA		
	Total	Attacked	Total	Attacked	
Pallas (*Mlo Ror1*)	34.3 ± 4.6	16.0 ± 2.2	33.9 ± 4.8	15.5 ± 1.4	6 (21)
Ingrid (*Mlo Ror1*)	51.0 ± 8.9	27.6 ± 8.7	49.9 ± 5.6	31.5 ± 7.8	3 (11)
A89 (mlo5 ror1)	34.4 ± 5.4	18.1 ± 4.0	34.1 ± 5.5	16.7 ± 3.8	5 (22)

[a] Four leaves were bombarded per shot. [b] Mean ± SE. [c] No. of independent experiments (shots in *n* experiments each for control and *RacB*-dsRNA).

of post-transcriptional gene silencing of endogenous *RacB*. In barley, high sequence identities of dsRNA and target genes are necessary for RNAi (Schweizer et al., 2000). However, because *RacB* is probably very similar to other barley *Rac* genes, we cannot exclude that we might have affected the expression of RAC proteins other than RACB by *RacB*-dsRNA.

The resistance inducing effect of *RacB*-dsRNA effect was somewhat stronger in cv Pallas than in cv Ingrid. Because RACB apparently plays a negative role in broad resistance to *Bgh*, different levels of broad resistance in cv Pallas and cv Ingrid might influence RACB activity. In the barley double-mutant A89 (*mlo5-ror1*), *RacB*-dsRNA did not interfere with resistance. Therefore, it appears that the function of a RAC protein is linked to elements of the MLO/ROR network. Because MLO and ROR1 are involved in broad resistance against *Bgh*, this finding suggests that *RacB*-dsRNA interferes with race-unspecific penetration resistance of barley against *Bgh*, and that the same processes underlying *mlo*-mediated resistance limit this effect. Because RACB and MLO are required for fungal entry in barley epidermal cells, we speculate that they might be linked functionally. It is interesting that functional RACB and functional MLO play negative roles in resistance to *Bgh*, whereas losses of RAC1 or MLO function lead to hypersusceptibility to the fungal parasite *M. grisea* (Jarosch et al., 1999; Ono et al., 2001). Thus, MLO and RAC G-proteins are signal transduction elements that play ambivalent roles in resistance to biotrophic *Bgh* and hemibiotrophic *M. grisea*.

The mechanism by which RAC interferes with penetration resistance needs to be elucidated. One possibility might be that RAC interacts with the cytoskeleton. In mammals, RAC activation is triggered by bacterial pathogens that invade nonphagocytic cells and in phagocytes during phagocytosis. Thereby, RAC is involved in actin reorganization processes during plasma membrane ruffling or bacterial engulfment (Knodler et al., 2001). Both processes appear to resemble the process of plasma membrane invagination during establishment of a fungal haustorium in a plant cell. If barley RAC is needed for plasma membrane invagination, loss of RAC function should lead to inhibition of haustorium formation, as shown here. We speculate that the *Bgh* triggers a RAC small GTP-binding protein and that this process depends on MLO allowing plasma membrane invagination as a prerequisite for establishment of compatibility. Also, active RAC could be involved in cytoskeleton organization processes that antagonize formation of cell wall appositions. Cytoskeleton reorganization appears to be required for penetration resistance of barley coleoptiles to nonhost pathogens such as *Erysiphe pisi* (Kobayashi et al., 1997).

RAC proteins are involved in activation of the $O_2^{\cdot-}$-generating NADPH oxidase complex (Bokoch, 1995; Hassanain et al., 2000). In previous studies, we have shown that enhanced $O_2^{\cdot-}$ generation in barley cells attacked by *Bgh* temporally and spatially coincided with successful penetration and haustorium formation, but not with processes resulting in penetration resistance. Resistant *mlo5* genotypes did not produce $O_2^{\cdot-}$ during the period of attempted penetration (Hückelhoven and Kogel, 1998; Kogel and Hückelhoven, 1999). Thus, it is tempting to speculate that barley RACB functions via activation of NADPH oxidase and that $O_2^{\cdot-}$ generation influences penetration resistance to *Bgh* negatively. In contrast to $O_2^{\cdot-}$, H_2O_2 accumulates at sites of formation of cell wall appositions in which *Bgh* sticks (Thordal-Christensen et al., 1997; Hückelhoven et al., 1999, 2000b). Thus, H_2O_2 is strictly associated with barley defense reactions. Together, the balance of $O_2^{\cdot-}$ and H_2O_2 might be crucial for accessibility of epidermal cells.

MATERIALS AND METHODS

Plant Materials, Pathogen, and Inoculation

The barley (*Hordeum vulgare*) lines Ingrid, Pallas, and the backcross line BCIngrid-*mlo5* were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen). Their generation was described previously (Kølster et al., 1986). The mutant A89 was obtained from Paul Schulze-Lefert (Max-Plank-Institute for Plant Breeding Research, Köln, Germany). Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (60 μmol m^{-2} s^{-1} photon flux density). The barley powdery mildew fungus, *Blumeria graminis* (DC) *Speer* f.sp. *hordei* Em. Marchal, race A6 (Wiberg, 1974) was inoculated onto barley primary leaves to give a density of 50 conidia mm^{-2}. *Bgh* was maintained on barley cv Siri under the same conditions.

Isolation of epidermal tissue for expression analysis was performed by scribing adaxial sides of leaf tips with a scalpel without harming the abaxial epidermis. Leaf tips were folded back and taken as a handle to peel off epidermal strips that were cut off the leaf tips and frozen in liquid nitrogen immediately.

Isolation of Barley *RacB*, Cloning, Sequencing, and Probe Generation

We isolated cDNA fragments by the use of one-step RT-PCR kits (Invitrogen, Carlsbad, CA or Qiagen, Hilden, Germany). A complex RNA pool out of barley seedlings was used as a template. RNA was isolated from cv Pallas at 3, 5, and 7 d after germination. In addition, RNA was isolated from cv Pallas and backcross lines bearing *mlo5*, *Mlg*, or *Mla12* at 1, 2, and 5 d after inoculation with *Bgh*A6 at the 7th d after germination. All isolated RNAs were diluted to a concentration of 1 μg μL^{-1} and they were pooled. Primers were designed using GenBank or expressed sequence tag database information for specific barley sequences or rice (*Oryza sativa*) sequences. To amplify a putative barley *RacB* cDNA, we designed primers from rice and barley sequences. Primers 5′-GGATCCGATGAGCGCGTCCAGGTT-3′ (from GenBank accession no. AF250327) and 5′-GTCGACCTTCGCCCTTGTTCTTTGTC-3′ (from GenBank accession no. BF260616) were suitable to generate a 642-bp RT-PCR product including 618-bp barley-specific sequence (GenBank accession no. AJ344223). We isolated cDNAs from gels and cloned them into pGEM-T-Vektor (Promega, Mannheim, Germany). cDNAs were sequenced from plasmids by use of the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing kit (Amersham Biosciences, Freiburg, Germany) and were analyzed for similarities in the GenBank database using the BLAST algorithm (Altschul et al., 1997). Because the 5′ end of the isolated complete *RacB* open reading frame contained primer-derived sequences, we carried out RACE. First-strand cDNA synthesis and RACE were carried out as suggested by the manufacturer (GeneRacer; Invitrogen, Karlsruhe, Germany). First strand cDNA synthesis started from mRNA that was isolated from total RNA using the Dynabeads mRNA Purification kit (Dynal, Hamburg, Germany) according to the manufacturer's instructions. Hot-start touch-down RACE-PCR included the GeneRacer 5′primer and the *RacB*-specific primer 5′-GGATCCGATGAGCGCGTCCAGGTT-3′. Touch-down PCR was carried out with initial denaturation (5 min at 94°C), five cycles at a 70°C annealing temperature, five cycles at 68°C, and 28 cycles at 66°C. Each annealing was followed by a 1-min primer extension at 72°C and a 30-s denaturation at 94°C. The final extension time at 72°C was 10 min. The resulting RACE product of approximately 400 bp was reamplified with the gene-specific primer and the 5′GeneRacer nested primer, and was then isolated, cloned, and sequenced as already described.

For probe generation, plasmids were amplified in *Escherichia coli*, isolated, and used for in vitro transcription using T7 or SP6 RNA polymerases and digoxygenin- or fluorescein-labeled nucleotides (DIG-Luminescence Detection kit; Roche Molecular Biochemicals, Mannheim, Germany).

RNA Extraction and RT-PCR

Total RNA was extracted from eight to 10 primary leaf segments (5 cm long) or from 20 epidermal strips (mentioned before) using RNA extraction buffer (Applied Genetechnology Systems, Heidelberg) according to the manufacturer's instructions. RNA contents of the extracts were measured by UV photometry and were adjusted after checking in ethidium bromide-stained gels taking rRNA bands as a measure.

The OneStep RT-PCR kit (Qiagen) was used for semiquantitative RT-PCR following the manufacturer's instructions. To estimate template amounts, the RT-PCR reaction was stopped during the exponential phase of amplification, maintaining initial differences in target transcript amounts. PCR products were separated in agarose gels, denatured, blotted on nylon membranes, and detected with specific nonradioactively labeled RNA probes according to the DIG System user's guide (Roche Molecular Biochemicals). Prior to immunodetection of DNA-RNA hybrids, blots were washed stringently two times for 20 min in 0.1% (w/v) SDS and 0.1× SSC (15 mM sodium chloride and 1.5 mM sodium citrate, pH 7.0) at 68°C.

The primers were: 5′-GTTCATCAAGTGCGTCACCGTG-3′ (5′ primer) and 5′-TTAGCTTCCTCAGTTCTTCCCTG-3′ (3′ primer) for a 387-bp *RacB* cDNA fragment; 5′-CGCGCCGCAGCCGAGTACGAC-3′ (5′ primer) and 5′-GTCACAAAAACA-CATGTAACC-3′ (3′ primer) for a 674-bp barley *BAS* cDNA fragment (GenBank accession no. Z34917); 5′-GGC-CGACATGCATTCACCAG-3′ (5′ primer) and 5′-CATCT-GATATTGCTGGGTCTG-3′ (3 ′ primer) for a 506-bp *OXLP* cDNA fragment (GenBank accession no. X93171); and 5′-CCAAGATGCAGATCTTCGTGA-3′ (5′ primer) and 5′-TTCGCGATAGGTAAAAGAGCA-3′ (3′ primer) for a 513-bp *Ubi* cDNA fragment (GenBank accession no. M60175).

Construction of pGFP:RACB

For expression of a GFP:RACB fusion protein, cDNAs of *GFP* (GFPemd-b in pGFP; Schweizer et al., 1999) and *RacB* were amplified from plasmids by PCR using primers with attached restriction sites. PCR products were cloned into pGEM-T, amplified in *E. coli*, digested using primer-specific restriction enzymes, isolated from gels, and cloned one after another in pGY1 (Schweizer et al., 1999). Primers were designed in a way that allowed cloning of *GFP* upstream of the *RacB* 5′ end under elimination of the *GFP* stop codon. The primers used were 5′-GGATCCATGGTGAGCAAGGGCGAG-3′ and 5′-GGATCCTTGTACAGCTCGTCCAT-3′ for *GFP* and the *RacB* primers already mentioned. Orientation of the inserts was checked by PCR.

Transient Transformation, RNAi, and Evaluation of Fungal Development

A transient transformation protocol originally developed for wheat (*Triticum aestivum*) to assess gene function

in the interaction with powdery mildew was used to induce RNAi via biolistic delivery of dsRNA into epidermal cells of barley leaf segments as described by Schweizer et al. (1999) and Schweizer et al. (2000; compare also Nielsen et al., 1999). For the transient transformation assay, plants were grown in a growth chamber at 24°C (20°C in the dark) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). In principle, 312 μg of 1.1-μm tungsten particles was coated with dsRNA (2 μg) together with pGFP (1 μg; GFP under control of cauliflower mosaic virus 35S promoter) as a transformation marker for each shot. ds*RacB* RNA was obtained by annealing of sense and antisense RNA synthesized in vitro (Schweizer et al., 2000). Leaf segments were bombarded with coated particles 4 h before inoculation with *Bgh*, race A6. Inoculation with 100 conidia mm^{-2} led to an attack rate of approximately 50% on transformed cells. Interaction outcome was judged subsequently by fluorescence and light microscopy. For each individual experiment, at least 100 interaction sites were evaluated. Transformed GFP-expressing cells were identified under blue light excitation. Three different categories of transformed cells were distinguished: (a) penetrated cells, which contained an easily visible haustorium; (b) cells that were attacked by a fungal appressorium but did not contain a haustorium; (c) and cells that did not contain a haustorium and were not attacked by *Bgh*. Cells that contained more than one haustorium were scored as one penetrated cell independent of the number of fungal penetration attempts. Cells with multiple attacks from *Bgh* without a haustorium were scored as one nonpenetrated cell. Stomata cells and stomata guard cells were excluded from the evaluation. Surface structures of *Bgh* were detected by light microscopy or by fluorescence staining of the fungus with 0.1% calcofluor (w/v in water) for 30 s.

Deviation of PE referring to average control PE was used as a measure for susceptibility of cells that were bombarded with *RacB*-dsRNA compared with those bombarded with control *TR*-dsRNA (human thyroid receptor-dsRNA; Fig. 3). In five independent experiments, *TR*-dsRNA did not change the PE of *Bgh* compared with water. Deviation of PE was calculated for each experiment as the number of penetrated cells divided by the total number of attacked cells (PE) minus average PE in the controls divided by average PE of the controls multiplied by 100.

Deviation of PE referring to individual control PE was used to compare the impact of RNAi in different genotypes (Fig. 4). Therefore, PE in each experiment with *RacB*-dsRNA was divided by PE of individual controls, normalized by subtraction of one and multiplication by 100.

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Chapter 8

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Functional analysis of barley RAC/ROP G-protein family members in susceptibility to the powdery mildew fungus

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Summary

Small monomeric G-proteins of the plant ras (rat sarcome oncogene product) related C3 botulinum toxin substrate (RAC)/Rho of plants (ROP) family are molecular switches in signal transduction of many cellular processes. RAC/ROPs regulate hormone effects, subcellular gradients of Ca^{2+}, the organisation of the actin cytoskeleton and the production of reactive oxygen intermediates. Therefore, we followed a genetic bottom-up strategy to study the role of these proteins during the interaction of barley (*Hordeum vulgare* L.) with the fungal biotrophic pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*). We identified six barley RAC/ROP proteins and studied their gene expression. Five out of six *Rac/Rop* genes were expressed constitutively in the leaf epidermis, which is the site of interaction with *Bgh*. None of the genes showed enhancement of mRNA abundance after inoculation with *Bgh*. After microprojectile mediated transformation of single barley epidermal cells with constitutively activated mutant RAC/ROP proteins, we found an RAC/ROP-specific enhancement of pathogen accessibility, tagging HvRACB, HvRAC3 and HvROP6 as host proteins potentially involved in the establishment of susceptibility to *Bgh*. Confocal laser scanning microscopy (CLSM) of green fluorescent protein (GFP):HvRAC/ROP-transformed cells revealed varying strengths of plasma membrane association of barley RAC/ROPs. The C-terminal CAAX motif for presumable prenylation or the C-terminal hypervariable region (HVR), respectively, were required for membrane association of the RAC/ROPs. Proper intracellular localisation was essential for HvRACB and HvRAC3 function. Together, our data support the view that different paths of host signal transduction via RAC/ROP G-proteins are involved in processes supporting parasitic entry into epidermal host cells.

Keywords: MLO, penetration resistance, powdery mildew, small RAC/ROP G-protein, susceptibility.

Introduction

In mammals, the Rho family of small monomeric GTPases contains RAC, cell division cycle protein 42 (CDC42) and ras homologue (RHO) proteins. In plants, however, only one subgroup, slightly more similar to the Rac than to the Rho family subgroup, exists and was designated Rac or Rho of plants (Rop; Hall, 1998; Valster *et al.*, 2000; Winge *et al.*, 1997; Zheng and Yang, 2000). All members of the Rho-family, including the plant-specific Rac/Rop subfamily share a similar five-loop domain architecture. These domains are responsible for binding of guanine nucleotides (GTP or GDP), hydrolysation of GTP to GDP (GTPase activity), binding of regulatory proteins, binding of downstream targets and subcellular localisation (Paduch *et al.*, 2001). The most variable part of the RAC/ROP proteins is the C-terminal hypervariable region (HVR), which regulates intracellular targeting and localisation. Small GTPases such as the RAC/ROPs are signal transduction proteins acting as molecular switches in a multitude of cellular processes. In response to extracellular signals, regulatory proteins (in mammals: guanine nucleotide exchange factors) support binding of GTP by the RAC/ROP protein, activating the G-protein, which then binds and activates downstream effectors. By hydrolysing GTP to GDP and Pi conformational changes are reverted and the protein becomes inactive again. The activity status of plant RAC/ROP proteins is adjusted most likely by receptor-like kinases, GTPase activating proteins and guanine nucleotide dissociation inhibitors (Baxter-Burrell *et al.*, 2002; Vernoud *et al.*, 2003). RAC/ROPs associate with the plasma membrane (PM) mediated by either prenylation or palmitoylation of cysteine residues in the C-terminal HVR (Ivanchenko *et al.*, 2000; Lavy *et al.*, 2002).

RAC/ROPs are involved in actin cytoskeleton remodelling, secondary wall formation, establishing of intracellular Ca^{2+} gradients, regulation of polar cell growth, production of reactive oxygen intermediates (ROIs) and the modulation of hormone signalling and gene expression (Valster *et al.*, 2000; Yang, 2002). All of these processes are expected to be linked to signal transduction and physiological changes during plant–microbe interactions. Cell polarity is of special importance during early interaction of plant cells and fungal or oomycete pathogens (reviewed by Schmelzer, 2002). As many parasitic fungi invade cells by highly localised secretion of cell-wall-degrading enzymes along with osmotic pressure, forcing fungal infection structures into host cells, the attacked host needs to focus its defence to the site of attempted penetration. Thereby, the plant cytoskeleton is rearranged to direct actin filaments and microtubules to the site of attack. This leads further cytoplasma aggregation and migration of the nucleus. These types of subcellular processes are assumed to be important for early defence and penetration resistance (Kobayashi *et al.*, 1997; Schmelzer, 2002). Inhibition of cytoskeleton rearrangement leads to facilitated parasitic access to plant cells. Despite this, invagination of the plant PM by biotrophic fungi and establishment of compatibility require membrane traffic and reorganisation of host cell architecture, processes that also dependent on rearrangement of the cytoskeleton. The function of RAC/ROPs has been linked to rearrangement of both the cytoskeleton and subcellular Ca^{2+} gradients during polar pollen tube and root hair growth (e.g. Camacho and Malho, 2003; Gu *et al.*, 2003; Jones *et al.*, 2002; Molendijk *et al.*, 2001). Intracellular Ca^{2+}, like the cytoskeleton, plays roles in both cellular accessibility and defence to fungal pathogens (Blume *et al.*, 2000; Kim *et al.*, 2002; Xu and Heath, 1998). In barley, the defence and cell death regulator membrane protein MLO interacts Ca^{2+}-dependently with calmodulin to completely fulfil its role in barley susceptibility to *Bgh* (Kim *et al.*, 2002).

Another role for RAC/ROP proteins is the regulation of ROI production. In mammals, RAC proteins are required for assembly of the NADPH oxidase complex that generates superoxide radical anions ($^{\cdot}O_2^-$) as signal or antimicrobial agent (Bokoch and Diebold, 2002). The role of RAC in NADPH oxidase assembly is the interaction with cytoplasmic subunits and/or direct interaction with the membrane protein GP91PHOX supporting electron flow (Bokoch and Diebold, 2002). Although the cytoplasmic subunits P47PHOX and P67PHOX do not exist in plants, constitutively activated (CA) maize RAC/ROP mutant proteins are able to induce ROI production in mammalian cell cultures (Hassanain *et al.*, 2000) and human CA RAC1 enhances ROI production in soybean cell suspensions in response to defence elicitors (Park *et al.*, 2000). Moreover, the involvement of RAC/ROP proteins in ROI production in plants is also given by the fact that overexpression of CA OsRAC1 leads to an enhanced hydrogen peroxide generation (Kawasaki *et al.*, 1999), hypersensitive cell death in response to virulent races of *Magnaporthe grisea* and enhanced resistance in rice (Ono *et al.*, 2001).

All these indications argue for an important participation of RAC/ROP proteins in regulation of plants defence against various pathogens. Indeed, importance of RAC/ROPs in defence mechanisms could be shown in at least three pathosystems. First, in the rice–rice blast fungus system (Ono *et al.*, 2001); second, in the rice–rice bacterial leaf blight system (Ono *et al.*, 2001); and third, in the interaction of barley with the barley powdery mildew fungus (Schultheiss *et al.*, 2002).

The barley powdery mildew fungus *Bgh* is an obligate biotrophic pathogen that attacks epidermal cells of barley (*Hordeum vulgare* L.). The crucial step of fungal invasion is the penetration of the cell wall followed by the establishment of a haustorium that does not destroy PM integrity. During penetration, superoxide radical anions ($^{\cdot}O_2^-$) are produced around the site of successful penetration and haustorium establishment (Hückelhoven and Kogel, 1998).

Resistance to the powdery mildew fungus is mediated by major genes such as the *powdery mildew resistance genes* a_x, Mla_x, or by loss of *powdery mildew resistance gene o* (MLO)-function in *Mlo*-mutant genotypes (e.g. *mlo5*-barley, Jorgensen, 1994). The latter is expressed exclusively via penetration resistance, which is accompanied by accumulation of hydrogen peroxide but not by detectable O_2^- generation (Hückelhoven and Kogel, 2003; Schulze-Lefert and Vogel, 2000). The wild-type MLO protein is a seven-transmembrane-protein reminiscent of G-protein-coupled receptors in animals and fungi (Devoto *et al.*, 1999). It could be excluded that MLO signalling in susceptibility to *Bgh* depends on heterotrimeric G-proteins (Kim *et al.*, 2002). However, HvRACB, a small monomeric G-protein of the RAC/ROP family, may be linked to the MLO-signalling pathway because the transient knock down by *HvRacB*-dsRNA interference strongly enhanced penetration resistance to *Bgh* in susceptible barley but not in lines bearing the required for mlo-specified resistance (*ror*)*1-2* mutant allele (Schultheiss *et al.*, 2002), which was discovered as a suppressor allele of *mlo*-mediated penetration resistance (Freialdenhoven *et al.*, 1996). This puts HvRACB as an upstream antagonist of the hypothetical ROR1 protein that was not yet identified. Interestingly, recent studies revealed that overexpression of barley BAX (BCL-2 associated X protein; BCL-2: B-cell lymphoma protein-2) Inhibitor 1, a putative cell death inhibitor without sequence similarity to MLO, is able to suppress *mlo* penetration resistance (Hückelhoven *et al.*, 2003). This underscores a possible link of penetration resistance, RAC/ROPs and cell death regulation.

In this study, we report about the identification of six barley RAC/ROP proteins and show RAC/ROP-specific support of barley susceptibility to the barley powdery mildew

fungus. To further examine the role of the RAC/ROP proteins in plant defence, we determined their subcellular localisation and show a link of RAC/ROP function and proper localisation at the PM.

Results

Isolation of barley Rac/Rop family cDNAs

In a previous study, we showed that the knock down of the barley RAC/ROP family protein HvRACB by dsRNA interference led to enhanced resistance of barley to *Bgh*. To start a detailed examination of the RAC/ROP family proteins, we accomplished an expressed sequence tag (EST) database search starting with the sequence information of *HvRacB* (Hückelhoven *et al*., 2001), and identified several ESTs that could be assembled to tentative mRNA sequences *in silico*. By using RT-PCR and rapid amplification of cDNA ends (RACE), we isolated the cDNAs of six different RAC/ROP homologous proteins (see Experimental procedures). Open-reading frames showed high aa sequence similarities between different barley RAC/ROPs of up to 90%. As a result of the lack of a common nomenclature for monocot RAC/ROPs, we named the six barley RAC/ROP proteins HvRAC1, HvRAC3, HvRACB, HvRACD, HvROP4 and HvROP6 according to the closest related homologue regardless of the species this protein originates from.

All barley-deduced RAC/ROPs amino acid sequences typically contain five domains for the presumed protein function (Figure 1). The GDP/GTP-binding domains and the GTPase domains are highly conserved among all six homologues. We could not observe any amino acid exchange within the core of these domains. Another completely conserved amino acid stretch belongs to the so-called effector-loop thought to be crucial for interactions with regulatory proteins (Valster *et al*., 2000). Two domains do not show high similarities between all barley RAC/ROP family members. The first one is the special effector-loop (or insert-region) that is probably responsible for downstream signalling. The second one is the C-terminal HVR that contains signals targeting the RAC/ROP protein to specific membranes. HvRACB and HvRACD contain typical prenylation signals (CAAX-boxes, for cysteine–aliphatic aa-aliphatic aa-X) at their C-terminus, whereas the HVRs of all other RAC/ROPs contain additional cysteine residues that might be palmitoylated (Ivanchenko *et al*., 2000; Lavy *et al*., 2002). The length of the C-terminal region also distinguishes the two type I RAC/ROPs, HvRACB and HvRACD, from the four type II RAC/ROPs, HvRAC1, HvRAC3, HvROP4 and HvROP6 (Figure 1).

An aa sequence comparison of the six barley RAC/ROPs with other cereal and *Arabidopsis* RAC/ROP proteins was used for a bootstrap analysis to construct an unrooted

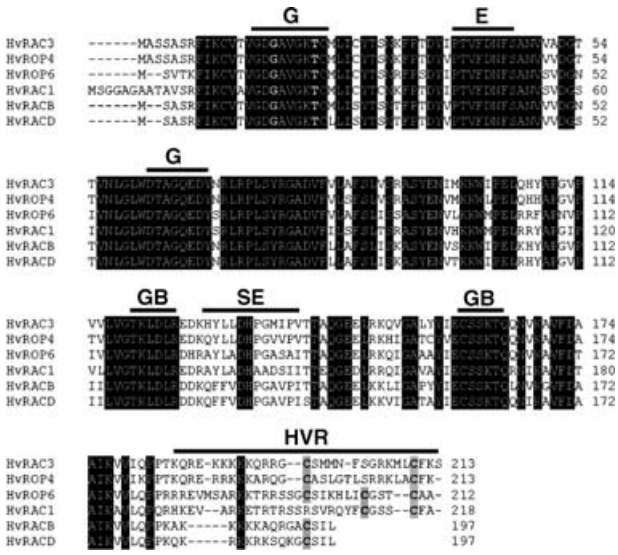

Figure 1. Amino acid alignment of barley RAC/ROP proteins.
Identical amino acids are boxed black, the potentially palmitoylated or prenylated C-terminal cysteines are shaded. Bars indicate conserved regions of RAC/ROP family proteins. The functional domains are: G, GTPase domain; E, effector loop; GB, GDP/GTP-binding domain; SE, special effector loop (insert region); and HVR, C-terminal hypervariable region. The glycine and threonine residues printed in bold (GTPase domain) were mutated to generate constitutively active or dominant negative mutants of the RAC/ROP proteins, respectively.

phylogenetic tree using the CLUSTALW program 1.83 (http://www.ebi.ac.uk/clustalw, Figure 2). Although there is a relatively high similarity between the barley RAC/ROP proteins, the phylogenetic analysis divide them into

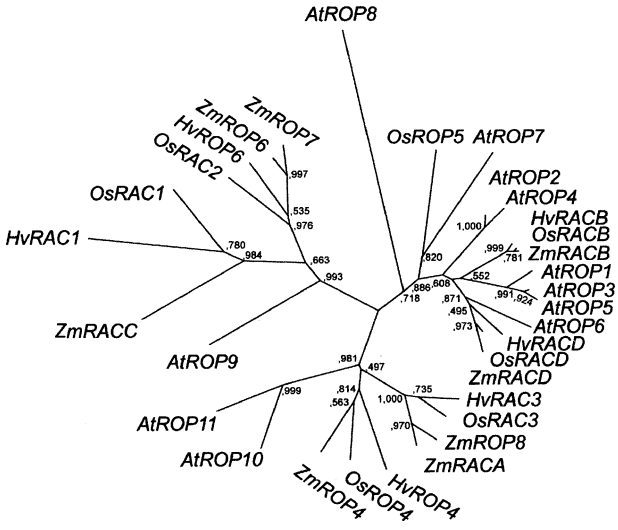

Figure 2. Unrooted phylogenetic tree of RAC/ROP proteins from barley, rice, maize and *Arabidopsis*.
The tree was constructed after bootstrap analysis using CLUSTALW 1.83 (CLUSTALW: http://www.ebi.ac.uk/clustalw). The sequence of the rice, maize and *Arabidopsis* RAC/ROP proteins were taken from GenBank. Abbreviations: At, *Arabidopsis thaliana*; Hv, *Hordeum vulgare*; Os, *Oryza sativa*; and Zm, *Zea maize*. AtROPs are named according to Yang (2002).

at least four subgroups, together with *Arabidopsis*, rice and maize RAC/ROPs. The type I RAC/ROPs, HvRACB and HvRACD of barley, rice and maize form a closely related subgroup together with six *Arabidopsis* ROPs. Figure 2 also demonstrates that the common ROP nomenclature suggested for *Arabidopsis* (Yang, 2002) is not applicable to cereal RAC/ROPs. Other cereal subgroups of RAC/ROPs contained: (i) RAC3, ROP8, RACA and ROP4; (ii) ROP6, ROP7 and RAC2; and (iii) RAC1 and RACC (Figure 2).

mRNA expression analysis of barley RAC/ROP proteins

We analysed the tissue-specific mRNA expression of barley RAC/ROP proteins during the interaction with *Bgh* by using RT-PCR. Total RNA was extracted from adaxial epidermal strips of first leaves from susceptible cv. Ingrid and the fully resistant backcross line Ingrid-*mlo5* by 24 h after inoculation (hai) with *Bgh.* All of the barley *Rac/Rops* were expressed in the mesophyll tissue (Figure 3). Five out of the six *Rac/Rops* were also expressed in the epidermal layer when compared to epidermis or mesophyll-expressed controls oxalate oxidase-like protein (*OXLP*) or ribulose bisphosphate carboxylase (*Rubisco*), respectively. *HvRac1* and *HvRac3* showed no tissue-specific expression and were detectable in mesophyll and epidermis to equal amounts. *HvRop6, HvRacD* and *HvRacB* signals appeared to be stronger with RNA from the epidermal cell layers when compared to the remaining leaf. *HvRop4* transcripts were detected only in the mesophyll and only after blotting of the RT-PCR products (Figure 3).

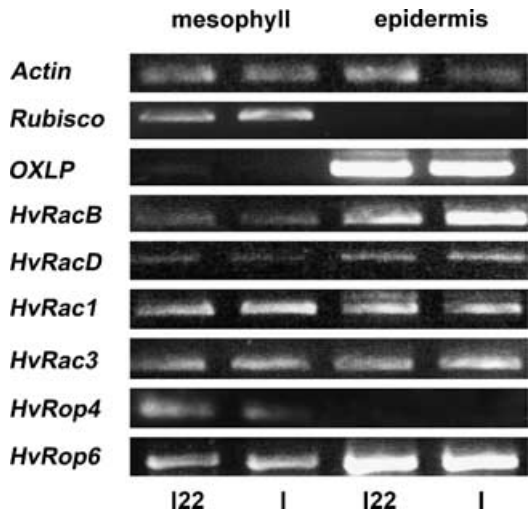

Figure 3. Expression profile of barley Rac/Rops during interaction with *B. graminis* f.sp. *hordei*.
Expression of the barley *Rac/Rop* genes in leaf epidermal and mesophyll tissue. Reverse transcriptase (RT)-PCR with RNA from cv. Ingrid (I) and Ingrid-*mlo5* (I22) at 24 hai with *Bgh*A6. For extraction of RNA, abaxial epidermal strips were separated from the mesophyll and adaxial epidermis (considered as mesophyll). *Actin* was selected as a marker for tissue unspecific gene expression. *Rubisco* was selected as positive control for gene expression in the mesophyll. *OXLP* (Wei *et al.*, 1998) was selected as positive control for gene expression in the epidermal cell layer. The expression of *HvRop4* was very weak so that we could detect a signal only after blotting of the RT-PCR product (inverted X-ray film shown).

To assess the influence of a fungal attack on the *Rac/Rop* mRNA levels, we isolated RNA from barley first leaves at 8, 14 and 24 hai and carried out RT-PCRs. Transcript levels of all epidermis-expressed *Rac/Rops* were either not upregulated remarkably or were slightly downregulated during *Bgh* attack (data not shown).

Functional analysis of RAC/ROP proteins

To further examine the role of barley RAC/ROP proteins in the interaction with *Bgh*, we conducted transient knock down and overexpression assays. We bombarded susceptible barley with tungsten particles coated with a marker gene construct and an expression vector carrying the test gene under control of the cauliflower mosaic virus 35S (CaMV35S) promoter . After 24 h, we inoculated the leaves with *Bgh* and evaluated the outcome of the interaction 40 hai by bright field and fluorescence microscopy. Penetration efficiency in controls, transformed only with GFP and the empty vector, amounted to 33% on average of all experiments. Transformation with an *HvRacB*-hairpin construct CaMV35S::*HvRacB*(antisense)-Intron-*HvRacB*(sense), which leads to expression of a dsRNA-hairpin provoking sequence-specific gene silencing of *HvRacB*, reduced the penetration efficiency (PE) of *Bgh* into epidermal cells by 21% compared to the control (Figure 4). This result corroborates earlier knock down experiments in which dsRNA-*HvRacB* produced *in vitro*, instead of the dsRNA-hairpin vector, was directly delivered into the host cells (Schultheiss *et al.*, 2002). However, the effect of the dsRNA-hairpin construct after 24 h of RNAi was somewhat weaker than the effect obtained by direct delivery of dsRNA and 4 h of silencing before inoculation.

To confirm the role of RAC/ROP proteins in powdery mildew resistance, CA mutants were generated by site-directed mutagenesis of the GTPase domain. The exchange of the GTPase intrinsic aa glycine to valine leads to the loss of the GTPase function, which should result in a CA, GTP-bound RAC/ROP protein (e.g. Kawasaki *et al.*, 1999; see Figure 1, bold G in GTPase domain). Overexpression of the CA mutant of HvRACB (HvRACB-V15) consistently resulted in increased susceptibility of wild-type barley. In six independent experiments, we observed a significant average increase of fungal PE by 45% (Figure 4). In contrast, overexpression of wild-type HvRACB or a dominant negative HvRACB-N20 mutant did not yield significant effects on susceptibility (Figure 4).

As *HvRacB*-dsRNA interference was inefficient in barley genotypes bearing the *ror1-2* mutant allele (Schultheiss *et al.*, 2002), we tested the effect of CA HvRACB-V15 in moderately susceptible *mlo5 ror1-2* and completely resistant

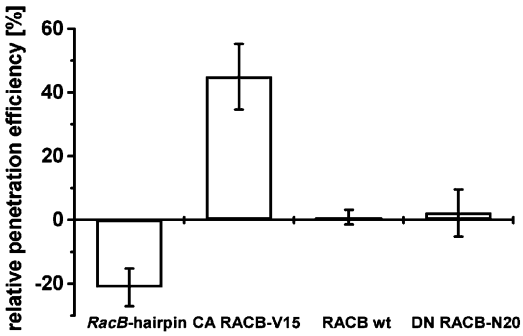

Figure 4. Impact of HvRACB protein overexpression and RNA interference on PE of *Bgh*.
Columns indicate the influence of overexpressed *HvRacB* constructs on the relative PE of *Bgh* in barley epidermal cells (susceptible cv. Ingrid, genotype *Mlo Ror1*). Error bars show standard errors. Columns represent the average deviation of PE from controls. Results were confirmed in at least five independent experiments. Negative and positive values of relative PE indicate reduced and enhanced PE compared to the controls (set as 0). Controls were transformed with the marker gene and the empty overexpression vector. Transformation of barley cells with RNAi inducing *HvRacB*-hairpin construct (*HvRacB*(as)-intron-*HvRacB*(s)) significantly reduced the PE of *Bgh* into barley cells (Students' *t*-test, $P = 0.028$), whereas overexpression of CA HvRACB-V15 enhanced the PE ($P < 0.001$). Overexpression of wild-type (wt) HvRACB or the dominant negative HvRACB-N20 did not change the PE compared to the control ($P = 0.607$ or $P = 0.862$, respectively). Absolute PE on controls was 33.2% in average.

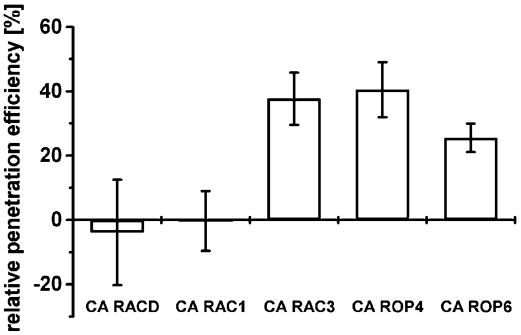

Figure 5. Impact of overexpression of five different CA RAC/ROP proteins on PE of *Bgh*.
Columns indicate the influence of overexpressed RAC/ROP proteins on the relative PE of *Bgh* in barley epidermal cells (cv. Ingrid). Error bars show SEs. Columns represent the average deviation of PE from controls. Significant results were confirmed in at least five independent experiments. Overexpression of the constitutive active forms of HvRAC3, HvROP4 and HvROP6 led to an enhanced PE of *Bgh* into barley epidermal cells ($P < 0.002$, $P = 0.009$ or $P = 0.011$, respectively), whereas the overexpression of CA HvRACD-V15 and CA HvRAC1-V23 did not change the PE of *Bgh* compared to control ($P = 0.560$ or $P = 0.878$, respectively). Absolute PE in controls was 31.2% on average.

mlo5 Ror1 mutant genotypes. In both genotypes, overexpression of CA HvRACB-V15 did not enhance susceptibility, contrasting the clear effect in wild-type barley (Table 1).

The second barley type I RAC/ROP protein HvRACD, which is 90% identical to HvRACB, did not exhibit any significant effect on barley accessibility to *Bgh*, when overexpressed in its CA form (Figure 5). This underscores RAC/ROP-specificity of the CA HvRACB overexpression effect.

Overexpression experiments of type II RAC/ROP are summarised in Figure 5. The constitutive active forms of type II RAC/ROPs HvRAC3-V17, HvROP4-V17 and HvROP6-V15 enhanced significantly the accessibility of epidermal cells to *Bgh* in each at least five independent experiments. Overexpression of HvRAC3-V17 enhanced PE by 38%, overexpression of HvROP4-V17 that is closely related to HvRAC3 (86% aa identity) enhanced PE by 40% and overexpression of HvROP6-V15 conferred a 26% higher PE of *Bgh*. In contrast, overexpression of the constitutively active HvRAC1-V23 had no influence on PE (Figure 5). None of the type II RAC/ROPs altered the number of living GFP-expressing cells (*Bgh*-attacked or non-attacked), which makes it unlikely that they are involved in *Bgh*-induced cell death reaction (data not shown).

Table 1 The overexpression effect of CA HvRACB-V15 on PE of *B. graminis* f.sp. *hordei* is influenced by the host genotype

Genotype	n[a]	Interaction sites	Relative PE[b]	P-value[c]
Mlo Ror1	6	804	+45%	0.0007
mlo5 Ror1	2	194	±0%[d]	0.5
mlo5 ror1-2	4	444	−13%	0.3

[a]number of independent experiments.
[b]average PE [%] compared to controls expressing GFP and empty vector (set as 0%).
[c]*P*-value, *t*-test.
[d]PE was zero in this resistant genotype both in control and CA HvRACB-V15 overexpression.

Role of the C-terminal localisation motifs

To identify the subcellular site of CA RAC/ROP activity, we performed intracellular localisation of N-terminal GFP-fusion proteins of CA HvRAC/ROPs. The fusion proteins were expressed in epidermal cells after biolistic transformation of leaf segments. The co-transformation with the red fluorescent protein DsRed under control of CaMV35S served as control for a protein that freely moves in the cytoplasm and into the nucleus (pe35AscloptRed, DsRed-C1; Dietrich and Maiss, 2002). We observed the localisation of DsRed and GFP fusion proteins 48–96 h after transformation by confocal laser scanning microscopy (CLSM).

Expression of the GFP protein alone resulted in evenly green fluorescence of the entire cytoplasm and nucleoplasm (Figure 6a). By comparing the GFP localisation with the distribution of DsRed in short epidermal cells, we found a nearly identical localisation of both fluorescent proteins. Similar localisation is visible in overlay pictures (Figure 6a merge) where pixels reflecting co-localised GFP and DsRed appear yellow.

Subcellular localisation of the type I RAC/ROP GFP:HvRACB-V15 is shown in Figure 6(b). Localisation of HvRACB-V15 was complex. We always observed GFP:HvRACB-V15 fluorescence at the PM. However, evaluation of the subcellular

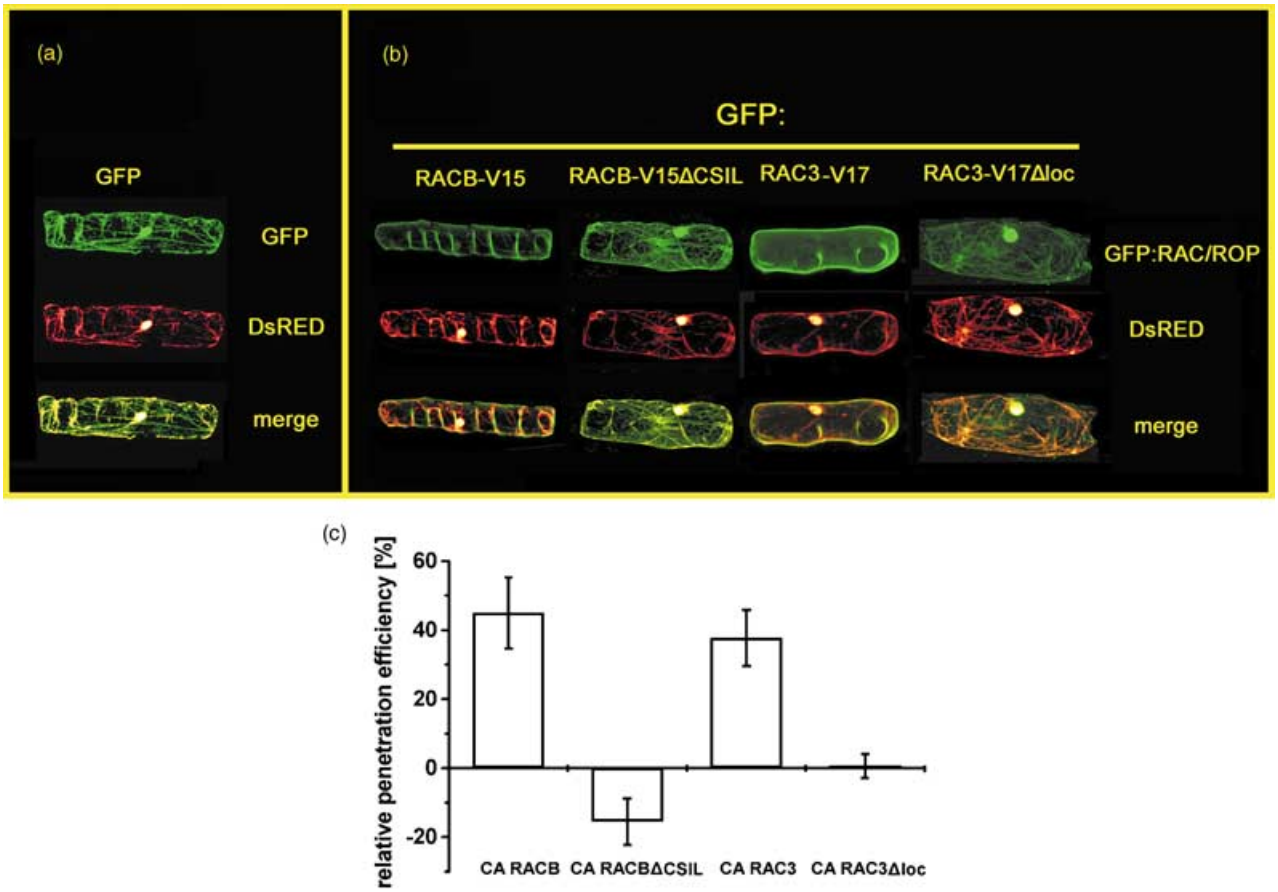

Figure 6. The C-terminal localisation motifs of HvRACB and HvRAC3 are essential for both protein localisation and function in susceptibility.
(a) Barley epidermal cells of leaf segments were transiently transformed with GFP:RAC/ROP (green) and DsRed (red). DsRed was co-transformed as nuclear and cytoplasmic localised control. Overlays of the GFP and the DsRed channels led to yellow colour if GFP and DsRed were co-localised in one pixel. Pictures represent whole cell projection of 20–30 optical cuts of 3 μm increments. Fluorescence patterns of GFP and DsRed after transformation of barley epidermal cells are similar. Both proteins are localised in the cytoplasm and the nucleus.
(b) Barley epidermal cells expressing DsRed and CA GFP:RAC/ROPs. Barley epidermal cells were transformed with GFP-RAC/ROP constructs (green) and DsRed (red). GFP:RACB-V15 and GFP:RAC3-V17 are located at the PM. The GFP-fusion mutants lacking the C-terminal localisation motifs, GFP-RACB-V15ΔCSIL and GFP-RAC3-V17Δloc, were only visible in the cytoplasm and within the nucleus.
(c) Impact of overexpression of HvRACB-V15ΔCSIL and HvRAC3-V17Δloc, lacking the C-terminal localisation motifs, on fungal PE. Each column represents the average of deviation of PE from control. Error bars show SEs. Overexpression of HvRACB-V15 and HvRAC3-V17 led to an enhancement of PE of *Bgh* by 45% ($P < 0.001$) and 38% ($P < 0.002$), respectively (see Figures 4 and 5). The overexpression of the respective mutants HvRACB-V15ΔCSIL and HvRAC3-V17Δloc lacking the C-terminal localisation motifs did not change the PE compared to control ($P = 0.093$ or $P = 0.919$, respectively).

localisation during the first 48 h after transformation also revealed an equally strong distribution of the GFP:HvRACB-V15 fusion protein throughout cytoplasm and nucleus (not shown). Surprisingly, evaluation at later time points after transformation, e.g. 72 h, revealed contrasting subcellular patterns of GFP:HvRACB-V15 distribution. In approximately 50% of the cells, cytoplasmic and nuclear green fluorescence completely disappeared, while the accumulation of the fusion protein at the PM was stable. The merge of confocal GFP- and DsRed-channel whole-cell projections of 20–30 optical cuts of 3 μm increments delivered epidermal cells with a green periphery and a red inside suggesting PM localisation of GFP:HvRACB-V15 (Figure 6b). In the remaining cells, strong GFP:HvRACB-V15 derived fluorescence in nucleus and cytoplasm decomposed, but was still detectable suggesting that the level of GFP:HvRACB-V15 expression might be responsible for specificity of the targeting process. Also, for strong GFP:HvRACB-V15 expressing cells, we could not rule out that HvRACB-V15 was partly attached to endomembranes, e.g. the endoplasmatic reticulum and the tonoplast (not shown). However, when we introduced a stop codon in front of the terminal CAAX motif of RACB (aa sequence: CSIL; Figure 1), the mutant GFP:HvRACB-V15ΔCSIL accumulated within the cytoplasm and the nucleus and its localisation could never be distinguished from that of DsRed (Figure 6b). As we never observed any GFP:HvRACB-V15ΔCSIL-derived fluorescence at the PM, we concluded that the C-terminal CSIL prenylation motif is required for PM-localisation of

HvRACB-V15. Expression of an N-terminal GFP-fusion of the second type I RAC/ROP, RACD-V15, resulted in distribution of green fluorescence mainly at the PM and in the nucleus whereas the cytoplasm was hardly labelled (data not shown). Expression of DsRed together with GFP:HvRAC3-V17 led to the normal cytoplasmic and nuclear distribution of the red fluorescence. In contrast, the GFP-derived fluorescence was only visible in the cell periphery at the PM (Figure 6b). The nucleus, which shined bright in the red channel, was hardly or not detectable in the GFP channel. The same was true for all other CA type II GFP:RAC/ROPs. Only GFP:ROP4-V17 was found to some extent in the nucleus but not in the cytoplasm (not shown). Together, barley type II RAC/ROPs were more strictly associated with the PM than were type I RAC/ROPs. None of the type II RAC/ROPs described here possess a typical CAAX motif. Instead, the HVR of these proteins contains cysteine residues that might be palmitoylated. Mutants lacking the putative localisation domain were constructed and expressed in barley epidermal cells. We constructed a GFP:HvRAC3-V17 mutant, which possessed a stop codon in front of the cysteine residue in aa position 198 (GFP:HvRAC3-V17Δloc). This results in an open-reading frame lacking both cysteine residues possibly responsible for PM localisation of the full-length HvRAC3. Localisation of the truncated GFP:HvRAC3-V17Δloc within epidermal cells differed clearly from that of the full-length GFP:HvRAC3-V17 (Figure 6b). The green fluorescence was dispersed throughout the cell instead of being restricted to the cell periphery. Nevertheless, there was a difference between the distribution of the fusion and the DsRed because GFP:HvRAC3-V17Δloc was most strongly detected in the nucleus.

To study whether the membrane attachment is important for signalling in the barley–powdery mildew fungus interaction, we performed overexpression analysis using the truncated HvRACB-V15ΔCSIL and HvRAC3-V17Δloc constructs. As already shown, overexpression of both full-length CA HvRACB and full-length CA HvRAC3 conferred enhanced susceptibility to barley epidermal cells (see Figures 4 and 5). In contrast, overexpression of the de-localised mutants HvRACB-V15ΔCSIL and HvRAC3-V17Δloc did not significantly change the basal resistance status of barley (Figure 6c). We rather observed a tendency for a resistance-inducing effect of HvRACB-V15ΔCSIL (Figure 6c), demonstrating that the C-terminal cysteine residues of HvRACB and HvRAC3 are required for both the specific localisation at the PM and the accurate function in barley powdery mildew fungus interaction.

Discussion

Small GTPases of the RAC/ROP family play important roles in cell growth and cell morphogenesis, and are involved in host–pathogen interactions (Gu *et al.*, 2003; Mathur and Hülskamp, 2002; Paduch *et al.*, 2001; Valster *et al.*, 2000; Yang, 2002; Zheng and Yang, 2000). We identified six, for the most part, formerly unknown barley RAC/ROP family proteins and examined their function and subcellular localisation in the barley–powdery mildew fungus interaction. Our data suggest that HvRACB, HvRAC3 and HvROP6 activate signalling cascades facilitating invasion of *Bgh* into barley epidermal cells and therefore are potential susceptibility factors. These proteins belong to different subgroups of the plant RAC/ROP family. This suggests that they function through different mechanisms.

All of the identified barley RAC/ROPs shared typical domains, such as GDP/GTP-binding and GTPase domains and the effector loop that is thought to be responsible for interaction with regulatory enzymes like GTPase-activating proteins (Moodie *et al.*, 1995). There are only two amino acid stretches lacking high similarities within the protein family. The first region is the special effector loop (or insert region) that is thought to be responsible for downstream signalling (Valster *et al.*, 2000). This insert region is likely not the only downstream signalling domain of barley RAC/ROPs because HvRACB and HvRACD, although functionally distinct, are identical within this domain. Moreover, *Arabidopsis* type I RAC/ROP proteins, which have different functions (Bischoff *et al.*, 2000; Cheung *et al.*, 2003), are very similar in the insert region (Valster *et al.*, 2000; Winge *et al.*, 1997). The second domain of the RAC/ROP proteins without high similarities is the HVR at the C-terminus, thought to be required for subcellular targeting (Bischoff *et al.*, 2000; Lavy *et al.*, 2002). Within this domain, the basic character of the polybasic region is conserved but not the amino acids themselves. Also, the post-translationally lipid-linked cysteine residues are largely conserved, whereas the surrounding sequences are variable.

By comparing the barley RAC/ROP proteins with those from *Arabidopsis*, maize and rice, several distinct subgroups can be formed (Figure 2). The construction of a phylogenetic tree showed that the six barley homologues can be classified into the subgroups formed together with rice and maize proteins. As in rice and maize, not more than each 7 or 8 members of RAC/ROP proteins were identified so far, we speculate that the barley RAC/ROP family is not much larger than six members, although *Arabidopsis* contains 11 homologues (Vernoud *et al.*, 2003). Interestingly, *Arabidopsis* RAC/ROPs show partly higher similarities to cereal RAC/ROPs than they show to each other, likely indicating functional conservation in all higher plants.

The comparison of the barley RAC/ROPs with known proteins from other species and the construction of the phylogenetic tree generated first hints on barley RAC/ROP functions. Some members of the closely related type I subgroup (AtROP1–AtROP6, HvRACB, HvRACD) are known to be regulators of the actin cytoskeleton (Fu *et al.*, 2001; Gu *et al.*, 2003) that is rearranged during the attack of powdery

mildew fungi on barley (Kobayashi *et al.*, 1997; K. Opalsky and R. Hückelhoven unpublished results).

Transient knock down of HvRACB via dsRNA interference led to enhanced resistance against the powdery mildew fungus. This effect was only detectable in wild-type barley but not in mutant genotypes *mlo5 ror1* and *Mlo ror1* (Schultheiss *et al.*, 2002 and unpublished results; unpublished *Mlo ror1* genotype kindly provided by Nicholas Collins, Sainsbury Laboratory John Innes Centre, Norwich, UK). This suggests that HvRACB is a susceptibility factor involved in the ROR1-dependent defence regulation. In the present study, we confirmed the role of HvRACB as a potential susceptibility factor in the interaction of barley with *Bgh*. However, overexpression of HvRACB-V15 did not break the strong resistance of *mlo5*/*Ror1* or the moderate resistance of *mlo5*/*ror1* mutant genotypes (Table 1). Together, we speculate that HvRACB functions as an antagonist of functional ROR1.

To demonstrate that RACB can promote susceptibility, it was necessary to overexpress the CA mutant because wild-type HvRACB had no effect on the interaction outcome (Figure 4). This may not surprise as RAC/ROP proteins are signal transduction proteins that normally need to be activated by extracellular stimuli. Interestingly, overexpression of the presumably dominant negative mutant HvRACB-N20 did not induce resistance contrasting the results for *HvRacB*-dsRNA interference (Figure 4). This might be explained by the fact that low levels of active endogenous RACB are sufficient to function in susceptibility so that competition by the dominant negative mutant is not strong enough to eliminate RACB function. In contrast, knock down of RACB might be able to reduce the abundance of RACB proteins sufficiently to interfere with its function in susceptibility. Alternatively, HvRACB-N20 could interact with RACB up- and downstream interacting components as well as inhibitors such as plant RHO-guanine nucleotide dissociation inhibitors leading to abolishment of effects. To further examine HvRACB function, we analysed the intracellular localisation of the CA HvRACB. HvRACB posses a polybasic domain followed by a typical C-terminal CAAX-box motif (CSIL). The final lysine residue is crucial for recognition by geranylgeranyltransferases instead of farnesyltransferases. Hence, the combination of these structures should result in geranylgeranylation (Caldelari *et al.*, 2001) and possible attachment to membranes (Thompson and Okuyama, 2000). Some type I RAC/ROP proteins were shown to be localised at the PM (Fu *et al.*, 2002; Nakanomyo *et al.*, 2002), whereas others are distributed all over the cell (Bischoff *et al.*, 2000). As expected for a CA RAC/ROP protein, we found GFP:HvRACB-V15 fluorescence not only at the PM but also a nuclear and cytoplasmic localisation of the fusion protein, albeit to a lesser extent (not shown). Bright fluorescing cells, which expressed high amounts of the fusion protein, showed a more intensive cytoplasmic and nuclear distribution of the fusion protein. Cells containing only small amounts of the GFP:HvRACB-V15 fusion always concentrated the fluorescence at the PM, especially when incubated for 3 or 4 days after transient transformation. We speculate that the membrane transport mechanism of HvRACB is not effective enough to target large amounts of proteins to the PM. In contrast to GFP:HvRACB-V15, the GFP:HvRACB-V15ΔCSIL fusion protein accumulated exclusively in the cytoplasm and in the nucleus (Figure 6b). In addition, overexpression of HvRACB-V15ΔCSIL did not result in enhanced susceptibility of barley to *Bgh*. We conclude that both the PM localisation and HvRACB function in host cell accessibility depend on the C-terminal CAAX-box motif. The induction of plant susceptibility mediated by CA HvRACB is a specific feature of this particular type I RAC/ROP protein, which is clearly demonstrated by the fact that overexpression of CA HvRACD-V15 had no effect, although HvRACD shows 90% aa identity to HvRACB (Figure 1).

It is known that the HvRACB-homologous type I RAC/ROP proteins influence the assembly of the cytoskeleton by various mechanisms. First, human Rho-GTPases re-modulate the actin cytoskeleton by regulating the activity of serine/threonine kinases such as p65PAK or p160ROCK (Hall, 1998). These kinases regulate the activity of actin modifying enzymes such as actin depolymerisation factors (ADFs; Maekawa *et al.*, 1999). The same pathway may be conserved in plants, as NtRAC1, a HvRACB homologous type I RAC/ROP protein from *Nicotiana tabacum*, mediates pollen tube growth by regulating the activity status of ADF1 (Chen *et al.*, 2003). The actin cytoskeleton can also be modified by RAC/ROP proteins via the phosphoinositol pathway (Kost *et al.*, 1999). Another RAC/ROP-dependent mechanism during pollen tube and root hair growth is the formation of a tip-focused Ca^{2+} accumulation that is needed for polar growth (Camacho and Malho, 2003; Fu *et al.*, 2001; Molendijk *et al.*, 2001). Calcium ions are also able to regulate the activity of the MLO protein via calmodulin (Kim *et al.*, 2002), which links back to susceptibility in the barley–*Bgh* interaction. It is likely that HvRACB is involved in at least some of these processes regulated by type I RAC/ROPs. The regulation of cell polarity might be of special interest, because invagination of the host PM during haustoria establishment within barley epidermal cells may represent a kind of 'inverted' tip growth induced by *Bgh* possibly via HvRACB. Alternatively, CA HvRACB such as other CA type I RAC/ROPs could induce isotropic cell growth, which antagonizes polar cytoskeleton focusing to site of attempted penetration crucial for penetration resistance (Kobayashi *et al.*, 1997).

With the exception of HvRAC1, all type II RAC/ROPs described here influence the barley–*Bgh* interaction. Overexpression of barley CA HvRAC3, HvROP4 and HvROP6 type II RAC/ROPs resulted in enhanced accessibility

(Figure 5). As HvROP4 mRNA was not detectable in the epidermis of barley leaves (Figure 3), the effect of CA HvROP4-V17 likely did not resemble an intrinsic signalling pathway. We speculate that the CA HvROP4 effect mimics the overexpression of the closely related HvRAC3 (Figure 5).

The molecular mechanism of enhanced cell accessibility and plant susceptibility by overexpression of HvRAC3 and HvROP6 is unclear. There is little known about function of type II RAC/ROPs in plants. The type II RAC/ROP OsRAC1 was shown to be involved in ROI production, cell death and disease resistance to *M. grisea* (Kawasaki *et al.*, 1999; Ono *et al.*, 2001). Kawasaki *et al.* (1999) provided evidence that OsRAC1 could be a regulator of an NADPH oxidase, as known for RAC2 and RAC1 from mammalian systems (Bokoch and Diebold, 2002). Like RAC/ROP proteins (Li *et al.*, 1999), the NADPH oxidase is involved in ROI production and tip-focused Ca^{2+} accumulation during polar root hair growth (Foreman *et al.*, 2003). The establishment of *Bgh*-haustoria in barley epidermal cells requires polar plant membrane growth, and during this process, generation of superoxide at the side of penetration has been detected. Interestingly, such a superoxide burst was not detectable in resistant barley preventing haustorium establishment such as *mlo5*-genotypes. Thus, appearance of $^{\cdot}O_2^-$ that is possibly produced by NADPH oxidase is linked to accessibility to *Bgh* (Hückelhoven and Kogel, 1998). ROIs from NADPH oxidase activity are required for cell growth by regulating different processes, including calcium uptake, which might feed back NADPH oxidase activity that is Ca^{2+} stimulated (Keller *et al.*, 1998; Sagi and Fluhr, 2001). Although participation of the NADPH oxidase in barley–*Bgh* interaction seems possible, we do not exclude other explanations, e.g. involvement of phytohormones like ABA as described for AtROP10 (Zheng *et al.*, 2002).

Surprisingly, overexpression of HvRAC1-V23 did not influence the resistance status of barley to *Bgh* (Figure 4). This appears in clear contrast to the effect of CA OsRAC1 on rice blast resistance (Kawasaki *et al.*, 1999; Ono *et al.*, 2001). Possibly, HvRAC1 represents a part of a defence machinery that is only effective against hemibiotrophic fungi such as *M. grisea*, but is not involved in repelling the biotrophic *Bgh*. Moreover, our experiments with the CA RAC/ROPs were carried out using susceptible barley lines whereas Ono *et al.* (2001) linked OsRAC1 with *R*-gene-mediated resistance. Finally, the similarity of OsRAC1, ZmRACC and HvRAC1 is relatively low compared to the similarities with in other subgroups (Figure 2). This may indicate some differences in function of barley and rice RAC1.

The expression of fusion proteins composed of N-terminal GFP and the CA type II RAC/ROPs resulted in a clear PM-associated green fluorescence (Figure 6b). The transport of barley type II RAC/ROPs to the PM corresponds with data obtained from *Arabidopsis* and maize. As previously shown, the membrane-targeting signal in the C-terminal hypervariable region of type II RAC/ROPs does not depend on a functional CAAX-box (Ivanchenko *et al.*, 2000; Lavy *et al.*, 2002). Especially the maize PM-located ZmROP6 exhibits very high similarities with HvROP6, even in the hypervariable region (22 of the last 31 aa are identical). Ivanchenko *et al.* (2000) showed that both cysteine residues (cys199 and cys206) upstream of the rudimentary CAAX-box are important for membrane association of ZmROP6, whereas the rudimentary CAAX-box (CAA) cysteine (cys210) could be mutated without changes in localisation. All barley type II RAC/ROPs possess only an incomplete or non-functional C-terminal CAAX-box, such as CAA (HvROP6) or CFKS (HvRAC3), but contain additional upstream cysteine residues, which could be palmitoylated. There is not much known about the function of type II RAC/ROPs, but it seems that a permanent PM association is required for their function. The strict and essential PM association can be seen as an additional hint that the downstream targets of the type II RAC/ROPs are also bound to the PM. This speculation is supported by the fact that CA HvRAC3 became non-functional when the C-terminal localisation domain was cut off (HvRAC3-V17Δloc, Figure 6c).

Finally, the question arises why host RAC/ROPs could act as susceptibility factors? It is not likely that the plant maintained *Rac*/*Rop* genes through evolution because of their function in helping fungal parasites to invade. The small G-proteins are involved in many signalling cascades, required for normal plant development. Some pathogens may trigger these pathways to cause a plant reaction allowing the pathogen to infect. Especially, the cytoskeleton is a primary target for animal pathogens trying to infect host cells (for review, see Boquet and Lemichez, 2003). The cytoskeleton can be either modified directly by toxins or cytoskeleton modulating proteins become deregulated. In the latter case, the targets of pathogen effectors are often small G-proteins of the Rho family (Boquet and Lemichez, 2003). Bacterial plant pathogens were shown to introduce effectors into the plant cell acting as pathogenicity factors (Abramovitch *et al.*, 2003). The *Pseudomonas* avirulence protein AvrPphB is a homologue of the *Yersinia* effector YopT that works as a protease cleaving small G-proteins (Shao *et al.*, 2002). It is imaginable that *Bgh* also effects the host RAC/ROP-signalling pathways to more easily infect the plant cell. For the future, it will be important to isolate the up- and downstream effectors of the different RAC/ROP proteins to uncover the signalling network, modulating cell accessibility to pathogen invasion.

Experimental procedures

Plant materials, pathogen and inoculation

The barley (*H. vulgare* L.) lines Ingrid and the backcross line BCIngrid-*mlo5* were obtained from Lisa Munk (Royal Veterinary

and Agricultural University, Copenhagen, Denmark). Their generation was described previously by Kølster *et al.* (1986). The mutant *mlo5 ror1-2* was obtained from Paul Schulze-Lefert (Max-Planck-Institute for plant breeding research, Cologne, Germany). Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} sec^{-1} photon flux density). The barley powdery mildew fungus, *Blumeria graminis* (DC) Speer f.sp. *hordei* Em. Marchal, race A6 (Wiberg, 1974) was inoculated onto barley primary leaves to give a density of 150 conidia mm^{-2}. *Bgh* was maintained on barley cv. Siri under the same conditions.

Isolation of barley Rac/Rop family cDNAs, cloning and sequencing

We isolated cDNA fragments by the use of one-step RT-PCR kits (Qiagen, Hilden, Germany) and a complex RNA pool described previously by Schultheiss *et al.* (2002). Primers were designed using GenBank or EST database information for specific barley EST sequences. For primer sequence and accessions, see Table 2. Details of the isolation of *HvRacB* were described by Schultheiss *et al.* (2002). For first sequence information of *HvRac1*, we carried out RT-PCR with a primer derived from the conserved GTPase-region and a Primer derived from a barley EST (BM097964), which only contain 3′ UTR sequence information. To access the complete 5′-region, we performed RACE (Schultheiss *et al.*, 2002).

cDNAs were isolated from gels and cloned into pGEM-T-Vector (Promega, Mannheim, Germany). cDNAs were sequenced from plasmids by use of the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham, Freiburg, Germany) and analysed for similarities in the GenBank database using the BLAST algorithm (Altschul *et al.*, 1997).

For transient transformation assays complete open-reading frames of the *Rac/Rop* cDNAs were subcloned into pGY1 (Schweizer *et al.*, 1999) that contains a 540-bp fragment of the CaMV35S promoter and terminator separated by a multiple cloning site. The constructs were cloned using the restriction sites linked to primers mentioned in Table 2. All constructs were checked by sequencing (see above).

We constructed a dsRNA expression vector by use of pJP26 (provided by Robert Dudler, Institute of Plant Biology, University of Zurich, Switzerland) containing the CaMV35S promoter followed by an intron in the multiple cloning site suitable for cloning PCR products with adapted restriction sites to give an antisense-intron-sense construct.

To generate the constitutively active and dominant negative RAC/ROP mutants, we used the Transformer™ Mutagenesis Kit (Clontech, Heidelberg, Germany) following manufacturers instructions. For each RAC/ROP, the glycine corresponding to G12 of human Rac1 was substituted by a valine, leading to the destruction of the GTPase domain, which results in a CA small G-protein (primer: see Table 2). The dominant negative HvRACB-N20 was obtained in the same way by exchange of a threonine (T20) for an asparagine (primer: see Table 2; Kawasaki *et al.*, 1999).

Construction of GFP:HvRAC/ROP-fusions

For expression of GFP:HvRAC/ROP fusion proteins, cDNAs of GFP (GFPemd-b in pGFP; Schweizer *et al.*, 1999) were amplified from plasmids by PCR using primers with attached *Bam*HI restriction sites in all three frames under elimination of the GFP stop codon (GFP-5′ primer: 5′-GGATCCATGGTGAGCAAGGGCGAG-3′; GFP-3′ primer (frame 1): 5′-GGATCCTTGTACAGCTCGTCCAT-3′; GFP-3′ primer (frame 2): 5′-GGATCCCTTGTACAGCTCGTCCAT-3′; GFP-3′ primer (frame 3): 5′-GGATCCCCTTGTACAGCTCGTCCAT-3′).

Table 2 Accession numbers and oligo-DNA primers used for RT-PCR, RACE and cDNA mutagenesis of barley *Rac/Rops*

HvRac/Rop (Accession number)	Primer	Sequence	EST Accession number[a]
RacB (AJ344223)	RacBs	5′-GGATCCGATGAGCGCGTCCAGGTT-3′	AJ290420, AF250327, BF260616
	RacBas	5′-GTCGACCTTCGCCCTTGTTCTTTGTC-3′	
	RacB-RACE	5′-GGATCCGATGAGCGCGTCCAGGTT-3′	
	CA RacB	5′-ACCGTGGGGGACGTCGCCGTCGGCAAGAC-3′	
	DN RacB	5′-GCGCCGTCGGCAAGAACTGCATGCTCATCT-3′	
	RacBΔCSIL	5′-GTCGACTCAAGCCCCCCTCTGCGCCTTTTTC-3′	
RacD (AJ439334)	RacDs	5′-GGATCCATGAGCGCATCTCGGTTC-3′	AV943381
	RacDas	5′-GTCGACGCGAGACACTGCAAAACAAA-3′	
	CA RacD	Identical to CA RacB	
Rac1 (AJ518933)	Rac1s	5′-GGATCCGCTGGAGAGGAGAGGAGAGG-3′	BM097964
	Rac1as	5′-GTCGACCCCATTTGGAGAACAACCAC-3′	
	GTPase	5′-CAGGTTCATCAAGTGCGT-3′	
	Rac1RACE	5′-GTCGGTGGGGAGCTTGTTGCAGGTGTA-3′	
	CA Rac1	5′-GCCGTGGGGGACGTCGCCGTGGGCAAGAC-3′	
Rac3 (AJ518932)	Rac3s	5′-GGATCCCGCGCGGCGAGCCATG-3′	BM816965
	Rac3as	5′-GTCGACGCAAGGAACCTTCTCTTTCATC-3′	
	CA Rac3	Identical to CA RacB	
	Rac3Δloc	5-CGTCGGGGATGATCAATGATGAAC-3′	
Rop4 (AJ439335)	Rop4s	5′-GGATCCTTCTCGTCCATTTAGCCGGC-3′	AV924458, AV836932
	Rop4as	5′-GTCGACTGATCACTTGAAGCATGCCAG-3′	
	CA Rop4	Identical to CA RacB	
Rop6 (AJ439333)	Rop6s	5′-GGATCCGTGGAGGCGCGGCGAGA-3′	BI957947, BM099394
	Rop6as	5′-CTGCAGCCATGCTTCATCTCCATAGTCA-3′	
	CA Rop6	Identical to CA RacB	

[a]ESTs served to create tentative consensus sequences for primer design.

GFP-PCR products were cloned in frame into the appropriate pGY1-RAC/ROP-construct (linearised with *Bam*HI). Orientation of the inserts was checked by PCR and the fusion constructs were sequenced.

RNA extraction and reverse transcription-polymerase chain reaction

Isolation of epidermal tissue for expression analysis was performed as described previously by Schultheiss *et al.* (2002).

Total RNA was extracted from 8–10 primary leaf segments (5 cm long) or from 20 epidermal strips using RNA extraction buffer (PEQLAB, Erlangen, Germany) according to the manufacturer's instructions. The OneStep RT-PCR kit (Qiagen, Hilden, Germany) was used for semi-quantitative RT-PCR following manufacturers instructions. To estimate template amounts the RT-PCR was stopped during the exponential phase of amplification, maintaining initial differences in target transcript amounts. PCR products were separated in agarose gels.

Primers. For semiquantitative RT-PCR of *Rac/Rop* family member's mRNA, we used the primers mentioned (Table 2). For *OXLP* cDNA fragment (506 bp; GenBank Accession X93171): 5′-GGCCGACATGCATTCACCAG-3′ (5′ primer) and 5′-CATCTGATATTGCTGGGTCTG-3′ (3′ primer); *Rubisco* cDNA fragment (607 bp; GenBank Accession U43493): 5′-CCCTGTCTTACCTTCCACCA-3′ (5′ primer) and 5′-GCGTGCAAAGATGTTTCTCAT-3′ (3′ primer); actin-like cDNA fragment (758 bp; GenBank Accession AJ234400): 5′-CTGTAGGAAATGGCTGACGG-3′ (5′ primer) and 5′-TCGGATCACCTGACCCAT-3′ (3′ primer).

Transient transformation and evaluation of fungal development

A transient transformation protocol, originally developed for wheat to assess gene function in the interaction with powdery mildew, was used to deliver overexpression constructs of the RAC/ROP proteins into epidermal cells of barley leaf segments as described by Schweizer *et al.* (1999) and Hückelhoven *et al.* (2003).

Barley leaf segments were bombarded with coated particles 24 h before inoculation with *Bgh*, race A6. Interaction outcome was judged 40 hai by fluorescence and light microscopy. For each individual experiment, at least 100 interaction sites were evaluated. Transformed GFP expressing cells were identified under blue light excitation. Penetration efficiency of *Bgh* was judged by the frequency by which *Bgh* was able to establish haustoria in transformed cells (Hückelhoven *et al.*, 2003). Surface structures of *Bgh* were detected by light microscopy or by fluorescence staining of the fungus with 0.3% calcofluor (w/v in 50 mM TRIS, pH 9) for 30 sec. Deviation of PE [%] was used as a measure for susceptibility of cells that expressed GFP and a test-gene (pGY1-RAC/ROP) compared to those transformed with GFP and empty vector (pGY1). PE [%] was calculated as number of penetrated cells divided by total number of attacked cells multiplied by 100. The deviation of PE was calculated as PE in cells expressing the testgene divided by PE of controls minus 1 and multiplied by 100.

Localisation of RAC/ROP proteins with CLSM

For localisation experiments, barley leaves were transformed with pGY1-GFP:HvRAC/ROP (1 μg) and pDsRed containing *DsRed* under control of CAMV35S (0.5 μg, pe35AscloptRed; Dietrich and Maiss, 2002) as described above.

The localisation of the GFP:HvRAC/ROP fusion protein was detected by CLSM (Leica TCS SP2, Leica Microsystems, Bensheim, Germany) 24–96 h after transformation. GFP:HvRAC/ROP was excitated with by a 488-nm laser line and detected at 505–530 nm. DsRed was excitated by 543 nm laser line and detected at 580–650 nm.

All new materials will be delivered freely to the research community. Economically relevant use is restricted by patent WO03020939.

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Accession numbers: *HvRacB*: AJ344223; *HvRacD*: AJ439334; *HvRac1*: AJ518933; *HvRac3*: AJ518932; *HvRop4*: AJ439335; *HvRop6*: AJ439333.

Special introduction to chapters 9-11:

The following three chapters deal with the role of the cell death regulator protein BAX Inhibitor 1 (BAX: BCL-2 associated X protein; BCL-2: B-cell lymphoma protein-2) in barley susceptibility to *Bgh*. The corresponding barley mRNA was isolated from leaf tissue and found to accumulate after powdery mildew attack and to transiently disappear after root treatment with DCINA. Importantly, over-expression of barley BAX Inhibitor 1 weakened background resistance of barley to *Bgh*, broke *mlo*-mediated penetration resistance and additionally broke non-host resistance to the wheat pathogen *Bgt*. BAX Inhibitor 1 is, besides MLO, the second protein sufficient to overcome complete penetration resistance of barley to *B. graminis*. Therefore, its potential role in basic compatibility to *B. graminis* is discussed and compared to that of MLO. In chapter 9, BAX Inhibitor 1 was further analysed based on its amino acid sequence and literature data. This tagged BAX Inhibitor 1 as an ancient cell death suppressor protein conserved in all higher eukaryotes, and traces its origin back to possibly prokaryotic ancestor proteins.

Chapter 9

Hückelhoven R, Dechert C, Kogel K-H (2003) Overexpression of barley BAX inhibitor 1 induces breakdown of *mlo*-mediated penetration resistance to *Blumeria graminis*. *Proc. Natl. Acad. Sci. USA*. 100: 5555-5560

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Overexpression of barley BAX inhibitor 1 induces breakdown of *mlo*-mediated penetration resistance to *Blumeria graminis*

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Cell death regulation is linked to pathogen defense in plants and animals. Execution of apoptosis as one type of programmed cell death in animals is irreversibly triggered by cytochrome *c* release from mitochondria via pores formed by BAX proteins. This type of programmed cell death can be prevented by expression of BAX inhibitor 1 (*BI-1*), a membrane protein that protects cells from the effects of BAX by an unknown mechanism. In barley, a homologue of the mammalian *BI-1* is expressed in response to inoculation with the barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*). We found differential expression of *BI-1* in response to *Bgh* in susceptible and resistant plants. Chemical induction of resistance to *Bgh* by soil drench treatment with 2,6-dichloroisonicotinic acid led to down-regulation of the expression level of *BI-1*. Importantly, single-cell transient overexpression of *BI-1* in epidermal leaf tissue of susceptible barley cultivar Ingrid led to enhanced accessibility, resulting in a higher penetration efficiency of *Bgh* on *BI-1*-transformed cells. In *Bgh*-resistant *mlo5* genotypes, which do not express the negative regulator of defense and cell death MLO, overexpression of *BI-1* almost completely reconstituted susceptibility to fungal penetration. We suggest that BI-1 is a regulator of cellular defense in barley sufficient to substitute for MLO function in accessibility to fungal parasites.

Programmed cell death (PCD) in animals and plants is involved in many developmental processes and stress responses. Animal apoptosis is a morphologically and biochemically defined type of PCD irreversibly triggered by cytochrome *c* release from mitochondria via pores in the outer mitochondrial membrane. This process is regulated by members of the Bcl-2 protein family that either support PCD, such as pore-forming BAX, or inhibit PCD, such as Bcl-2. BAX, Bcl-2, and their relatives are not present in plants. However, screening in yeast identified another mammalian antagonist of BAX (1). This antagonist was designated BAX inhibitor 1 (BI-1), and functional plant homologues of BI-1 were identified recently (2–5). BI-1 can interact with Bcl-2 but not with BAX, and it is localized at the endoplasmic reticulum and the nuclear envelope rather than at mitochondria (1, 3).

The hypersensitive reaction (HR) of plants to avirulent pathogens restricts pathogen growth effectively and includes a characteristic PCD of one or a few cells at the site of pathogen invasion (6–9). Interestingly, although plant *BI-1* expression can inhibit BAX-induced PCD in yeast and *Arabidopsis*, overexpression of *Arabidopsis BI-1* in resistant *RPM1* plants appeared not to interfere directly with the hypersensitive cell death reaction induced by avirulent *Pseudomonas syringae* pv. *tomatae*. However, because *Arabidopsis BI-1* is expressed in response to multiple stress treatments, BI-1 might play a role in protecting plants from stress-induced metabolic perturbations (4) or types of PCD different from that induced by a bacterial pathogen.

The biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) establishes a compatible interaction with barley by formation of haustoria in penetrated living cells. During pustule development and maintenance, a certain leaf area around the emerging fungal colony remains green while the rest of the leaf undergoes an early senescence process. This "green island" effect illustrates massive pathogen-induced changes of cell death regulation resulting in cell death suppression in invaded cells and leaf senescence. Although it is self-evident that the HR stops development of biotrophic pathogens, it is not unequivocally proven that cell death is required for resistance. Therefore, manipulation of plant cell death regulation could be a tool for understanding the role of cell death in pathogen defense.

The HR is under genetic control, and some physiological features of HR resemble those of animal PCD (6–9). Moreover, BAX-induced cell death in tobacco is similar to HR (10). Leaf cell death control and defense control seem to be linked. For instance, cell death control mutants such as *Arabidopsis lsd1* show both spontaneous cell death and broad-spectrum resistance (11). Also, Alvarez *et al.* (12) reported that onset of broad systemic acquired resistance in *Arabidopsis* is associated with development of microlesions. Barley lines carrying recessive mutant *mlo* alleles of the *Mlo* locus, similar to *lsd1*, show spontaneous leaf cell death and broad-spectrum resistance to *Bgh* (13, 14). Thus, the functional barley MLO protein is a negative control element of cell death and of defense responses to *Bgh*. Cell-survival mechanisms mediated by MLO probably negate plant defenses against *Bgh*, thereby allowing infection by the biotrophic fungus. However, *mlo* genotypes are highly susceptible to the hemibiotrophic pathogen *Magnaporthe grisea* and to necrosis-inducing culture filtrate from *Bipolaris sorokiniana* (15, 16). Kim *et al.* (17) recently demonstrated a link between MLO and calmodulin function. They suggested that negative MLO control of defense mechanisms against *Bgh* might be responsible for limited susceptibility to other pathogens, tagging MLO as a central modulator of antagonistic plant defense mechanisms. MLO structure is reminiscent of plasma membrane receptors that interact with heterotrimeric G proteins. However, MLO is not likely to depend on heterotrimeric G proteins (17) but possibly on small G proteins to fulfill its function in powdery mildew susceptibility. Down-regulation of the barley small GTP-binding protein RACB by RNA interference leads to enhanced penetration resistance to *Bgh* (18). This effect depends on *Ror1*, which is also required for *mlo*-mediated resistance (18, 19).

We present here a functional study on the implication of *Bl-1* in disease resistance by an expression analysis of *BI-1* in barley lines that are differently resistant to *Bgh* and by transient overexpression of *BI-1* in barley epidermal cells during interaction with *Bgh*.

Materials and Methods

Plants, Pathogens, and Inoculation. The barley (*Hordeum vulgare* L.) lines Ingrid, Pallas, and the corresponding backcross lines

Abbreviations: BCP, backcross Pallas; *Bgh*, *Blumeria* (*Erysiphe*) *graminis* f.sp. *hordei*; BI-1, BAX inhibitor 1; CIR, chemically induced resistance; DCINA, 2,6-dichloroisonicotinic acid; PCD, programmed cell death; HR, hypersensitive reaction; PE, penetration efficiency.

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BCP*Mla12*, BCP*mlo5*, and BCIngrid-*mlo5* (I22) were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen). Their generation was described previously (20). Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (60 μmol m^{-2} s^{-1} photon flux density). Barley powdery mildew fungus *B. graminis* (DC) Speer f.sp. *hordei* Em. Marchal, race A6 was inoculated onto barley primary leaves to give a certain density of conidia. We used 5 conidia mm^{-2} for inoculation after chemical induction of resistance and macroscopic evaluation of induction success, 50 conidia mm^{-2} for gene expression studies, and 150 conidia mm^{-2} for gene function assessment on transformed leaf segments. *Bgh* was maintained on cv. Golden Promise under the same conditions.

Chemical Treatment. 2,6-Dichloroisonicotinic acid (DCINA, Syngenta AG, Basel), formulated as 25% active ingredient with a wettable powder carrier, was applied to 4-day-old barley seedlings of cultivar Pallas as a soil drench. The compound was used at a final concentration of 8 mg·$liter^{-1}$ soil volume. The suspensions were prepared with tap water. Soil drench with a wettable powder suspension served as a control.

RNA Extraction and Expression Analysis. Total RNA was extracted from 8–10 primary leaf segments (5 cm long) by using RNA extraction buffer (AGS, Heidelberg) according to the manufacturer's instructions. For Northern blots, 10 μg of total RNA from each sample were separated in agarose gels and blotted by capillary transfer to positively charged nylon membranes. Detection of mRNAs was performed according to the DIG system user's guide with Digoxygenin-labeled antisense RNA probes (5). Before immunodetection, blots were washed stringently two times for 20 min in 0.1% (wt/vol) SDS/0.1× SSC at 68°C.

To detect low-level transcripts, we used the One-Step RT-PCR kit (Qiagen, Hilden, Germany) for semiquantitative reverse transcription PCR following the manufacturer's instructions. We used a low cycle number of 20 that maintained different transcript levels during the exponential amplification phase but did not allow cDNA detection in agarose gels by ethidium bromide staining. Hence, cDNAs were separated in agarose gels, denatured, blotted on nylon membranes, and detected with specific nonradioactively labeled RNA probes by using standard protocols and stringent conditions. Hybridization, washing, and immunodetection were performed as described for Northern blotting. Primers were 5′-ccaagatgcagatcttcgtga-3′ (5′ primer) and 5′-ttcgcgataggtaaaagagca-3′ (3′ primer) for a 513-bp *Ubi* cDNA fragment (GenBank accession no. M60175) and 5′-atggacgccttctactcgacctcg-3′ (5′ primer) and 5′-gccagagcaggatcgacgcc-3′ (3′ primer) for a 478-bp *BI-1* cDNA fragment (accession no. AJ290421).

Transient Transformation and Evaluation of Penetration Efficiency. A transient transformation protocol originally developed for wheat was used to transform barley via biolistic delivery of expression vectors into epidermal cells of leaf segments as described by Schweizer *et al.* (21). In general, each shot consisted of 312 μg of 1.1-μm tungsten particles with 0.3 μg of pGFP (GFP the under control of the CaMV ^{35}S promoter) (21) together with 0.7 μg of empty vector or *pBI-1* containing *BI-1* under the control of CaMV ^{35}S promoter. *BI-1* was cloned into the *Sal*I site of pGY-1 via restriction sites linked to oligo DNA primers that were used to amplify *BI-1*. Resulting *pBI-1* and antisense *pasBI-1* were sequenced to confirm that the original ORF was unchanged. To induce posttranscriptional gene silencing via RNA interference, particles were coated with dsRNA as described (18, 22).

Leaf segments were bombarded with coated particles 4 h before inoculation with *Bgh*, race A6. Inoculation with 150 conidia mm^{-2} led to ≈50% of transformed cells attacked by the fungus. The outcome of the interaction was evaluated subsequently by fluorescence and light microscopy. For each experiment, a minimum of 100 interaction sites was evaluated. Transformed GFP-expressing cells were identified under blue light excitation. Three different categories of transformed cells were distinguished: penetrated cells that contained a haustorium, cells that were attacked by a fungal appressorium but did not contain a haustorium, and cells that were not attacked by *Bgh*. Cells that contained more than one haustorium or that contained haustoria but less than fungi attacked were scored as one penetrated cell. Cells with multiple attack from *Bgh* without a haustorium were scored as one unpenetrated cell. Stomata and stomatal guard cells were excluded from the evaluation. *Bgh* was detected by light microscopy or by fluorescence staining of the fungus with 0.3% calcofluor (wt/vol in water) for 30 s.

Penetration efficiency was calculated as number of penetrated cells divided by number of attacked cells multiplied by 100 and used as a measure for resistance of bombarded cells.

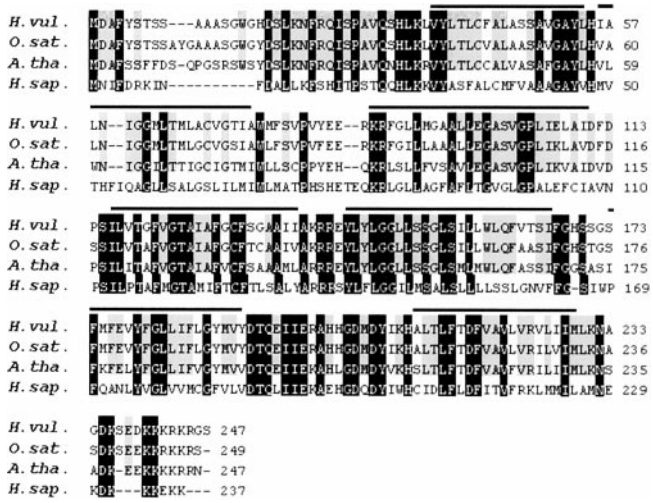

Fig. 1. Comparison of deduced amino acid sequences of barley (*H. vulgare*, GenBank accession no. CAC37797), rice (*Oryza sativa*, accession no. Q9MBD8), *Arabidopsis thaliana* (accession no. Q9LD45), and human (*Homo sapiens*, accession no. AAB87479) BI-1 proteins. Black-shaded amino acids are identical in all sequences. Gray-shaded amino acids are identical only in plant homologues. Bars indicate the seven predicted transmembrane domains in HvBI-1.

Results

Characterization of the BI-1 Amino Acid Sequence. The ORF of the barley *BI-1* gene (GenBank accession no. AJ290421) encodes 247 aa (5). The deduced protein of ≈25 kDa is very similar to the homologue of rice (88% identical, 98% similar) and *Arabidopsis* (75% similar) and 53% similar to the human BI-1 protein (Fig. 1). The barley BI-1 amino acid sequence contains presumably seven putative transmembrane domains with the C terminus in the cytosol (TMpred prediction, www.ch.embnet.org/software/TMPRED_form.html; ref. 23).

Expression of *BI-1* in Response to *B. graminis*. In a previous study, we showed that a barley *BI-1* homologue is expressed in early response to attack by *Bgh* (5). Here, we studied *BI-1* expression during compatible and incompatible interactions of near-isogenic barley backcross Pallas (BCP) lines bearing no functional resistance gene, the major resistance gene *Mla12* mediating the HR after race-specific recognition of AvrMla12 from fungal race *Bgh*A6 (6, 24), or the recessive null mutant *mlo* allele *mlo5* mediating broad penetration resistance (13, 17, 19, 24).

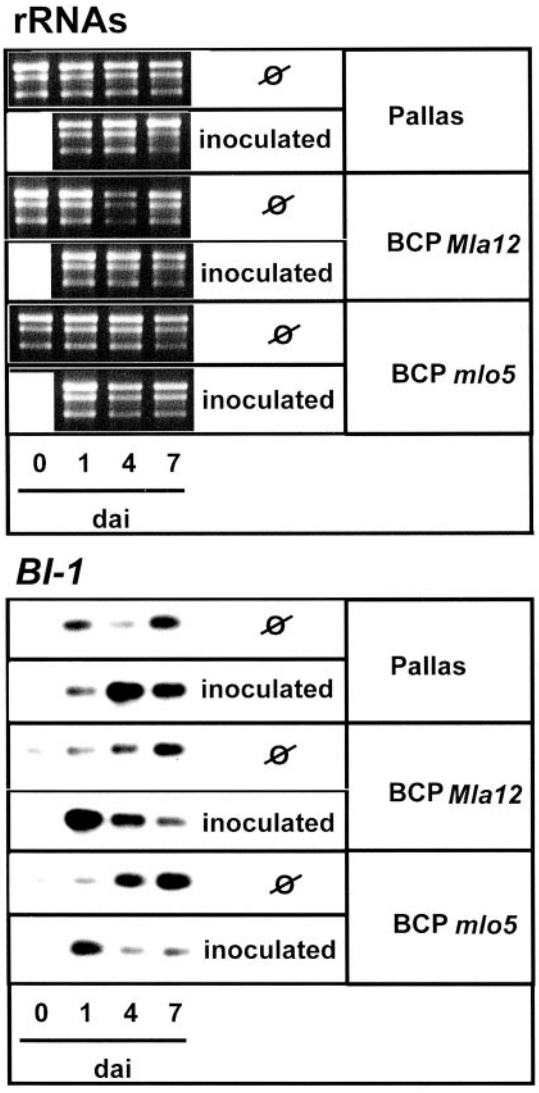

Fig. 2. *BI-1* expression in resistant and susceptible barley lines. cDNA gel blot analysis. cDNAs were synthesized by RT-PCR from total RNA. RNA was isolated from susceptible Pallas, resistant BCP*Mla12*, or resistant BCP*mlo5* at 0 (immediately before inoculation), 1, 4, and 7 days after inoculation with *Bgh* and in parallel from noninoculated control plants (Ø). RT-PCR for *BI-1* was carried out with 20 cycles under specific conditions. We checked loading of RNA (0.5 μg) by rRNA staining with ethidium bromide in gels. Repetition of the experiment led to similar results.

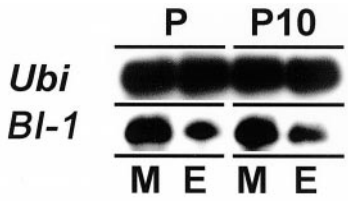

Fig. 3. *BI-1* is expressed in mesophyll tissue. cDNA gel blot analysis. RT-PCR analysis with RNA from Pallas (P) and BCP*Mla12* (P10) by 24 h after inoculation with *Bgh*A6 is shown. For extraction of total RNA, abaxial epidermal stripes (E, inoculated site of the leaves) were separated from the mesophyll and adaxial epidermis (M). *Ubiquitin 1* (*Ubi*) was selected as a marker for tissue-unspecific gene expression. RT-PCR was carried out with 30 cycles under specific conditions.

Expression of *BI-1* was analyzed during the first 7 days after dense inoculation with conidia of *Bgh*A6. We selected the pathogenesis-related protein 1b gene to confirm defense-related gene expression in the near-isogenic lines. This marker gene was expressed in response to *Bgh* in all lines (not shown). Starting with the same RNA, we analyzed *BI-1* expression by RT-PCR and cDNA blotting. We detected constitutive *BI-1* expression in all lines. Expression tended to increase slightly with leaf age and changed remarkably in response to *Bgh* (Fig. 2). *Bgh*-induced expression of *BI-1* occurred early in BCP*Mla12* and BCP*mlo5*, whereas *BI-1* transcript accumulation was delayed in the susceptible parent Pallas. Expression of *BI-1* at 1 day after inoculation was strongest in BCP*Mla12*, closely correlating with the onset of HR (24).

Early expression of *BI-1* in response to *Bgh* posed the question as to whether *BI-1* is expressed in the leaf epidermis, the only tissue in direct contact with fungal infection structures. Therefore, RNA was isolated from stripped epidermal and remaining leaf tissue of *Bgh*A6-inoculated Pallas and BCP*Mla12* 1 day after inoculation. RT-PCR and cDNA gel blots revealed that barley *BI-1* was expressed mainly in mesophyll tissue when compared with *Ubiquitin 1*, which is constitutively expressed equally in epidermis and mesophyll tissue (Fig. 3). We detected a low level of *BI-1* transcripts in epidermal tissue, whereas other genes showed epidermis-dominant expression in the same plants (data not shown; ref. 18).

Expression of *BI-1* in Chemically Induced Resistance. We investigated *Bl-1* expression in plants that were treated with the resistance-inducing compound DCINA. Four-day-old plants were soil-drench treated with 8 mg of DCINA per liter of soil volume and inoculated 3 days later with a low density of *Bgh* conidia suitable to macroscopically estimate the efficacy of chemically induced resistance (CIR). Plants expressing CIR showed ≈70% less mildew colonies than control plants treated with the unloaded carrier substance (Fig. 4*A*). Northern blots and cDNA blots were carried out to compare *BI-1* transcript accumulation during the onset and expression of CIR with the transcript accumulation of the CIR marker gene *Bci4* (25). As expected, *Bci4* expression was up-regulated in response to DCINA treatment. In contrast, *BI-1* was down-regulated 1–3 days after chemical treatment (Fig. 4*B*). The low inoculation density used in this experiment was not sufficient to induce strong *BI-1* expression in response to inoculation, whereas *PR1b* was clearly induced (data not shown). However, *BI-1* expression recovered in chemically induced plants after inoculation.

***BI-1* Overexpression Induces Accessibility to *Bgh*.** For gene function assessment, we performed transient *BI-1* overexpression in barley epidermal cells by biolistic transformation and subsequent microscopic analysis of the interaction of *Bgh* with transformed cells (Fig. 5 *A* and *B*; refs. 18 and 21). In six independent experiments, overexpression of *BI-1* in susceptible barley cultivar Ingrid resulted in significantly enhanced penetration efficiency (PE) of *Bgh*. The average PE was significantly enhanced from 47% to 72% (165% of the controls) on cells expressing *BI-1* compared with control cells (Fig. 5*C*). In independent experiments using an antisense construct, the average PE of *Bgh* on *pasBI-1*-bombarded cells was reduced relatively by 12% compared with cells bombarded with an empty vector (Fig. 5*D*). However, the PE on cells transformed with *pasBI-1* was not significantly different from that on controls. We obtained similar results, i.e., weak but not significant induction of resistance, by cobombardment of *pGFP* together with dsRNA of *BI-1*, which should induce sequence-specific RNA interference and thus down-regulation of *BI-1* (18, 22). On Ingrid, coexpression of sense or antisense *BI-1* with GFP did not change the number of transformed GFP-expressing cells per shot, indicating that BI-1

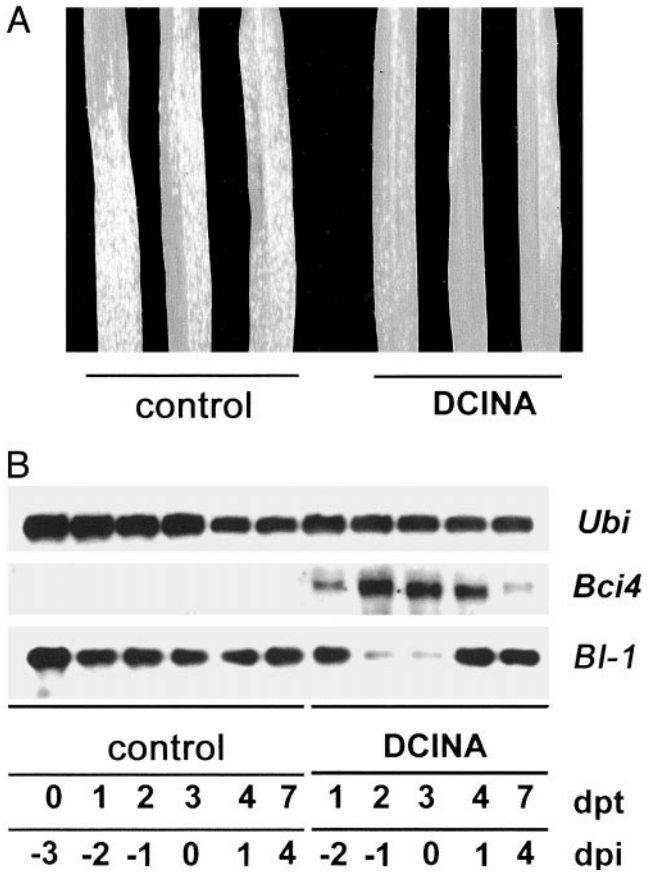

Fig. 4. *BI-1* expression is repressed during chemical induction of resistance. (*A*) Chemically induced resistance in barley cultivar Pallas to *Bgh*. Barley first leaves treated with DCINA show less powdery mildew pustules than control leaves treated with the carrier substance wettable powder as a soil drench. (*B*) RNA (*Bci4*, 10 μg of RNA) and cDNA blots. RNA was extracted 0, 1, 2, and 3 days after soil drench treatment (dpt) with DCINA or the carrier substance wettable powder and additionally 1 and 4 days postinoculation (dpi, corresponding to 4 and 7 dpt). RT-PCR (*Ubi*, *BI-1*) was carried out with 20 cycles under specific conditions. Repetition of the experiment led to similar results.

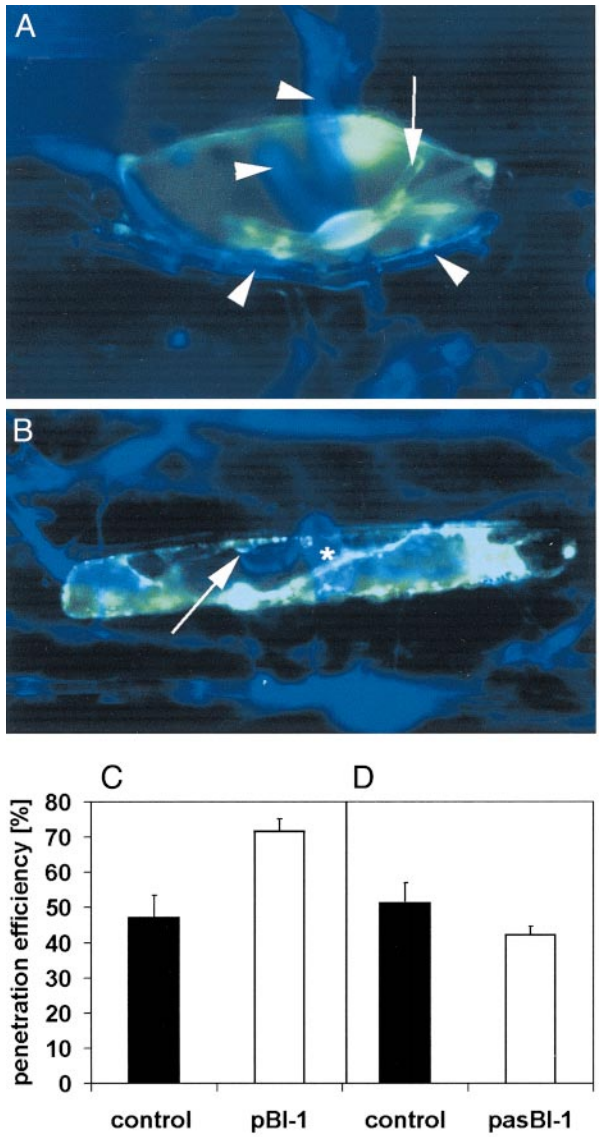

Fig. 5. Overexpression of *BI-1* induces supersusceptibility. (*A*) GFP-expressing cell that was penetrated by *Bgh*. The fungus formed a haustorium with finger-like protuberances (arrow) and elongated secondary hyphae on the leaf surface (arrowheads). Surface structures were stained with calcofluor and visualized by UV-light excitation. GFP and calcofluor images were merged in PHOTOSHOP. (*B*) GFP-expressing cell that was attacked (arrow) but not penetrated by *Bgh*. (*C*) Average penetration efficiency of *Bgh* in six independent experiments with *Bgh* on barley cultivar Ingrid. PE of *Bgh* was enhanced significantly ($P < 0.01$, Student's *t* test) in cells that were bombarded with *pBI-1* compared with cells that were bombarded with empty control *pGY1*. (*D*) Penetration efficiency of *Bgh* on cells that were bombarded with antisense-*BI-1* (*pasBI-1*) was evaluated in independent experiments with *Bgh*. PE of *Bgh* was reduced nonsignificantly ($P > 0.05$) on cells that were bombarded with *pasBI-1* compared with cells that were bombarded with empty control *pGY1*. Columns represent average values of independent experiments. Bars represent standard errors.

did not alter cell survival during the first 2 days after transformation (not shown).

Induction of supersusceptibility by the putative cell death guard protein BI-1 is reminiscent of MLO function in barley (17, 26). Therefore, we transformed epidermal cells of *Bgh*-resistant *mlo5* barley to test whether BI-1 interferes with *mlo5*-mediated resistance. In our experimental system, *mlo5* genotypes in the background of cv. Pallas or cv. Ingrid were slightly accessible to *Bgh*. In seven independent experiments, we found that penetration efficiencies in control GFP cells ranged from 0% to 11% (minimum–maximum). Strikingly, *BI-1* overexpression reconstituted accessibility in *mlo5* barley close to a level that is typically seen in susceptible (*Mlo*) lines. Average penetration efficiency of *Bgh* on Ingrid-*mlo5* and Pallas-*mlo5* leaf segments was enhanced from 4% to 23% and from 6% to 33%, respectively (Fig. 6). This is equivalent to relative 520% and 510% of controls, respectively.

Discussion

We have shown here that the putative cell death regulator BI-1 is involved in the powdery mildew resistance of barley. We demonstrated pathogen-induced *BI-1* expression, DCINA-induced *BI-1* repression, and supersusceptibility to *Bgh* induced by *BI-1* overexpression. Most importantly, barley BI-1, when overexpressed in *mlo5* barley, was sufficient to induce accessibility to *Bgh*. Thus, BI-1 is a suppressor protein of *mlo*-mediated penetration resistance.

Stress response and cell death regulation in plants is not well understood. Many approaches to isolate pro- or antiapoptotic homologues of the animal Bcl-2 family from plants failed, and because the *Arabidopsis* genome sequence is now available, one may assume that plants lack homologous proteins. Nevertheless, there are some similarities of plant and animal PCD that indicate

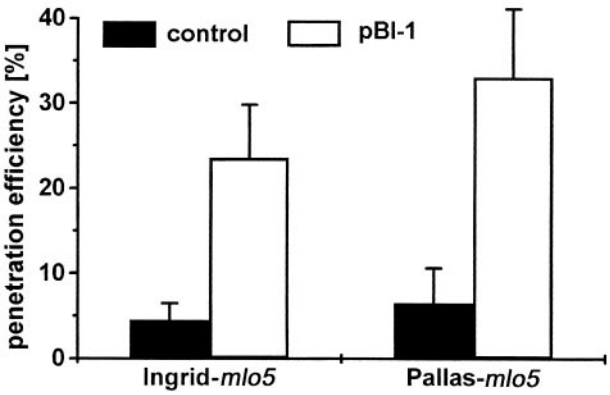

Fig. 6. Overexpression of *BI-1* induces breakdown of *mlo5*-mediated penetration resistance. Penetration efficiency of *Bgh* was evaluated in three to four independent experiments with *Bgh* on barley cultivar Ingrid-*mlo5* or Pallas-*mlo5*. PE of *Bgh* was enhanced significantly ($P < 0.05$) in cells that were bombarded with *pBI-1* compared with cells that were bombarded with empty control *pGY1*. Columns represent average values of at least three independent experiments. Bars represent standard errors.

common elements in both systems. For instance, reactive oxygen intermediates, cysteine proteases, DNA degradation, and some morphological changes seem to take part in animal and plant PCD. Identification of some plant homologues of animal cell death suppressors, for instance BAG (Bcl-2 associated athanogene), DAD (defender against apoptotic death), and BI-1, indicates common elements of negative cell death control for eukaryotes. BI-1 proteins are highly conserved among humans, animals, and plants. Barley BI-1 is very similar to rice and *Arabidopsis* BI-1 proteins that were shown to inhibit BAX function in yeast and *Arabidopsis* (2–4). However, the mechanism by which BI-1 inhibits PCD is unknown. Further homologues of BI-1 have been identified in *Arabidopsis*, and it will be interesting to see whether they have cell death-suppressing capacity (8).

Bgh-induced expression of barley *BI-1* correlated with early defense against *Bgh* in resistant barley as well as with pathogen development in susceptible barley. Therefore, we speculate that BI-1 is generally involved in cell survival at sites of fungal attack. BI-1 might be involved in both restriction of HR-associated cell death and fungus-induced cell survival. Cell survival and *Bgh* resistance are antagonistically regulated in barley. This is most prominently demonstrated by the fact that loss of MLO function leads to both *Bgh* resistance and spontaneous cell death (14). The functional *Mlo* gene is expressed in response to pathogens, wounding, reactive oxygen intermediates, and during leaf aging (27). A similar expression profile was found for *BI-1* (refs. 4 and 5; Fig. 2; unpublished results). Because *BI-1* overexpression induced susceptibility even in a *mlo*-mutant genotype, BI-1 similar to MLO might support a survival pathway, which negatively interferes with penetration resistance. The fact that overexpression of BI-1 mediated susceptibility independent from MLO, although they share no sequence similarity, shows that BI-1 acts independently or downstream from MLO.

The CIR response to *Bgh* correlates with enhanced epidermal cell death, papillae formation, and highly localized H_2O_2 accumulation (24, 28). The onset of CIR was accompanied by down-regulation of *BI-1*, whereas overexpression of BI-1 resulted in induced susceptibility (Figs. 4–6). This finding supports the notion that BI-1 is a negative regulator of penetration resistance to *Bgh* and strengthens the hypothesis that cell death control and plant defense against biotrophic pathogens are negatively linked. Accordingly, resistance, induced in barley by DCINA, is not effective against the toxin-producing and thus cell death-promoting fungal pathogen *B. sorokiniana* (J. Kumar and K.-H.K., unpublished results). Transient expression of antisense-*BI-1* or *BI-1*-specific RNA interference did not influence fungal penetration efficiency strongly. This can be explained by the tissue-specific expression pattern of BI-1 that is weakly expressed in the epidermis (Fig. 3). Therefore, single-cell epidermal *BI-1* silencing may not be sufficient to abrogate BI-1 function. Alternatively, BI-1 protein might show a low turnover rate explaining insufficiency of transient gene silencing.

MLO was detected mainly in the plasma membrane (29), whereas BI-1 was visualized as a fusion with GFP in endomembranes, particularly in ER and the nuclear envelope (refs. 1 and 3 and unpublished results). These findings question a physical interaction of MLO and BI-1. We speculate that BI-1 is a mesophyll teammate of epidermis-expressed MLO (27). Interestingly, although MLO is epidermis-specific, *mlo* mutants show spontaneous cell death especially in the mesophyll (30). Possibly, MLO-dependent signal exchange between epidermal and mesophyll tissue is required for BI-1 function in cell death control. Future studies need to be done to show whether overexpression of BI-1 in the mesophyll prevents barley from pleiotrophic *mlo* effects that have agronomic impact (13).

The role of BI-1 in susceptibility of wild-type barley is not yet clear. *BI-1* overexpression induced supersusceptibility to *Bgh* (Fig. 5*C*), similar as MLO overexpression does (17). More importantly, *BI-1* overexpression induced breakdown of *mlo5*-mediated penetration resistance. Sanchez *et al.* (4) reported that AtBI-1 overexpression in *RPM1-Arabidopsis* was insufficient for suppression of HR triggered by avirulent *P. syringae* pv. *tomatae*. Plant *BI-1*, when expressed in human fibrosarcoma cells, induced *apoptosis*-like PCD instead of preventing it, possibly by competing with the functional mammalian BI-1 (31). Our finding that barley BI-1 is able to substitute for the cell death suppressor MLO as a suppressor of *Bgh* penetration resistance supports the idea that BI-1 inhibits a specific, although unidentified, type of endogenous plant PCD. Interestingly, besides BI-1, two antioxidants, a soybean ascorbate peroxidase and a tomato glutathione *S*-transferase, as well as nuclear AtEBP, a protein that is known to be ethylene responsive, are able to suppress BAX-induced cell death in yeast (32–34). Barley and *Arabidopsis* BI-1 genes are responsive to pathogen challenge and wounding, which are both associated with oxidative stress. Together, BI-1 might be a redox-responsive cell death regulator involved in senescence processes similar as suggested for MLO (27). BAX and oxidative stress lead to pore formation in mitochondrial membranes finally triggering PCD (8). It was speculated that BI-1 could interfere with such a pore formation or forms by itself cell death-antagonistic ion channels (1, 4, 8). Recently, it was shown that antisense down-regulation of tobacco BI-1 accelerated cell death in BY-2 cells on carbon starvation (35). Mitochondria integrate diverse cell death signals including carbon starvation in plants such as in animals, and association of heterologously expressed BAX with plant mitochondria induces HR-like PCD (8, 10). However, it is not understood how cytochrome *c* release from mitochondria contributes to PCD in plants. Also, the role of cytochrome *c* in triggering plant defense is unclear. Based on its cellular localization, BI-1 should not directly interact with mitochondrial membranes. Possibly, BI-1 controls cellular levels of reactive oxygen intermediates, which accumulate both upstream and downstream of mitochondrial pore formation (8). In barley, this assumption is supported by the fact that resistance to *Bgh* is closely linked to H_2O_2 accumulation, whereas successfully invaded cells are completely bare of H_2O_2 (24).

The ambivalence of the MLO function in different pathosystems requires breeders to take the different infection strategies of plant parasites into account when they produce transgenic, pathogen-resistant plants. Because *mlo5* barley is highly susceptible to *M. grisea* and to toxins of *B. sorokiniana* (15, 16), one may

look at functional MLO as a resistance factor to hemibiotrophic and necrotrophic fungi. In the same direction, BI-1 could contribute to resistance against necrotrophic pathogens, as shown for the heterologous expression of cell death suppressors such as Bcl-2 or p35 in tobacco or tomato, respectively (36, 37).

This study demonstrates the contribution of BI-1 to regulation of plant defense to a pathogen. The fact that BI-1 proteins are able to suppress cell death in animals and plants as well as penetration resistance in barley indicates conserved overlapping pathways that regulate PCD and defense responses, possibly in all higher eukaryotes.

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Chapter 10

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Chapter 11

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BAX Inhibitor-1, an ancient cell death suppressor in animals and plants with prokaryotic relatives

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BAX Inhibitor-1 (BI-1) was originally described as testis enhanced gene transcript in mammals. Functional screening in yeast for human proteins that can inhibit the cell death provoking function of BAX, a proapoptotic Bcl-2 family member, led to functional characterisation and renaming of BI-1. The identification of functional homologues of BI-1 in plants and yeast widened the understanding of BI-1 function as an ancient suppressor of programmed cell death. BI-1 is one of the few cell death suppressors conserved in animals and plants. Computer predictions and experimental data together suggest that BI-1 is a membrane spanning protein with 6 to 7 transmembrane domains and a cytoplasmic C-terminus sticking in the endoplasmatic reticulum and nuclear envelope. Proteins similar to BI-1 are present in other eukaryotes, bacteria, and even viruses encode BI-1 like proteins. BI-1 is involved in development, response to biotic and abiotic stress and probably represents an indispensable cell protectant. BI-1 appears to suppress cell death induced by mitochondrial dysfunction, reactive oxygen species or elevated cytosolic Ca^{2+} levels. This review focuses on the present understanding about BI-1 and suggests potential directions for further analyses of this increasingly noticed protein.

Keywords: BAX Inhibitor-1; *Blumeria graminis* f.sp. *hordei*; hypersensitive reaction; mitochondria; MLO; pathogen invasion; protease.

Introduction

Programmed cell death (PCD) is a mechanism eukaryotes have developed to remove unwanted, excessive, infected or damaged cells to maintain the integrity of the remaining organism. PCD is mainly discussed for multicellular organisms but individual cell death may also serve to maintain stability of a population of unicellular eukaryotes. PCD is a physiological energy consuming process under genetic control. The dying cell itself contributes to it in a well-defined way, which distinguishes PCD from necrosis that is a non-physiological process out of control. Disturbance of the PCD machinery leads to developmental damage, disease and tumour genesis. Thereby, genetic abnormalities as well as pathogenic influence can lead to loss of PCD control. A lot of mechanisms of PCD in animals are well studied. One important mechanism that leads to a morphologically and biochemically defined type of PCD, apoptosis, can be triggered by BAX proteins, that interfere with mitochondrial function. Animal apoptosis is irreversibly activated by cytochrome *c* release from mitochondria *via* pores in the outer mitochondrial membrane. Members of the Bcl-2 protein family that either support PCD, such as pore forming BAX, or inhibit PCD, such as Bcl-2, regulate this process. Cytochrome *c* released from mitochondria binds to apoptosis activating factor 1 (APAF-1) that activates caspase-9 initiating a proteolytic caspase cascade and apoptosis.[1] Apoptosis commonly involves cytoplasmic shrinkage, cleavage of poly ADP ribose polymerase, inter-nuleosomal DNA degradation, chromatin condensation, nuclear fragmentation, phosphatidyl serine exposure on the outer plasma membrane site, membrane blebbing and cell fragmentation in apoptotic bodies.

Besides BAX, high concentrations of oxidants and cytosolic Ca^{2+} can lead to release of cytochrome *c* and other proapoptotic proteins such as apoptosis-inducing factor from mitochondria. The latter mechanisms appear to be conserved in plants where, in spite of that, BAX proteins as well as the entire Bcl-2 family are apparently not present. Anyway, mammalian Bcl-2 family members are cross-functional in plant cell death regulation. Mouse BAX can induce plant cell death, and antiapoptotic Bcl-2 family members can inhibit pathogen induced plant cell death.[2–5] Similar, the baculovirus caspase inhibitor p35 can suppress fungus as well as virus induced plant cell death.[6,7] This demonstrates that the physiology of cell death is similar in animals and plants.

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Plant intrinsic PCD occurs in several developmental processes and in stress responses. For instance, the development of xylem vessels, aerenchyma, endosperm or senescence includes PCD mechanisms. Plant PCD shares some biochemical features with animal apoptosis such as cytochrome *c* release, caspase-like activities, DNA laddering and changed protein phosporylation patterns.[8,9] Furthermore, many pathways of plant and animal PCD regulation cross at mitochondria.[1,10] However, like in animals, plants do not express a single type of PCD but different types that occur during distinct developmental phases and in interaction with the environment. Therefore, it is difficult to compare PCD in animals and plants in general. Additionally, besides some striking similarities, plant and animal PCD are also clearly different in some aspects. For instance, plasma membrane rupture after vacuole collapse is typical for many types of plant PCD whereas the plasma membrane remains intact during animal apoptosis. Apoptotic bodies are not observed *in planta* and the plant cell wall prevents engulfment by neighbouring cells. However, one may assume that proteins commonly involved in PCD in both kingdoms lead back to a type of PCD that was already present in ancient eukaryotes before divergence of animals and plants. Among general PCD regulators only a few are conserved in animals and plants. Defender against death, Bcl-2 associated anthanogene and BAX Inhibitor-1 (BI-1) are examples for such proteins. BI-1 is discussed here because it has been proven to be functional in animals, plants and yeast and therefore might be an ancient PCD regulator of general importance for cellular homeostasis.

Discovery and expression patterns of BI-1

BI-1 was first cloned from adult rat testis as so-called testis enhanced gene transcript (TEGT) and subsequently identified in mouse and human. Southern analysis predicted that BI-1 is a single copy gene present in all kinds of vertebrates. It was mapped to rat chromosome 7, mouse chromosome 15, swine chromosome 5 and human chromosome 12q12–q13.[11–13] Rat BI-1 transcript sizes differ due to the presence of two alternative polyadenylation sites at the 3′ end. BI-1 expression can be driven from two alternative TATA-less promoters resulting in transcripts with different first exons in the 5′ untranslated regions. The distal rat BI-1 promoter is ubiquitous whereas the proximal promoter is testis-specific. Expression from the distal promoter appears to be developmentally regulated since BI-1 expression peaked during late prenatal and early postnatal lung development, when the rate of apoptotic death is high. Since the rat BI-1 promoter region includes several putative p53 tumour suppressor, Myb and Wilms tumour suppressor sites, cell-death-linked expression of BI-1 appears likely.[14] Future studies have to show whether the regulatory elements are functional as activator or repressor signals for BI-1 expression. *In situ* localisation of BI-1 transcripts or of BI-1 promoter activity should provide further evidence for BI-1 expression in tissues of enhanced cell death or survival.[15] The fact that BI-1 transcripts were preferentially isolated from tissues with high cell death rates indicates a possible function for BI-1 in general cell survival. The finding that BI-1 is highly expressed in tumour cells such as COST anaplastic large cell lymphoma cells or prostate tumour cells supports this view.[15,16] In general, BI-1 appears to be over-expressed in several malignant tumours (Table 1). This tags BI-1 as a cell death regulator potentially involved in deregulation of apoptosis in tumour cells.

Table 1. Expression patterns of BI-1 in mammalian tissues

Species, cell line/tissue sample	Expression level[a]	References
Human prostate cancer/epithelial cells	↑	15, 57
Human breast cancer	↑	58
Human brain tumor/glioma	↑	59
Human ALK-positive anaplastic large cell lymphoma	↑	16
Adult rat testis	↑	11, 14
Developing rat lung	↑	14

[a]Besides the high levels of BI-1 expression mentioned here, BI-1 is widely expressed in many tissues *in vivo*.[17]

A plant BI-1-like protein from *Arabidopsis thaliana* was described already by Xu and Reed (1998)[17] and shortly afterwards, plant cDNAs with even higher similarity to human BI-1 (up to approximately 45% identical and 65% similar amino acids out of 234 aligned) were isolated from various species including *Arabidopsis thaliana, Oryza sativa, Hordeum vulgare, Brassica napus, Brassica oleracea and Nicotiana tabacum.*[18–23]

Plant BI-1 is expressed in various tissues, during aging and under several stresses. First report about expression of BI-1 in rice and *Arabidopsis* came from Kawai and co-workers (1999) who isolated BI-1 cDNAs by a candidate gene approach using EST sequences.[21] Rice BI-1 appears to be a single-copy gene that is expressed in plant roots and shoots. Later, it was found that *Arabidopsis BI-1* expression is responsive to wound and pathogen stress. *BI-1* gene expression was activated during interaction with either virulent or avirulent bacteria (*Pseudomonas syringae* pv *tomato*). This response was reduced but still measurable in signal transduction mutants that either contained the *eds1* or the *coi1* mutation responsible for loss of pathogen resistance gene function or responsiveness to the pathogen-resistance inducing phytohormone jasmonate, respectively. This suggested a role for BI-1 in plant pathogen response because both mutants are compromised in resistance to certain subsets of pathogens.[22]

Table 2. Expression patterns of B-1 in stressed and diseased plant tissues

Plant species	Organ/stress	Expression level[a]	Reference
Arabidopsis thaliana	Leaves/inoculation with *Pseudomonas syringae* pv. *tomatae*	↑	22
Arabidopsis thaliana	Leaves/wounding	↑	22
Arabidopsis thaliana	Cell suspension/salicylic acid	↑	29
Arabidopsis thaliana	Cell suspension/H_2O_2	↑	29
Oryza sativa	Cell suspension/cell wall elicitor from *Magnaporthe grisea*	↓	26
Oryza sativa	Leaves/inoculation with *Magnaporthe grisea*	↑↓	26
Hordeum vulgare	Leaves/inoculation with *Blumeria graminis* f.sp. *hordei*	↑	20, 24
Hordeum vulgare	Aging leaves	↑	24
Hordeum vulgare	Young leaves/soil drench with 2,6-dichloroisonicotinic acid[b]	↓	24
Lycopersicon esculentum	Cucumber mosaic virus D satellite RNA	↑	25
Nicotiana tabacum	Senescing flower	↑	19
Brassica napus	Senescing flower	↑	19
Brassica oleracea	Senescing flower	↑	23

[a]↑, ↓, enhanced or reduced BI-1 expression levels relative to non-stressed controls.
[b]Plant disease resistance inducing chemical.

Pathogen responsive expression of plant BI-1 was confirmed in barley upon infection with the barley powdery mildew fungus. BI-1 is early expressed in resistant and somewhat delayed in susceptible plants indicating a possibly multivalent role for the protein in the host response to microbes.[20,22,24] Tomato BI-1 expression is induced in plants accumulating H_2O_2 and other defence compounds as well as in plants undergoing systemic cell death after cucumber mosaic virus D satellite RNA infection.[25] Expression was especially high in infected and damaged tissues supporting the hypothesis that BI-1 could be involved in plant cell death restriction.[20,25] Matsumura and co-workers detected down-regulation of rice BI-1 in cell culture response to an elicitor preparation derived from cell walls of the pathogenic fungus *Magnaporthe grisea.* Inoculation of rice leaves with virulent fungal spores resulted in biphasic up- and interim down-regulation of BI-1 expression.[26] Down-regulation of BI-1 expression was also reported for barley treated with the disease-resistance inducing compound 2,6-dichloroisonicotinic acid that functions as an analogue of the plant hormone salicylate.[24] Since such a treatment provokes pathogen resistance involving H_2O_2 accumulation and the hypersensitive cell death response,[27] one can speculate that BI-1 down-regulation lowers the cell death threshold of plants. Vice versa, BI-1 induction might be involved in cell survival at wound sites or during controlled tissue disintegration in senescing tissue. This is underscored by the observation that senescent flowers of tobacco and oilseed rape as well as aging barley leaves show enhanced BI-1 transcript levels.[19,23,24] However, so far no plant BI-1 promoter elements have been analysed that might help to understand BI-1 expression patterns. Interestingly, the plant hormone salicylate can induce cell death in mammalian cells, too, and it is discussed as a chemotherapeutic agent.[28] Possibly, salicylate can influence mammalian BI-1 expression. Surprisingly, BI-1 expression can be induced in *Arabidopsis* cell suspensions by both H_2O_2 and salicylic acid.[29]

On the whole, plant and animal BI-1 appears to be expressed during tissue development and stress responses, situations in which cell death has to be controlled strictly to avoid inappropriate tissue suicide. In infectious diseases and tumour development, inappropriate BI-1 expression might contribute to disturbed cell death reactions (Tables 1 and 2). It would be interesting to learn whether BI-1 is generally expressed in pathogen response in higher eukaryotes and whether plant B-1 is expressed in plant tumours induced by parasitic organisms.

BI-1 function in cell death regulation and disease resistance

BI-1 function as a cell death suppressor was first described after screening for human proteins that can inhibit cell death induced by mouse BAX expression in yeast.[17] Importantly, BI-1 suppressed apoptosis induced by BAX also in human 293 kidney cells. Additionally, apoptosis induced by growth factor withdrawal in GM701 fibroblasts could be suppressed by over-expression of a fusion of BI-1 and the green fluorescing protein marker (GFP). However, in contrast to the caspase inhibitor XIAP, but similar to BCL-2, BI-1 over-expression was not sufficient to protect 293 cells from cell death induced by expression of the tumour necrosis factor family receptor FAS (CD95). This suggested that BI-1 does not directly interfere with FAS-induced caspase activation, which can bypass the mitochondrial PCD pathway. Interleukin-3 withdrawal induced apoptosis of FL5.12 lymphocytes is also suppressed by BI-1-GFP in a similar amount as by BCL-2. Finally, drug induced cell death by etoposide and staurosporine is

limited by over-expression of BI-1-GFP. In reciprocal experiments, suppression of BI-1 expression by an antisense strategy induced apoptosis-like cell death in 293 cells.[17] In a different approach, BI-1 was isolated as a suppressor of SW480 cell death induced by tumour necrosis factor-related apoptosis inducing ligand (TRAIL), which has been suggested as potential therapeutic agent against cancer because it kills cells in many tumour lines but obviously does not affect most healthy cells. In independent experiments, protection against TRAIL could not be confirmed questioning a general role of BI-1 in survival after TRAIL treatment.[30] However, RNA interference, induced by small BI-1 dsRNAs, led to specifically reduced BI-1 expression, caspase-3 activation and spontaneous apoptosis in different prostate carcinoma cells lines. This strongly supported the view that BI-1 is not only an expression marker for prostate cancer but might be a potential target for novel therapeutic approaches.[15]

Interestingly, BI-1 also protects HeLa cells from apoptosis induced by the obligate intracellular bacterial pathogen *Chlamydia psittaci*. Apoptosis induced by *C. psittaci* appears thereby to involve BAX activation but to be caspase-independent since it could not be inhibited by the broad-spectrum caspase inhibitor zVAD. Furthermore, caspase-3 appeared not to be activated in *C. psittaci* infected cells.[31] The fact that BI-1 apparently can inhibit both caspase-dependent and caspase-independent cell death in mammalian cells shows that BI-1, though not suppressing all kinds of PCD, is a broad cell death suppressor. Host cell death suppression and activation take part in pathogenesis of intracellular bacteria and it is obvious that obligate pathogens corrupt host cell death regulation for their own use. In this regard, one may speculate that pathogenic bacteria induce BI-1 expression or repression in different stages of interaction with their host cells.

Plant BI-1 is able to suppress mammalian BAX induced cell death in yeast, 293 kidney cells, and plants.[3,19,21,22,29,32] This is of special importance since it demonstrates both structural and functional conservation of BI-1 and tags BI-1 as an ancient cell death suppressor apparently developed before evolution of eukaryotic kingdoms. Surprisingly, plants possess BI-1 although no BAX or other BCL-2 family members can be identified in plant genomes. This and the fact that rice BI-1 suppresses an intrinsic plant cell death response to a fungal pathogen elicitor,[26] strongly suggests that BI-1 originally evolved as a cell death suppressor that was not specialised on regulation of BAX induced cell death. Together with the observation, that BI-1 over-expression does neither interfere with BAX protein synthesis nor BAX localisation, this supports the assumption that BI-1 works downstream of BAX in animals and downstream of mitochondria in plants.[17] BI-1 localisation in the ER supports the alternative view that BI-1 could also regulate PCD independent from mitochondria that might be triggered by ER stress. In this regard, it is interesting that BAX can directly interact with the ER during apoptosis induction.[33]

Astonishingly, the plant BI-1 from *Arabidopsis* induces apoptosis-like cell death in human fibrosarcoma HT1080 cells, which can be blocked by co-transfection with human BI-1, XIAP or antiapoptotic members of the Bcl-2 family. This indicated that plant BI-1 was not simply toxic to HT1080 cells but rather interfered with intrinsic cell death control most likely by competition with human BI-1.[34] In contrast, plant BI-1 from tobacco and oilseed rape can inhibit BAX-induced cell death in human 293 cells in a dose dependent manner.[19] Future studies will show whether different behaviour of different cells transfected with plant BI-1 is caused by a certain competence of cell lines or is specific for each plant BI-1. Sequence comparison of human and *Arabidopsis* BI-1 might help to identify amino acids that could be targets for mutation to derive potential dominant negative human BI-1 mutants as a molecular tool to study BI-1 function.

The same *Arabidopsis* BI-1 that induces cell death in HT1080 cells suppresses BAX induced cell death in yeast and *Arabidopsis*.[3,34] The fact that heterologous BAX can induce cell death in *Nicotiana* species and *Arabidopsis* suggests functional conservation of mitochondrial dysfunction induced PCD though plants lack BAX-homologues.[4,8–10] Carbon starvation in plants is one of the PCD signals that are most likely integrated by mitochondria.[10] Accordingly, antisense downregulation of tobacco BI-1 induces accelerated cell death in BY-2 cells under carbon starvation.[18] BAX mediated plant cell death depends on the C-terminal transmembrane (TM) domain of BAX further supporting the idea that association with mitochondria or the ER is essential for cell death induction.[3,4] In plants, BAX induces DNA laddering, cytoplasmic shrinkage, chloroplast membrane deterioration and defence gene expression typical for different types of plant cell death during development or pathogen response.[3,4,8–10] Together, BI-1 appears to be potent in suppressing cell death induced by a multitude of stimuli in eukaryotes from different kingdoms (Table 3).

Plant mutants that show spontaneous cell death, also called lesion mimic mutants, often show enhanced resistance to certain pathogens.[35] This can be linked either to a status of constitutive induced resistance when mutants spontaneously express defence responses or to a lowered threshold for a hypersensitive reaction that involves a programmed cell death restricting pathogen development at the site of invasion. In some mutants, cell death and resistance can be phenotypically uncoupled, for instance when resistance and cell death are expressed in different developmental stages. The barley *Mlo*-mutation confers broad spectrum resistance to the obligate biotrophic leaf pathogen powdery mildew fungus *Blumeria graminis* in young seedlings and leads to spontaneous leaf cell death

Table 3. Examples of PCD suppressed or controlled by BI-1 in mammals, plants and yeast

Species/organ, cell line	PCD controlled by BI-1	Reference
Homo sapiens/293	Mouse BAX-induced	17, 19
Homo sapiens/GM701	Serum deprivation-induced	17
Homo sapiens/ FL5.12	Staurosporine-induced	17
Homo sapiens/ FL5.12	Etoposide-induced	17
Homo sapiens/HeLa	*Chlamydia psittaci*-induced	31
Homo sapiens/SW80	TRAIL-induced[a]	30
Homo sapiens/PC-3, LNCaP, DU-145, 293	Spontaneous	15, 17
Saccharomyces cerevisiae	Mouse BAX-induced	3, 17, 19, 21, 22, 32
Saccharomyces cerevisiae	Heat shock	32
Saccharomyces cerevisiae	H_2O_2-induced	32
Arabidopsis thaliana/entire plants	Mouse BAX-induced	3
Nicotiana tabacum/BY-2 cells	H_2O_2-induced	29
Nicotiana tabacum/BY-2 cells	Salicylate-induced	29
Nicotiana tabacum/BY-2 cells	Starvation-induced	18
Nicotiana tabacum/leaf discs	Cold shock	32
Nicotiana tabacum/leaf discs	Heat shock	32
Oryza sativa/cell suspension	Fungal cell wall elicitor-induced	26
Oryza sativa/cell suspension	Salicylate-induced	26

[a]Results could not be confirmed in independent experiments.

and early senescence in adult leaves.[36] However, pathogen resistance is mediated by a penetration resistance mechanism involving secretion of defence compounds and localized H_2O_2 accumulation instead of hypersensitive cell death.[27,36,37] On the first view, this appears to separate disease resistance from cell death regulation but over-expression of barley BI-1 in leaf cells attacked by the *B. graminis* abolishes penetration resistance and allows fungal development on *mlo*-barley. This may indicate that cell death regulation and defence regulation in plants are linked even if pathogen resistance does not depend on a cell death mechanism. Over-expression of MLA resistance proteins of the CC-NBS-LRR class, that normally confer race-specific resistance to *B. graminis* by a hypersensitive cell death reaction, leads to penetration resistance strengthening the connection between cell death regulation and non-cell death defence mechanisms.[38] Plant resistance proteins share the so-called NBS-ARC motif with APAF1.[39] The function of such R proteins apparently depends on the SCF ubiquitination complex that may control proteolysis of cell death inhibitors.[40] In animal PCD, ubiquitination and removal of pro- or antiapoptotic proteins is pivotal for cell death regulation.[41]

Over-expression of BI-1 in barley also induces cellular susceptibility to the wheat pathogen *Blumeria graminis* f.sp. *tritici* that normally cannot grow on barley. This indicates that virulence of obligate pathogens with a limited host range might depend on their ability to suppress cell death during establishment of pathogenesis.[42] Together, cell death regulator proteins such as BI-1 appear to be central regulators of pathogen defence. Thereby, they seem to finetune stress responses and cell death. However, it is not clear whether BI-1 controls defence and cell death independently or by a common mechanism.

BI-1 protein properties, subcellular localization and potential BI-1 functions

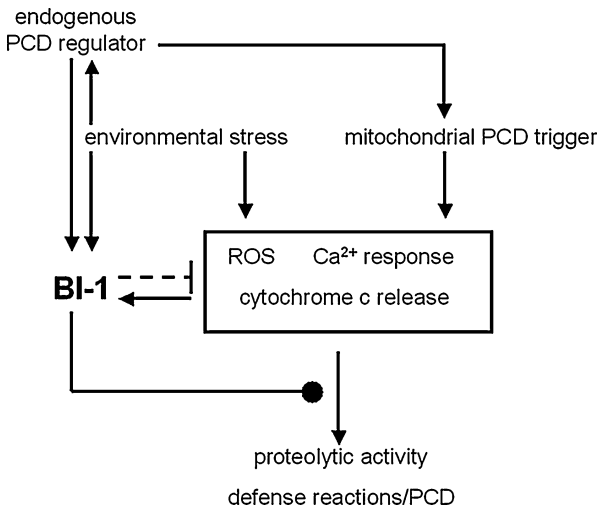
BI-1 is a small TM protein of 25–27 kDa with 6–7 TM domains. The protein was detected in the nucleus and the heavy membrane fraction of 293T cells, including rough ER, mitochondria and lysosomes. Both C- and N-terminal fusion proteins with the green fluorescing protein were imaged in the ER in continuum with the nuclear envelope in mammalian and plant cells.[3,17,19] *In vitro* translation of BI-1 mRNA required a microsomal fraction supporting association of BI-1 with the ER during protein synthesis.[43] Two findings support that the charged C-terminus of BI-1 is outside of the ER. First, Bolduc *et al*. (2003) found that digitonin digestion of tobacco cells, which permeabilises the PM, but not endomembranes, allowed anti GFP-immnuodetection of BI-1-GFP (C-terminal GFP fusion) in the ER.[19] Second, most eukaryotic BI-1 proteins comprise RXR and/or KKXX-like amino acid sequences close to the C-terminus representing motifs that typically mediate ER retention of membrane proteins with the C-terminus in the cytoplasm.[44] Interestingly, the charged C-terminus of BI-1 that might be

also involved in protein-protein interaction is important for cell death regulation.[29,32,34] Besides the C-terminus, there are no obvious amino acid stretches of BI-1 that are likely to interact with other proteins. The non-TM regions of BI-1 are relatively short. This together with the presence of a few charged residues within the predicted TM domains led to the assumption that BI-1 could function as a pore or ion channel.[17,19]

Since human BI-1 does not physically interact with BAX and no clear association of BI-1 with mitochondria could be demonstrated thus far, a physical interaction of BI-1 and mitochondria seems not very likely.[3,17,19] However, BI-1 can interact with other human cell death regulators, *e.g.* Bcl-2 and Bcl-X_L, apparently via their BH4 domain that is specific for antiapoptotic Bcl-2 family members.[17] BI-1 might affect other signalling pathways downstream of mitochondria as well, thereby monitoring mitochondrial functions without physical interaction with these organelles. Alternatively, mitochondria might physically interact with the ER or BI-1 could be translocated under stress conditions.

Taking into account that the ER functions as a calcium reservoir and that a possible ion channel activity of BI-1 was proposed, BI-1 could be implicated in the regulation of cytosolic calcium and/or the redox status.[17,19,24,26] This could also explain why BI-1 functions in plants where no Bcl-2 family members are present but calcium and redox homeostasis are implicated in cell survival. Recently, Kawai-Yamada and co-workers from the Uchimiya laboratory have shown, that *Arabidopsis* BI-1 can protect tobacco BY-2 cell cultures from cell death induced by H_2O_2 supporting a role of BI-1 in redox regulation.[29] Similar, BI-1 protected plant cells from salicylic acid induced cell death, which is also linked with H_2O_2 accumulation.[8,26,29,45] Matsumura and associates have shown that the same type of rice cell death that is inhibited by BI-1 can be blocked by treatment with diphenylene iodonium, an inhibitor of the superoxide anion radical producing NADPH oxidase.[26] However, in *Arabidopsis*, mammalian BAX induced intracellular $O_2^{\bullet -}$ generation that could not be blocked by over-expression of BI-1 although cell death was strongly inhibited. This can be explained if BI-1 functions downstream of oxidative stress.[29] In a different approach, plant proteins other than BI-1, that can interfere with BAX function, have been discovered by screening in yeast. Among these, three proteins are antioxidants, a Fe-superoxide dismutase, a cytosolic ascorbate peroxidase and a glutathione S-transferase/peroxidase.[46–48] This indicated that the BAX induced cell death functioning in yeast and plants includes an essential pertubation of the redox homeostasis of the dying cell. Indeed, BAX induces accumulation of reactive oxygen intermediates in yeast, BI-1 protects yeast from H_2O_2-induced cell death and redox dependent mammalian PCD is conserved in mitochondria and prokaryotes.[32,49–51]

Figure 1. Hypothetical involvement of BI-1 in PCD regulation in eukaryotes. Both endogenous factors and environmental stress can induce PCD in eukaryotes. Reactive oxygen species (ROS), high levels of cytosolic Ca^{2+} and cytochrome *c* release can induce PCD and stress reactions both alone or in combination with each other. Finally, PCD depends on proteolytic activities, which may be directly or indirectly controlled by BI-1 function. BI-1 apparently works downstream or independent from cytochrome *c* release. However, BI-1 expression might be regulated by stress factors and upstream PCD regulators and/or activated by a mechanism, which monitors mitochondrial function, ROS and/or Ca^{2+} levels.



Over-expression of plant BI-1 alone is sufficient to inhibit mammalian cell death in a dose-dependent manner.[19] This stand-alone function may indicate that BI-1 does not require biophysical or biochemical activation by upstream signals, but is regulated mainly at the expression level. However, today no clear pictures of BI-1 protein topology and the mechanism of BI-1 action exist. To develop hypotheses about the mode of BI-1 action, it will be further important to uncover more proteins that physically and/or functionally interact with BI-1. This is especially important for plant research since it may discover plant Bcl-2 analogue PCD regulators. Alternatively, plant BI-1 homologues could functionally substitute for Bcl-2 proteins since in the *Arabidopsis* genome two close BI-1 relatives and further proteins of similar membrane topology can be identified (see also *Arabidopsis* membrane protein library at http://www.cbs.umn.edu/arabidopsis/).[10]

Hypothetical mechanisms of BI-1 involvement in PCD mechanisms are summarized in Figure 1.

BI-1 homologues in eukaryotes, prokaryotes and viruses

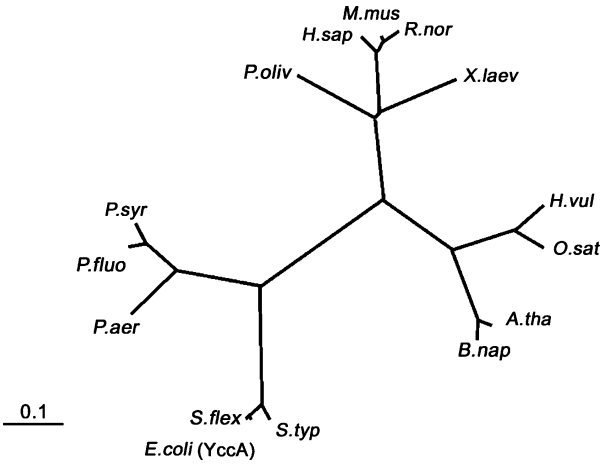
Besides higher eukatyotes, unicellular eukaryotes such as *Plasmodium falciparum* and *Cryptosporidium parvum* possess a

Figure 2. BI-1 amino acid pattern. Sequences were taken from Interpro PS01243 at EBI (http://www.ebi.ac.uk/index.html).[56] GenBank accessions: *Homo sapiens*, CAA53472 aa 96-125; *Rattus norvegicus*, CAA53470 aa 95-124; *Paralichthys olivaceus*, AAF61067 aa 96-125; *Drosophila melanogaster*, AAL13606 aa 99-128; *Oryza sativa*, BAA89540 aa 102-131; *Arabidopsis thaliana*, BAA89541 aa 101-130; *Escherichia coli*, CAA25218 aa 87-117; *Haemophilus influenzae*, AAC21722 aa 87-117; *Pseudomonas aeruginosa*, BAA02671 aa 88-119.

BI-1-like protein sequence. These proteins show 29–35% identity and about 50% similarity with human BI-1. It would be interesting to learn as to whether these proteins are cell death regulators in protozoa. Eukaryotic BI-1 proteins share a common amino acid pattern with bacterial proteins (Figure 2). This pattern starts in the third of seven predicted TM domains and ends in the fourth TM domain. One bacterial protein containing this pattern is the *E. coli* YCCA. BI-1 has also an overall domain architecture quite similar to YCCA.[12] YCCA interacts with the ATP and Zn^{2+} dependent metalloprotease FtsH, a member of the AAA ATPase superfamily that is also present in eukaryotes. It was speculated that YCCA regulates FtsH chaperone or protease function.[52] If such a function would be conserved in eukaryotes, this would give rise to speculations that BI-1 is part of a protein quality control or degradation machinery. Accordingly, plant chloroplastic FtsH homologues are functional in cell death regulation during the hypersensitive pathogen response.[53] Presumably, BI-1 is of bacterial or endosymbiotic origin and was introduced into the eukaryotic genome by horizontal gene transfer. A similar evolution was suggested for other PCD regulators, e.g. caspases and NTPases.[54]

Astonishingly, even viruses code for proteins with a domain architecture similar to BI-1. This may indicate that BI-1 has been corrupted during evolution by pathogens to reprogram a living host cell. Similarly, Bcl-2 homologues can be identified in the sequence of Epstein-Barr-Virus and other viruses.[55] The overall domain architecture of BI-1 is reflected in the Interpro accession IPR006214 covering far more than 100 proteins from different species.[56] Figure 3 shows a phylogenetic tree of a selection of these proteins. Protein architecture conservation provides an additional hint that BI-1 is an ancient protein and might be of prokaryotic origin. This was recently underscored by the finding that an *E. coli* YCCA-BI-1 homologous protein could partially protect yeast from mammalian BAX-induced cell death.[32]

Figure 3. Unrooted phylogenetic tree of proteins with domain architecture similar to BI-1. Proteins were identified via BLASTP at NCBI (http://www.ncbi.nlm.nih.gov/) or Interpro at EBI (http://www.ebi.ac.uk/index.html) and aligned and clustered by ClustalW at EBI (http://www.ebi.ac.uk/clustalw/).[56,60,61] GenBank accessions: *Homo sapiens*, CAA53472; *Mus musculus*, BAB31892; *Rattus norvegicus*, CAA53470; *Xenopus laevis*, AAH47131, *Paralichthys olivaceus*, AAF61067; *Hordeum vulgare*, CAC37797; *Oryza sativa*, BAA89540; *Arabidopsis thaliana*, BAA89541; *Brassica napus*, AAK73101; *Pseudomonas fluorescence*, ZP_00083444; *Escherichia coli*, CAA25218; *Shigella flexneri*, AAN42600; *Salmonella typhimurium*, AAL20018; *Pseudomonas syringae*, NP_793116; *Pseudomonas aeruginosa*, BAA02671.



Conclusions

The BAX inhibitor-1 is functionally and structurally conserved in eukaryotes. It might stem from an early eukaryotic ancestor or even from a prokaryotic or endosymbiotic origin. This assumption is underscored because BI-1 is cross-functional in mammals and plants. In members of both kingdoms, it is expressed in tissues where cell death suppression is either deregulated such as in tumour cells or required to keep stress-induced or developmental PCD under control. Therefore, BI-1 might serve as a molecular expression marker for both proper and pathogenesis-related cell death regulation. Since BI-1 is a potent cell death suppressor, it represents an excellent tool to study cell death mechanisms. Understanding BI-1 function might draw back to an evolutionary old cell death regulation process, which is fundamental for all eukaryotes. The potential endosymbiotic origin of BI-1 additionally opens a door to understand the interaction of eukaryotes with obligate intracellular pathogens and symbionts. This may open up a new strategic field for plant protection measures by specifically targeting plant PCD factors. Finally, therapeutic interference with BI-1

function might be a novel strategy to treat both infection and malignant human diseases.

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Special introduction to chapters 12-14:

Chapters 12-14 deal with non-host resistance and its link to host resistance to *B. graminis*. In this regard, the studies concentrate mainly on the role of *mlo*, *ror1* and *ror2* in response to *Bgt* and *Bgh*. Besides this, a set of different barley accessions was compared for their non-host resistance phenotypes in response to *Bgt*. Taken together, a similar role for *ror1* and *ror2* in host and non-host resistance to *B. graminis* was observed. Including literature data, the effect of *mlo* on non-host resistance seemed to depend on the aggressiveness of the *Bgt* isolate used. Additionally, a multiplicity of different non-host defence reactions of different barley accessions provoked the assumption that non-host resistance to *Bgt* is based on multiple defence pathways in barley.

Chapter 12

Hückelhoven R, Dechert C, Kogel K-H (2001) Non-host resistance of barley is associated with a hydrogen peroxide burst at sites of attempted penetration by wheat powdery mildew fungus. *Mol Plant Pathol* 2: 199-205

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Non-host resistance of barley is associated with a hydrogen peroxide burst at sites of attempted penetration by wheat powdery mildew fungus

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SUMMARY

In barley, non-host resistance against the wheat powdery mildew fungus (*Blumeria graminis* f.sp. *tritici, Bgt*) is associated with the formation of cell wall appositions and a hypersensitive reaction in which epidermal cells die rapidly in response to fungal attack. In the interaction of barley with the pathogenic barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei, Bgh*), these defence reactions are also associated with accumulation of H_2O_2. To elucidate the mechanism of non-host resistance, the accumulation of H_2O_2 in response to *Bgt* was studied *in situ* by histochemical staining with diaminobenzidine. H_2O_2 accumulation was found in cell wall appositions under appressoria from *Bgt* and in cells undergoing a hypersensitive reaction. A mutation (*mlo5*) at the barley *Mlo* locus, that confers broad spectrum resistance to *Bgh*, did not influence the barley defence phenotype to *Bgt*. Significantly, *Bgt* triggered cell death on *mlo5*-barley while *Bgh* did not.

INTRODUCTION

The most common and effective durable resistance is non-host resistance that prevents the majority of plants from becoming infected by most potential pathogens. However, in most cases the mechanisms involved in non-host resistance are poorly understood. The same is true for factors controlling the fact that grasses are usually infected by only one appropriate *forma specialis* of the powdery mildew fungus *Blumeria graminis* (DC) Speer (syn. *Erysiphe graminis* DC). For instance, *Blumeria graminis* f.sp. *hordei* Em. Marchal (*Bgh*) establishes a compatible interaction with susceptible barley plants but not with any other grass. Similarly, inappropriate *formae speciales* of *Blumeria graminis* and other powdery mildew fungi such as *Erysiphe pisi* or *Erysiphe cichoracearum* do not infect barley (Johnson *et al.*, 1982; Kobayashi *et al.*, 1990, 1997; Tosa *et al.*, 1990). On the one hand, species-specificity might be caused by the fact that some plant species are poor substrates for particular pathogens. On the other hand, features that make the plant surface inhospitable, or lack of fungal pathogenesis or host compatibility factors, as well as active defence responses, may contribute to incompatibility to different degrees in each non-host–pathogen interaction (Johnson *et al.*, 1982; Schulze-Lefert and Vogel, 2000).

Barley is a non-host for the wheat powdery mildew fungus, *Blumeria graminis* f.sp. *tritici* (*Bgt*). When attacked by this inappropriate *forma specialis* of the fungus, barley expresses a broad range of defence responses similar to those observed after inoculation with an avirulent race of *Bgh*. These responses include the formation of cell wall appositions, hypersensitive reaction (HR) in attacked and/or penetrated epidermal cells, accumulation of autofluorescent material in attacked cells and pathogenesis-related host gene expression (Carver *et al.*, 1992; Gregersen *et al.*, 1990; Peterhänsel *et al.*, 1997; Tosa and Shishiyama, 1984; Vallélian-Bindschedler *et al.*, 1998). A typical feature of effective defence against *Bgh* in barley is the accumulation of H_2O_2 at sites of fungal attack, occurring in two or three phases depending on the barley genotype (Hückelhoven *et al.*, 2000a; Thordal-Christensen *et al.*, 1997). The first phase is associated with the attachment of the primary germ tube to the leaf surface, the second phase occurs at sites of attempted fungal penetration from appressoria, and the third phase is closely linked to the onset of HR. Whereas phase I and II are localized to subcellular sites near the germ tubes, phase III is a whole cell response. In barley expressing penetration resistance to *Bgh*, phase II predominates and occurs mainly as H_2O_2 accumulation in effective cell wall appositions (papillae) subtending appressoria and in vesicles, cytoplasm and cell wall regions surrounding the attack site.

Mutations at the barley *Mlo* locus lead to broad-spectrum resistance against *Bgh* and have concomitant pleiotropic effects. In *mlo*-barley, such as backcross lines of Ingrid or Pallas bearing *mlo5* alleles, the *Bgh*-triggered oxidative burst results in up to 95% of cells reacting by accumulating H_2O_2 in papillae beneath appressoria. This kind of oxidative burst is not followed by a

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hypersensitive reaction (HR) within the attacked epidermal cells (Hückelhoven *et al.*, 1999, 2000b). The view that MLO may function as an element controlling the cellular redox status is supported by the strength of this H_2O_2 burst, along with the finding that *mlo*-barley also reacts with enhanced H_2O_2 accumulation following treatment with toxins of *Cochliobolus sativus* (Kumar *et al.,* 2001). This may also explain why *mlo*-barley forms papillae and expresses defence genes so massively in response to *Bgh* attack, and why *Mlo*-mutations are also associated with spontaneous leaf cell death. Furthermore, a lower elicitation threshold might be required to trigger these defence responses, so that that *mlo*-barley may be physiologically 'primed' for response to *Bgh* attack (Büschges *et al.*, 1997; Hückelhoven *et al.*, 2000b; Jørgensen and Mortensen, 1977; Jørgensen, 1994; Peterhänsel *et al.*, 1997; Stolzenburg *et al.*, 1984; von Röpenack *et al.*, 1998; Wolter *et al.*, 1993). Disturbed redox regulation in *mlo*-lines may also be involved in hypersusceptibility to the necrotrophic pathogen *Magnaporthe grisea* (Jarosch *et al.,* 1999). However, the interaction of barley with biotrophic pathogens other than *Bgh* does not seem to be altered in *Mlo*-mutants (Jørgensen, 1992) questioning a general role for the effects of MLO on cell redox status in the control of defence responses against fungal attack. In this study, we aimed to elucidate the role of H_2O_2 and *Mlo* in the interaction of barley with *Bgt*. We report here that the non-host resistance of barley to *Bgt* is associated with H_2O_2 accumulation in papillae and HR cells, and that this oxidative burst is not affected by the function of *Mlo* during early interaction stages.

RESULTS

Fungal development and barley response

To study the non-host response of barley, we inoculated primary leaves of barley (*Hordeum vulgare*) with *Bgt* (the wheat powdery mildew fungus) isolate A95 (*Bgt*A95). For a microscopic evaluation of the host oxidative burst at 18 h after inoculation (hai) and 22 hai we injected a solution of diaminobenzidine (DAB) which is polymerized *in situ* at sites of H_2O_2 accumulation (Thordal-Christensen *et al.*, 1997). Almost all the germinated spores formed appressoria, so cases where they failed to do so were ignored during the quantitative evaluation of interaction phenotypes. Dark brown DAB polymers could easily be detected subcellularly in cleared leaves, and different patterns of staining could be distinguished and quantified. It was easy to differentiate cells with subcellular staining from those showing whole-cell staining (Fig. 1A,B). The latter was most often associated with HR, as confirmed by either discontinuity of cytoplasmic strands or whole-cell autofluorescence (Görg *et al.*, 1993; Hückelhoven *et al.*, 2000a; Koga *et al.*, 1990). Subcellular staining was observed in papillae beneath primary germ tubes, beneath the appressoria (Fig. 1B,D), in anticlinal cell walls near papillae (Fig. 1C) or in mesophyll cells beneath interaction sites (not shown). Identical staining patterns indicating H_2O_2 accumulation have previously been described in the interaction of barley with *Bgh* (Thordal-Christensen *et al.,* 1997; Hückelhoven *et al.*, 2000b). Subcellular staining was most often observed in cells that remained alive until fixation (no whole-cell autofluorescence, apparently continuous cytoplasmic strands). We distinguished two categories of interaction sites associated with arrested *Bgt* development: barley cells either formed papillae and survived attack or they showed HR. Occasionally, cells showing HR had been penetrated by *Bgt* so that a rudimentary haustorium was visible within the dead cell (Fig. 1E,F). Interestingly, this was observed in both *Mlo* and *mlo5* barleys, even though *mlo5*-barley was not penetrated by natural European isolates of the barley powdery mildew fungus.

Quantitative analysis of non-host reactions of *Mlo*- and *mlo5*-genotypes

To compare the non-host response of wild-type *Mlo*- and *mlo*-barley quantitatively, we counted the frequencies of sites with effective papilla or HR on primary leaves of cv. Pallas (*Mlo*) and a backcross line of Pallas (P22, *mlo5*) as well as cv. Ingrid (*Mlo*) and I22 (*mlo5*) (Fig. 2). We used the cvs. Pallas and Ingrid to confirm that observed phenotypes are independent of the genetic background of the *Mlo* locus.

At most interaction sites fungal germlings were arrested before they had penetrated and the attacked cells remained alive. This phenotype was seen in the range of 70% to 84% of interaction sites and its frequency was independent of genotype and time of fixation (18 or 22 hai, Fig. 2). All other penetration attempts (16% to 30%) were associated with HR (Fig. 2) and again the frequency of this response was independent of genotype and time of fixation. By light microscopy, it was not possible to distinguish whether HR at particular interaction sites was a consequence of penetration by *Bgt* or a secondary response after papilla formation. However, in some cases penetration could be confirmed because immature haustoria of *Bgt* were clearly visible within HR cells (Fig. 1F).

When barley lines Pallas and P22 were inoculated with the appropriate *forma specialis* (*Bgh*) the fungus often penetrated Pallas successfully whereas this was never observed on P22. Haustoria of *Bgh* within living Pallas cells reached a size which easily allowed their detection by light microscopy at 22 hai (see Hückelhoven *et al.*, 1999). Evaluation at 22 hai revealed successful penetration in Pallas at about 48% of the interaction sites (Fig. 3). The remaining 52% of fungal germlings failed to penetrate as indicated by the absence of a visible haustorium. Penetration resistance of 35% of attacked cells was associated with the formation of a papilla which appeared to arrest fungal growth (designated as effective papilla). Of the attacked cells, 17% underwent HR. On P22 all fungal penetration attempts

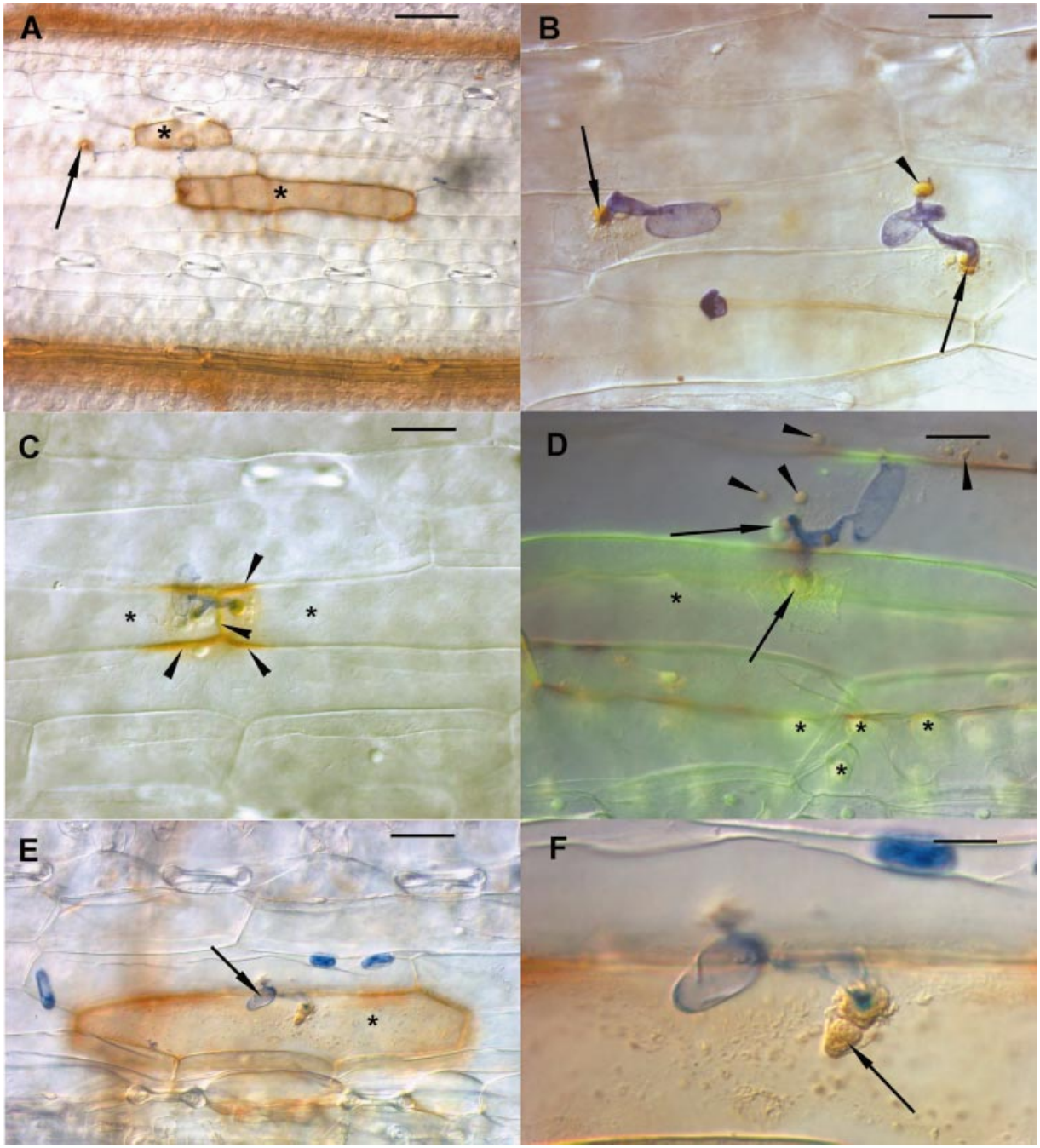

Fig. 1 (A–F) Microscopic detection of H_2O_2 accumulation at interaction sites of barley and *Blumeria graminis* f.sp. *tritici*. (A) Overview of a leaf cutting of cv. Pallas at 22 hai. Brown DAB polymers indicating H_2O_2 accumulation are visible in cells which underwent HR (asterisks) and in a papilla and surrounding halo (arrow). DAB polymerization takes place at sites of fungal attack and in leaf vessels visible at the top and the bottom of the picture. Bar = 60 µm. (B) Two interaction sites where fungal development was stopped during the penetration attempt in P22 at 22 hai. DAB staining is visible in papillae beneath the primary and appressorial germ tubes (arrowhead and arrows). Bar = 18 µm. (C) Interaction site where a fungal germling attacked two neighbouring cells (asterisks). Anticlinal cell walls close to the penetration attempts are stained intensively with DAB (arrowheads). Bar = 25 µm. (D) Superimposed brightfield and fluorescence micrographs of an interaction site 22 hai in P22. DAB staining is visible at papillae beneath the appressorium and in vesicles at the papilla and anticlinal cell walls (arrowheads). Autofluorescence is visible in papillae (arrows) and cell walls in the attacked cell and underlying mesophyll cells (asterisks). Bar = 18 µm (E and F) Whole-cell H_2O_2 accumulation on P22 at 22 hai. The fungal germling (arrow in E) penetrated the papilla and caused HR of the attacked cell (asterisk) which has a granulated cytoplasm. Papilla and the haustorial initial (arrow in F) are stained intensely with DAB. Bar = 36 µm and 12 µm in E and F, respectively.

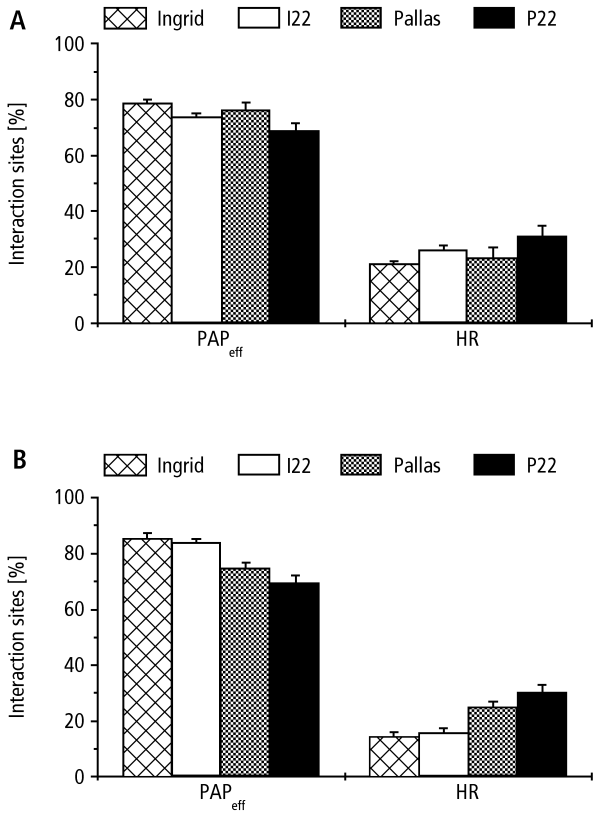

Fig. 2 (A, B) Defence responses of the non-host barley lines Ingrid, I22, Pallas and P22 at 18 hai (A) and 22 hai (B) with *Blumeria graminis* f.sp. *tritici*. Interaction sites where a fungal germling failed to penetrate are designated PAP_{eff} (effective papilla). None of these sites showed whole-cell autofluorescence. Interaction sites where the entire attacked cell showed autofluorescence and/or cytoplasm was apparently granulated were counted as hypersensitive reaction (HR). Each column represents 400–500 interaction sites on 4–5 leaves (100 sites each) with standard error bars. Repetition of the experiments led to similar results.

failed. Less than 1% of attacked cells underwent HR (Fig. 3) and these cells never contained visible haustoria.

Frequencies of H_2O_2 accumulation patterns

In all barley–*Bgt* interactions, papillae or cytoplasmic aggregates beneath primary germ tubes and appressoria were the predominant sites of DAB staining, and therefore H_2O_2 accumulation. The proportion of cases with DAB polymers visible at one or both of these sites ranged between 40% and 70% (Fig. 4). Anticlinal cell walls near papilla were also intensely stained, especially in Ingrid and I22 at 18 hai. The percentage of epidermal cells, that showed whole-cell DAB staining, ranged from 23% to 30% at 18 hai and from 16% to 30% at 22 hai. Frequencies of these events were not significantly different between *Mlo* and *mlo5* genotypes (Fig. 4.). Thus, comparing Figs 2 and 4, there was a very close

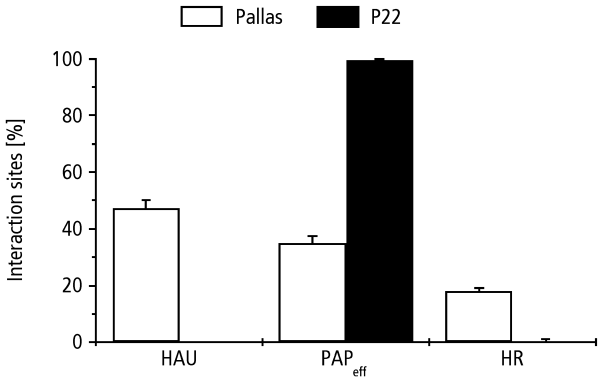

Fig. 3 Fungal development and defence responses of barley lines Pallas and P22 22 hai with *Blumeria graminis* f.sp. *hordei*. Successful penetration events which resulted in formation of an immature haustorium in the attacked cell are summarized as HAU. PAP_{eff} and HR as in Fig. 2. Each column represents 300 interaction sites on three leaves (100 sites each) with standard errors bars.

correlation between HR frequency and whole cell DAB staining in these genotypes.

DISCUSSION

Two main questions have been addressed by these investigations. First, is the non-host interaction of barley and *Bgt* associated with an oxidative burst as demonstrated for the interaction of barley and its appropriate pathogen *Bgh*? Secondly, is this non-host interaction influenced by the function of *Mlo*?

Regarding the first question, we show here that H_2O_2, previously found to be linked to defence of barley against *Bgh*, also accumulates in the non-host response of barley to *Bgt* (Figs 1 and 4). Interestingly, Bestwick *et al.* (1998) showed that non-host resistance of lettuce against *Pseudomonas syringae* pv *phaseolicola* is also associated with increased peroxidase activities, H_2O_2 accumulation and HR. In this study, H_2O_2 was detected in the cell wall at bacterial attachment sites and in the cytoplasm of cells undergoing HR. Recently, Mellersh and Heath (2001) found that non-host defence of cowpea to *Erysiphe cichoracearum* is associated with H_2O_2 accumulation in papillae and halos. We detected H_2O_2 accumulation at similar sites in barley attacked by *Bgt*.

The spatial distribution of H_2O_2 accumulation in the barley–*Bgt* interaction was also similar to that found in response to *Bgh* (Fig. 1). H_2O_2 accumulation in cell wall appositions beneath *Bgt* primary germ tubes, appressoria and in entire attacked cells, indicate temporal occurrence of the oxidative burst in two or three phases similar to those found after inoculation with an appropriate *forma specialis*. Whole-cell H_2O_2 accumulation was also detected in penetrated cells. In the interaction of barley and *Bgh*, whole-cell H_2O_2 accumulation in penetrated cells is usually limited to interactions which follow the gene-for-gene model, e.g. *Mla12-AvrMla12* (Flor, 1971; Hückelhoven *et al.*, 2000a).

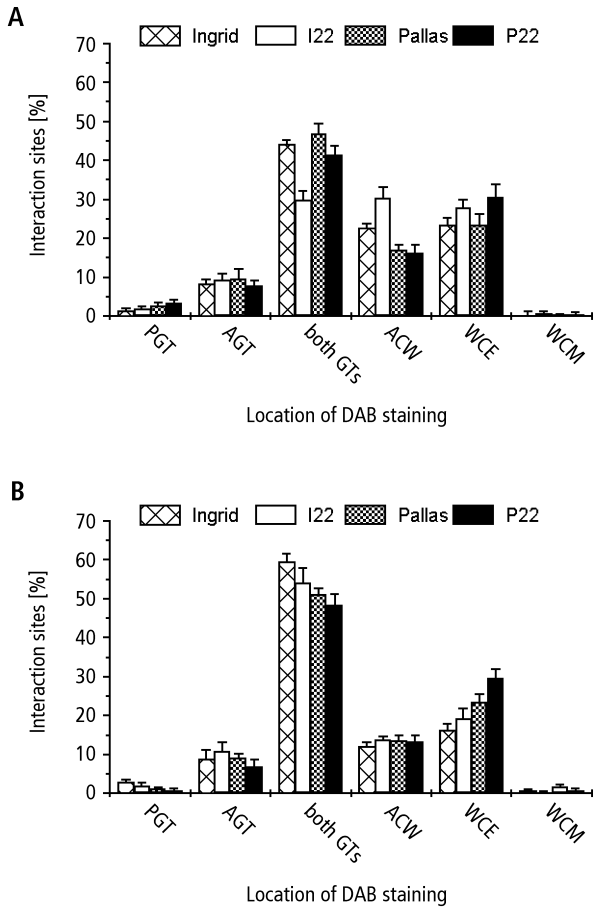

Fig. 4 (A, B) H_2O_2 accumulation at interaction sites of the non-host barley lines Ingrid, I22, Pallas and P22 at 18 hai (A) and 22 hai (B) with *Blumeria graminis* f.sp. *tritici*. H_2O_2 accumulation visualized by DAB staining was detected beneath primary germ tubes alone (PGT), appressorial germ tubes alone (AGT), both germ tubes (both GTs), in anticlinal cell walls close to penetration attempts (ACW), in whole cells in the epidermis (WCE) or in whole cells in the mesophyll (WCM). Each column represents 400–500 interaction sites on 4–5 leaves (100 sites each) with standard error bars. Repetition of the experiments led to similar results.

Some isolates of *Bgt* are able to form mature haustoria and some conidia on barley cv. Turkey 290 (Tosa and Shishiyama, 1984). Therefore, it seems possible that a basic compatibility exists between all *formae speciales* of *Blumeria graminis* and all grass species. This would suggest that non-host resistance to inappropriate *formae speciales* may be determined by gene-for-gene relationships (Heath, 2001). Support for this suggestion comes from the fact that crosses of *Bgt* with *Blumeria graminis* f.sp. *agropyri* led to an F1 generation that segregated for four major genes determining avirulence on wheat via corresponding wheat genes (e.g. Tosa, 1992).

Regarding the question on the role of MLO in non-host defence, Peterhänsel *et al*. (1997) found that I22 inoculated with *Bgt* isolate JIW28 did not undergo epidermal HR, whereas we found that I22 responded to *Bgt*A95 with the same frequency of HR as the wild-type Ingrid. The findings of Peterhänsel *et al*. (1997) indicate that the effect of *mlo5* can be epistatic over some non-host determinants at the *forma specialis* level, just as it can be over gene-for-gene interactions in barley-*Bgh* systems such as *Mla8-AvrMla8*. However, our results from the interaction of *mlo5*-barley with *Bgt*A95 suggest an alternative view in this system where the inappropriate *forma specialis* caused HR and the appropriate *forma specialis* did not. In addition, we occasionally found that *Bgt*A95 penetrated *mlo5*-barley and formed immature haustoria before the cells underwent HR and showed whole cell DAB staining. Thus, at least the isolate *Bgt*A95 as an inappropriate pathogen was able to overcome typical *mlo5*-mediated penetration resistance that was totally effective against the appropriate *forma specialis*. Together, our data show clearly that *mlo5*-barley is able to undergo epidermal HR as a response to *B. graminis* attack and therefore this response is not necessarily suppressed by the presence of the *mlo* allele. This raises the question of whether a lack of epidermal HR in *mlo*-barley during attack by *Bgh* is a result of response suppression by the fungus or because the appropriate *forma specialis* fails to trigger the HR. Non-host HR in *mlo5*-barley clearly indicates that *Mlo* function is not required for epidermal HR following *B. graminis* attack. This points to host species-specific suppression of epidermal HR by *Bgh* on *mlo5*-barley. Although evidence for post penetration cell death suppression by *Blumeria graminis* also exists (Hückelhoven *et al*., 1999; Lyngkjær and Carver, 1999; Wäspi *et al*., 2001), a number of arguments support the idea that HR is simply not triggered by *Bgh* on *mlo5*-barley: low levels of HR on susceptible wild-type barley are triggered by race nonspecific components which are released during the fungal penetration attempt but before haustorium formation (Fig. 3). *Bgh* is stopped on *mlo*-barley in an early stage of this penetration attempt, probably before a threshold amount of HR elicitors are released. This is further indicated by Ingrid-*mlo-ror* double mutants which allow greater progress of *Blumeria graminis* development than I22, in addition to showing more HR than I22 (Hückelhoven *et al*., 2000b; Peterhänsel *et al*., 1997). Why *Bgt*A95 progresses further on *mlo5*-barley than *Bgh* remains unanswered.

EXPERIMENTAL PROCEDURES

Plants, pathogens and inoculation

The barley (*Hordeum vulgare* L.) cv. Ingrid (*Mlo*), the backcross line BCIngrid-*mlo5* (I22), cv. Pallas (*Mlo*) and BCPallas-*mlo5* (P22) were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Their generation was described previously (Kølster *et al*., 1986). Plants were grown in a growth chamber at 18 °C with 60% relative humidity and a photoperiod

of 16 h (60 μmol/m²/s photon flux density). The inappropriate fungus *B. graminis* f.sp. *tritici* (field isolate, Aachen, Germany, 1995, designated *Bgt*A95), was inoculated on to 7-day-old barley primary leaves to give a density of 10–20 conidia/mm². The appropriate barley powdery mildew fungus *B. graminis*. f.sp. *hordei*, race A6 (Wiberg, 1974) was inoculated on to barley primary leaves in the same manner. *Bgh* was maintained on cv. Golden Promise under the same conditions. *Bgt*A95 was maintained on wheat cv. Kanzler in the greenhouse.

Microscopy of infection structures and H_2O_2 cytochemistry

Leaf fixation [in 0.15% trichloroacetic acid (w/v) in ethyl-alcohol:chloroform (4 : 1; v/v)], bright-field microscopy and fluorescence microscopy was performed as described by Hückelhoven and Kogel (1998). Whole-cell autofluorescence and discontinuity of cytoplasmic strands were taken as a reliable measure of cell death (Görg *et al*., 1993; Hückelhoven *et al*., 2000a; Koga *et al*., 1990).

Because the defence reactions of short and long epidermal cells are different, only short cells directly adjacent to stomata (cell type A) and short cells not directly adjacent to stomata (type B) were evaluated, whereas long epidermal cells covering vascular tissue (type C) were excluded (for leaf epidermal cell distribution see Koga *et al*., 1990). Host cells with only one attempted penetration were evaluated exclusively to avoid misinterpretation due to induced effects.

H_2O_2 was detected using the DAB-method as described previously (Hückelhoven *et al*., 1999; Thordal-Christensen *et al*., 1997). 0.5 mg/mL DAB were dissolved in water (pH 3.8, HCl) and injected into barley primary leaves 2 h before fixation (Hagborg, 1970).

Different patterns of DAB staining were evaluated quantitatively. Where staining at individual interaction sites did not show a totally clear pattern, the site was classified according to the most intense stain reaction at that site. For example, if there was intense staining at the primary germ tube contact site, and staining was faint at the appressorium contact site, the site was considered to show only a primary germ tube reaction.

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Chapter 13

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Mechanistic and genetic overlap of barley host and non-host resistance to *Blumeria graminis*

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SUMMARY

Non-host resistance of barley to *Blumeria graminis* f.sp. *tritici* (*Bgt*), an inappropriate *forma specialis* of the grass powdery mildew fungus, is associated with formation of cell wall appositions (papillae) at sites of attempted fungal penetration and a hypersensitive cell death reaction (HR) of single attacked cells. Penetration resistance and HR are also typical features of race-non-specific and race-specific resistance of barley to the appropriate *Blumeria graminis* f.sp. *hordei* (*Bgh*), raising the question of whether genotypic differences in the cellular response of barley to *Bgt* are detectable. First, we analysed fungal penetration frequencies and HR in different barley accessions known to show altered non-host resistance. In genotypes with limited resistance to inappropriate cereal rust fungi, we concomitantly detected low penetration resistance to *Bgt* and significant differences of HR rates during attack from *Bgt*. Second, we tested barley mutants known to show altered host responses to *Bgh*. The *rar1*-mutation that suppresses many types of race-cultivar-specific resistances did not influence the non-host response of the *Bgt*-isolate used in this study. However, mutants of *Ror1* and *Ror2*, two genes required for full race non-specific penetration resistance of *mlo*-barley to barley powdery mildew fungus, exhibited altered defence response to *Bgt*, including higher frequencies of fungal penetration. On these mutants, growth of the inappropriate fungus was arrested subsequent to penetration by HR. Together, the data show that barley defence response to the wheat powdery mildew fungus is determined by similar factors as race-specific and race-non-specific resistance to appropriate *Bgh*.

INTRODUCTION

Plants are resistant to the attack from the majority of pathogenic micro-organisms. Plant pathogens have to overcome basic non-host resistance to establish a compatible interaction with a certain host (Heath, 1981; Thordal-Christensen, 2003). Once basic compatibility is established, the microbe has to cope with race-cultivar-specific and with race-cultivar non-specific resistance of its host. In the interaction of barley with the barley powdery mildew fungus, *Blumeria graminis* f.sp. *hordei* (*Bgh*), penetration resistance and the hypersensitive cell death reaction (HR) are involved in these types of resistance (Jørgensen, 1994). The inappropriate *forma specialis*, *Blumeria graminis* f.sp. *tritici* (*Bgt*), triggers a set of defence reactions that are hardly distinguishable from those triggered by avirulent *Bgh* (Carver *et al*., 1992; Hückelhoven *et al*., 2001; Kita *et al*., 1981; Tosa and Shishiyama, 1984; Tosa *et al*., 1990; Trujillo *et al*., 2004; Vallélian-Bindschedler *et al*., 1998).

As early as 1978, Hiura described the hybridization between *formae speciales* and the segregation of pathogenicity on different cereal hosts (Hiura, 1978). Tosa and Shishiyama (1984) also reported on differences between barley cultivars in the interaction with *Bgt*. Moreover, segregation analysis revealed involvement of major genes in the determination of the interaction outcome of grasses and inappropriate *formae speciales* of *Blumeria graminis* (Matsumura and Tosa, 1995; Tosa, 1992). Thus it appears possible that *forma specialis* non-host resistance relies on several recognition processes principally following the gene-for-gene model (Flor, 1971). In the barley–*Bgh* interaction, papilla formation is predominant in race non-specific resistance that is either partial or, when mediated by *mlo*-mutant alleles, complete. In most cases of race-specific resistance, HR rather than penetration resistance is observed, although penetration resistance can also be mediated by *R*-genes (Jørgensen, 1994). Whereas most race-specific Mla_x-mediated resistance responses require function of the genetic element *Rar1* (*required for Mla12-specified resistance 1*), broad *mlo*-mediated penetration resistance to *Bgh* is dependent on *Ror1* and *Ror2* (*required for mlo-specified resistance*) (Collins *et al*., 2003; Freialdenhoven *et al*., 1994, 1996; Hückelhoven *et al*., 2000a; Shirasu *et al*., 1999; Torp and Jørgensen, 1986). Mutation of *Ror* genes leads to partial loss of penetration resistance to *Bgh*. Interestingly, an *Arabidopsis* mutant

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with limited non-host penetration resistance to *Bgh* was recently shown to be mutated in the *Ror2* homologue *Pen1* gene that codes for a syntaxin, most probably involved in exocytosis of defence compounds (Collins *et al.*, 2003). In barley, such mutants are partly compromised in their ability to accumulate H_2O_2 at sites of fungal attack (Hückelhoven *et al.*, 2000b; Piffanelli *et al.*, 2002). H_2O_2 apparently is essential for effectiveness of penetration resistance to various fungal pathogens and onset of HR (reviewed by Hückelhoven and Kogel, 2003; Mellersh *et al.*, 2002).

Recently, Peart *et al.* (2002) have provided evidence for a common molecular basis of host and non-host resistance by silencing the RAR1 interactor SGT1 in *Nicotiana benthamiana.* This led to loss of *R*-gene-mediated resistance and non-host resistance to tobacco mosaic virus and different bacterial pathogens. It was also demonstrated that loss of *EDS1* (*enhanced disease susceptibility 1*) function together with pharmacological inhibition of actin rearrangement is sufficient to induce partial susceptibility of the non-host *Arabidopsis* to the wheat powdery mildew fungus *Bgt* (Yun *et al.*, 2003). The involvment of EDS1 in *R*-gene-mediated disease resistance (Falk *et al.*, 1999) supports the view that pathogen recognition and active host defence but not lack of basic compatibility factors determine non-host resistance of *Arabidopsis* to grass powdery mildew (Yun *et al.*, 2003). Supporting this idea, the sugar metabolism inhibitors D-mannose and 2-deoxy-D-glucose induced susceptibility to inappropriate *formae speciales* in barley, oat and wheat (Zeyen *et al.*, 2002).

However, the contribution of *R*-gene-like factors to non-host resistance is not well understood and may differ from one non-host system to another. In the interaction of *Arabidopsis* with the inappropriate rust fungus *Uromyces vignae*, for instance, the salicylic acid pathway rather than upstream EDS1 appears to be important for non-host resistance (Mellersh and Heath, 2003).

In this study, we provide evidence for multiple defence pathways involved in barley non-host resistance to *Bgt*.

RESULTS

Qualitative microscopic analysis of the barley–*Bgt* interaction

To elucidate further what stops inappropriate *B. graminis* on non-host barley, we carried out a detailed microscopic analysis of the interaction of different barley genotypes and the wheat powdery mildew isolate *Bgt*A95. Leaves were stained with the H_2O_2-sensitive dye 3,3-diaminobenzidine (DAB) and fixed at 18, 24, 30 and 48 h after inoculation (hai) and at 10 and 13 days after inoculation (dai). We observed varying degrees of fungal development in association with different types of plant defence responses.

The following general interaction phenotypes were distinguished: at 18 hai, cells attacked by *Bgt* reacted with cytoplasm aggregation and deposition of cell wall appositions (papillae) at sites of fungal attack. At a few interaction sites, we detected immature haustoria indicating that first penetration took place before 18 hai. Also, as early as 18 hai, HR was visible as whole-cell autofluorescence or whole-cell DAB staining, both being closely associated with HR (Görg *et al.*, 1993; Hückelhoven *et al.*, 2000a).

Most successful cell wall penetrations took place between 18 and 24 hai. During the same period, HR occurred in cells that either contained a visible haustorium or apparently resisted penetration. By 48 hai, mature haustoria with finger-like protrusions were found. At these sites, the fungus developed elongated secondary hyphae (ESH) between 24 and 48 hai. By 48 hai, cells of all genotypes that had supported fungal growth could be categorized into those that appeared not to react and those showing local cell wall reactions indicated by DAB staining or autofluorescence. Alternatively, penetrated cells showed HR that was normally accompanied by whole-cell DAB staining.

By 10 dai, most fungal germlings with ESH had stopped growing. Arrest of growth was associated with abnormal development of haustoria and fungal surface structures, and more often haustorium or whole-cell autofluorescence restricted to the attacked cell (data not shown).

As late as 13 dai, no sporulation could be observed with the naked eye on any barley line. However, on barley line SUSPMUR (see below), very few conidiophores with single basal cells carrying conidia were microscopically detected at 10 and 13 dai (data not shown). Together, the observations indicated various expressions of defence against inappropriate *Bgt*. Differences between lines were clearly discernible at 48 hai and therefore we quantified interaction phenotypes at this time-point.

Quantitative microscopic analysis of the interactions of barley lines SUSPMUR, SUSPTRIT and Trigo Biasa with *Bgt*

This study aimed to answer the question of whether non-host resistance of barley to *Bgt* is determined by similar factors as resistance to the appropriate pathogen, *Bgh*. A second question dealt with the analysis of barley genotypes with partial loss of non-host resistance to rusts in regard to their non-host resistance to powdery mildew. In a first set of experiments, we densely inoculated different barley lines with *Bgt*A95 and quantitatively analysed leaf segments fixed at 48 hai. In a first analysis, the cultivar Ingrid was compared with barley accessions SUSPTRIT and SUSPMUR, which exhibit full seedling susceptibility to the rust fungi *Puccinia triticina* and *P. hordei–murini*, respectively. SUSPTRIT and SUSPMUR were developed in a recurrent selection programme for increased levels of susceptibility of these two inappropriate rust species (Atienza *et al.*, 2004). Rare barley accessions were identified that were somewhat susceptible to *P. triticina* and *P. hordei–murini*, respectively (Niks *et al.*, 1996). Additionally, we included the Indonesian barley line Trigo Biasa, which is an

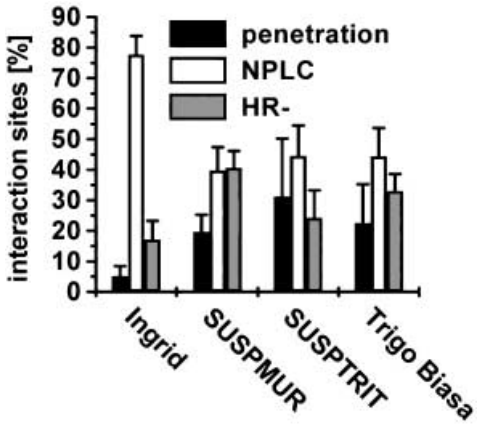

Fig. 1 Fungal development and plant defence reactions on different barley lines inoculated with *Bgt*A95. Indicated barley lines were densely inoculated and fixed at 48 hai for microscopic analysis. Black columns: interaction sites where penetration was confirmed by detection of an immature or mature haustorium within the first attacked cell (including cells that underwent HR, see Fig. 2). White columns: interaction sites where the fungus failed to penetrate and the attacked cell appeared intact (non-penetrated living cells, NPLC). Grey columns: interaction sites with HR of cells without detectable penetration (HR–). Columns represent means of four leaves (100 sites each). Vertical bars indicate standard errors.

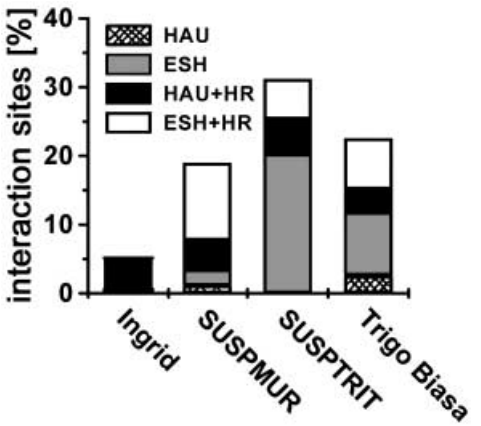

Fig. 2 Fungal development and HR frequencies at sites of successful penetration (black columns in Fig. 1) on different barley lines inoculated with *Bgt*A95. Indicated barley lines were densely inoculated and fixed at 48 hai for microscopic analysis. Cross-hatched columns: living cells with haustorium but without fungal growth. Grey columns: living cells with haustorium and ESH but without HR. Black columns: penetrated cells with HR but without ESH. White columns: penetrated cells that had allowed development of ESH prior to HR.

ancestor of both SUSPTRIT and SUSPMUR and shows relatively high susceptibility to inappropriate rust species (Niks *et al.*, 1996). Ingrid represents a typical cultivated barley genotype susceptible to most appropriate *Bgh* isolates. Cultivar Ingrid was highly resistant to *Bgt*A95, as indicated by a very high frequency of interaction sites where fungal growth was arrested in the cell wall (non-penetrated living cells, 78%). Penetration was observed at a low frequency of 5% of all interaction sites (Fig. 1). In 70% of all attacked cells that had undergone HR, no visible haustoria formation was detectable (Figs 1 and 2). Results on Ingrid were nearly identical to those found in cultivar Pallas (data not shown, and Hückelhoven *et al.*, 2001).

SUSPMUR, SUSPTRIT and Trigo Biasa showed significantly less non-penetrated living cells (NPLC) as compared with Ingrid (Student's *t*-test, $P < 0.01$), and penetration rates were significantly higher in SUSPMUR and SUSPTRIT than in Ingrid ($P < 0.05$; Figs 1 and 2). HR rates of apparently non-penetrated cells (HR–) differed strongly between the genotypes, e.g. SUSPMUR showed significantly more HR– than Ingrid and SUSPTRIT ($P < 0.05$, Fig. 1).

A closer view of penetrated cells allowed us to differentiate different types of post-penetration responses. At 48 hai, we distinguished between penetrated cells that contained immature haustoria and those that contained mature haustoria and supported development of ESH. Both stages of haustoria development could be observed in cells that mounted HR or stayed alive (Fig. 2). On Ingrid, where penetration was rare, all penetrated cells died before ESH could develop (Fig. 2).

All other genotypes supported some hyphal growth of *Bgt*; of these, the highest frequency of ESH without HR was observed in SUSPTRIT (65% of penetrated cells) and lowest in SUSPMUR (11% of penetrated cells). By contrast, ESH in association with HR was most often seen in SUSPMUR (60% of penetrated cells) that, remarkably, also had the highest rate of HR without a detectable penetration (Fig. 2).

Ror genes determine non-host penetration resistance

Because non-host penetration resistance was predominant in some barley genotypes, we were interested in the extent to which *Ror*-mutations influence the interaction outcome of barley with *Bgt*A95. *Mlo* and *Ror1*/*Ror2*-genes modulate the interaction of barley not only with *Bgh* but also with *Bgt* (Elliott *et al.*, 2002; Peterhänsel *et al.*, 1997). We inspected the cytological events in the new barley genotype *Mlo ror1–2* (Collins *et al.*, 2003). Penetration into epidermal cells subsequently undergoing HR was observed more often in *Mlo ror1* (genetic background Ingrid × Sultan-5, $P < 0.01$) than in *Mlo Ror1* genotypes (genetic background Ingrid; Fig. 3). The frequency of HR, which is considered to be at least partly dependent on the progress of the fungal penetration attempt, was much higher in *ror1* than in *Ror1* genotypes (Fig. 3, $P < 0.001$). We could exclude an influence of the genetic backgrounds of the genotypes by comparison of Sultan-5 and Ingrid, which showed nearly identical interaction types with *Bgt* (Figs 1 and 4).

A similar effect was observed at 30 hai when we compared *Ror2*- and *ror2*-genotypes in an independent experiment (genotypes: *mlo5 Ror2, mlo5 ror2*, Table 1). *Ror2* genotypes showed

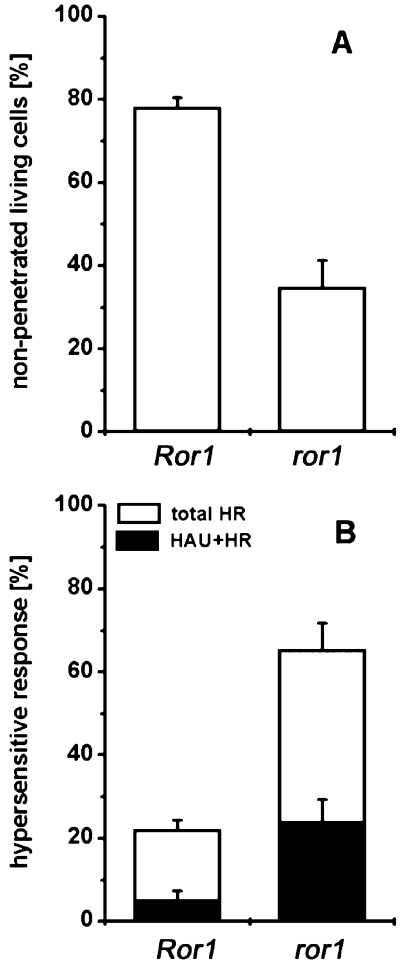

Fig. 3 Fungal development and plant defence reactions on different *Ror1*-genotypes inoculated with *Bgt*A95. Indicated barley genotypes (background Ingrid for *Ror1* and Sultan-5 × Ingrid for *ror1*) were densely inoculated and fixed at 48 hai for microscopic analysis. (A) Frequencies of interaction sites where the fungus failed to penetrate and the attacked cell appeared intact. (B) Frequencies of interaction sites with HR. HR cells either contained a visible fungal haustorium (black columns) or did not. Vertical bars indicate standard errors.

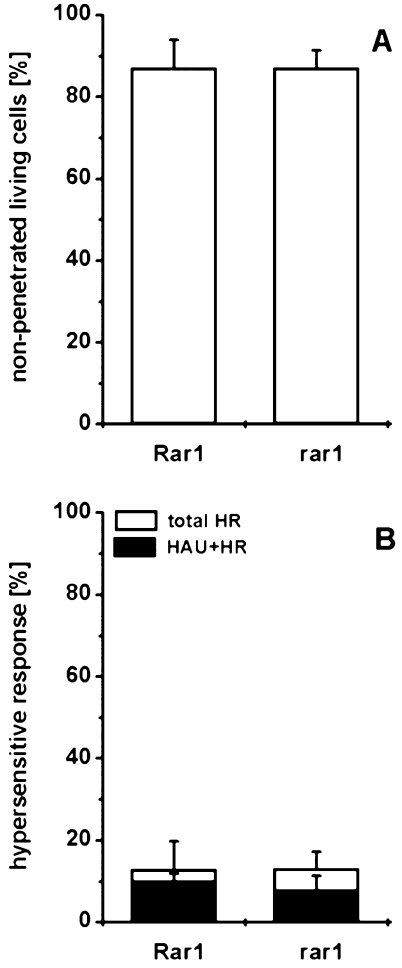

Fig. 4 Fungal development and plant defence reactions on different *Rar1*-genotypes inoculated with *Bgt*A95. Indicated barley genotypes (background Sultan-5) were densely inoculated, and fixed at 48 hai for microscopic analysis. (A) Frequencies of interaction sites where the fungus failed to penetrate and the attacked cell appeared intact. (B) Frequencies of interaction sites with HR. HR cells either contained a visible fungal haustorium (black columns) or did not. Vertical bars indicate standard errors.

much more NPLC ($P < 0.001$) and less HR ($P < 0.001$) than *ror2* genotypes when analysed in an *mlo5* genetic background.

Rar1 is not required for complete barley non-host resistance to *Bgt*A95

Because the post-penetration HR was common in all barley lines under attack from *Bgt*A95, and major genes might be involved in barley non-host resistance to *Bgt* (Tosa, 1992), we addressed the question of whether the *Rar1* gene is involved in non-host resistance to *Bgt*. The *Rar1* gene product is involved in many kinds of *R*-gene-mediated powdery mildew resistance in barley. We compared wild-type *Rar1* with mutant *rar1* (allele *rar1–2*) genotypes

Table 1 *Bgt* development and defence responses of two barley genotypes* differing in *Ror2* allele in recessive *mlo5* background at 30 hai.

	Interaction sites (%, ± SD)	
Interaction type	*mlo5 Ror2*	*mlo5 ror2*
Penetration†	1.0 ± 1.7	7.3 ± 3.4
NPLC‡	87.3 ± 4.4	26.1 ± 6.5
HR§	11.7 ± 3.4	66.6 ± 7.3

*Backcrosses and mutations in the background of cv. Ingrid.
†All penetration sites showed post-penetration HR.
‡Non-penetrated living cells.
§HR summarizes here all sites of HR without visible haustoria.

during their non-host reaction to *Bgt*. The *Rar1* wild-type parent line Sultan-5 showed a similar non-host response to *Bgt* as Ingrid or Pallas at 48 hai (Fig. 3, and Hückelhoven *et al*., 2001). At more than 80% of interaction sites, cells were not penetrated and survived fungal attack. The rest of the cells mounted an HR. We confirmed that most of the cells showing HR were penetrated. A similar frequency of post-penetration HR cells was observed in *Rar1* and *rar1*. Likewise, the total frequency of HR did not differ (Fig. 4). ESH was never observed either on Sultan-5 or on *rar1*-mutants.

DISCUSSION

Non-host resistance of cereals to inappropriate f.sp. of grass powdery mildew is known to be associated with penetration resistance and single-cell HR. Our results underline that non-host resistance of barley to *Bgt* can be associated with several mechanisms of cellular defence being expressed at clearly distinguishable stages of fungal development. This suggests that non-host resistance of barley to *Bgt* is established not only by lack of compatibility factors or determinants of penetration resistance and early HR but also by different determinants of post-penetration resistance, leading to late hypersensitive cell death and/or restricted fungal development.

The barley genotypes used here showed a maximum of 30% penetration, indicating that penetration resistance plays a major role in non-host resistance to *Bgt* (Fig. 1). However, SUSPMUR, SUSPTRIT and Trigo Biasa, which are susceptible to different inappropriate rust fungi (Atienza *et al*., 2004; Niks *et al*., 1996), were also relatively accessible to penetration by *Bgt*. Therefore, one may speculate that limited restriction of both inappropriate rust fungi and inappropriate powdery mildew fungi is based on the same genetic factors. Ingrid, SUSPMUR and Trigo Biasa are all highly susceptible to the barley powdery mildew (data not shown), indicating that differences observed in non-host defence patterns (Fig. 2) are caused by non-host resistance rather than by background susceptibility of these lines. SUSPTRIT showed *Bgh* race A6-induced necrosis and supported only little mycelium development, probably because it carries a corresponding *R*-gene (data not shown).

Interestingly, EDS1, being essential for Toll and interleukin-1 receptor-like nucleotide binding leucine-rich repeat class *R*-mediated resistance, was discovered to play a role in non-host penetration resistance of *Arabidopsis* to *Bgt* (Falk *et al*., 1999; Yun *et al*., 2003). The barley factors responsible for non-host rust susceptibility are presently under study via QTL analysis in barley (R. Niks, unpublished data).

When established as a single-cell compatible status on barley at 48 hai, *Bgt* either was stopped by cell death or it abnormally developed and generally failed to sporulate by 13 dai (data not shown). Occasionally, *Bgt* was able to form single conidiophores on SUSPMUR but no macroscopically visible symptoms developed. This indicates that *Bgt* was able to obtain sufficient nutrients from barley to develop secondary hyphae. Furthermore, it was shown that induced accessibility to *Bgt* by pre-inoculation with *Bgh* allows sporulation of *Bgt* on barley (Olesen *et al*., 2003; Ouchi *et al*., 1974). The fact that over-expression of the barley defence suppressors MLO and Bax Inhibitor-1 enhances penetration efficiency of *Bgt* on barley (Eichmann *et al*., 2004; Elliot *et al*., 2002; Hückelhoven *et al*., 2003) underlines that defence reactions rather than lack of compatibility factors are responsible for poor infection of barley by *Bgt*.

All barley lines showed a certain amount of post-penetration HR to *Bgt* before 48 hai. Because post-penetration resistance is typical for many Mla_x-mediated forms of race-specific powdery mildew resistance (Freialdenhoven *et al*., 1994; Hückelhoven *et al*., 1999, 2000a; Peterhänsel *et al*., 1997), one might assume that major genes are also involved in post-penetration HR to *Bgt*. However, it appears likely that several *R*-gene-like host factors rather than single *R*-genes are involved in non-host resistance to inappropriate *formae speciales.* This is supported by the fact that crosses of different *formae speciales* of *B. graminis* led to an F1 generation that segregated for several major genes determining avirulence on wheat via corresponding wheat genes (Matsumura and Tosa, 1995; Tosa, 1992).

We tested the possible dependence of non-host resistance on RAR1, which is a convergence point for various *R*-gene-mediated defence reactions (Freialdenhoven *et al*., 1994; Schulze-Lefert and Vogel, 2000). The *rar1*-mutants that are suppressed in many types of Mla_x-mediated resistances were fully resistant to *Bgt*A95 and not distinguishable from the parent *Rar1* line. This indicates that RAR1 is not required for barley non-host resistance to *Bgt*A95, although it cannot be excluded that other isolates of *Bgt* may develop better on *rar1* than on *Rar1* barley. Alternatively, major genes involved in non-host resistance to *Bgt* may differ in their dependencies on RAR1, but this effect is most probably masked as a result of the genetic complexity of non-host resistance.

Mlo and *Ror1/Ror2*-genes are known regulators of race non-specific penetration resistance of barley to its appropriate pathogen *Bgh*. Whereas the MLO protein is a negative regulator of penetration resistance, *Ror1* and *Ror2* gene products are required for penetration resistance. The *mlo5*-mediated resistance is a very effective penetration resistance and little HR is observed. The ROR2 protein appears to be involved in exocytosis of defence compounds necessary for penetration resistance (Collins *et al*., 2003; Freialdenhoven *et al*., 1996). The role of *Mlo* and *Ror* genes has been studied in non-host resistance to *Bgt*. The double-mutant *mlo5 ror1* and the *Mlo Ror1* wild-type showed similar degrees of penetration and cell death, whereas *mlo5 Ror1* was less often penetrated and showed less HR when attacked by certain isolates of *Bgt* (Peterhänsel *et al*., 1997). Our results with the newly generated *Mlo ror1* genotype (Collins *et al*., 2003) support these findings and uncouple the *ror1* effect from the *mlo* effect

in non-host resistance to *Bgt*. The *ror1* genotype was more often penetrated and showed much more HR. This indicates that HR was expressed as a second line of defence if penetration resistance failed due to *ror1*. Similar results were found in *ror2* mutants attacked by *Bgt* (Table 1; Peterhänsel *et al*., 1997) and for *Arabidopsis pen1* mutants. Both barley and *Arabidopsis* mutants showed less penetration resistance but more HR and whole-cell H_2O_2 accumulation when challenged by *Bgt* or *Bgh*, respectively (Collins *et al*., 2003; R. Hückelhoven, unpublished results). The *Pen1*/*Ror2* gene encodes a syntaxin-like protein that is probably involved in membrane dynamics during apoplastic defence. Vesicle accumulation near the site of attempted penetration was observed in both host and non-host response to *B. graminis* and is impaired in *ror2* genotypes (Collins *et al*., 2003; Hückelhoven *et al*., 1999, 2001). Therefore, ROR2 might be an important component of the secretion machinery for apoplastic defence (Collins *et al*., 2003).

R-gene like compounds are most likely to be involved in HR activation after penetration by *Bgt*. Interestingly, Peterhänsel *et al*. (1997) have shown that penetration resistance mediated by *mlo5* suppresses HR mediated by race-specific *Mla8* in the barley–*Bgh* interaction. When penetration resistance was impaired in *ror* mutants, HR reappeared as a post-penetration defence response. However, we cannot exclude that *Bgt*-triggered HR of the *Ror*-mutants might be a secondary effect of the *ror1- or ror2*-genotypes. Under such a scenario, *Ror* gene products would have a control function in directing the plant defence into the apoplast and the mutants might accumulate defence compounds in the symplast, finally killing the attacked cell. However, because *Mlo ror2* and *Mlo ror1* genotypes show enhanced support of the appropriate pathogen *Bgh* and little HR (Collins *et al*., 2003; U. Beckhove, unpublished results), HR appears not to be a general response of these *ror*-mutants. Together, enhanced HR in the *ror*-genotypes is indicative of a second line of defence activated in barley cells successfully penetrated by *Bgt*.

Our results support the view that there is a mechanistic overlap between non-specific host and non-host resistance of barley to powdery mildew fungi (Collins *et al*., 2003). Because this is so far only obvious for early defence responses, it will be interesting to learn how post-penetration non-host defence is determined in cereals.

MATERIALS AND METHODS

Plants, pathogens and inoculation

The barley (*Hordeum vulgare* L.) cv. Sultan-5 and the mutant M100 (*rar1–2*) were obtained from J. Helms Jørgensen (Risø National Laboratory, Roskilde, Denmark). Their development was as described previously (Torp and Jørgensen, 1986). Barley cv. Ingrid (*Mlo*), the backcross line BCIngrid-*mlo5* (I22), cv. Pallas (*Mlo*) and BCPallas-*mlo5* (P22) were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Their generation was as described previously (Kølster *et al*., 1986). The barley mutant genotypes *mlo5 Ror1 ror2*, *mlo5 ror1–2 Ror2* and *Mlo ror1–2 Ror2* were obtained from Paul Schulze-Lefert (Max-Planck-Institute for Plant Breeding, Cologne, Germany) and Nicholas Collins (Sainsbury Laboratory, John Innes Centre, Norwich, UK), respectively. Their generation was as described previously (Collins *et al*., 2003; Freialdenhoven *et al*., 1996). SUSPMUR and SUSPTRIT were generated as described by Atienza *et al*. (2004). Trigo Biasa was collected in Indonesia. Plants were grown in a growth chamber at 18 °C with 60% relative humidity and a photoperiod of 16 h (60 μmol/m^2/s^1 photon flux density). The inappropriate fungus *B. graminis* f.sp. *tritici* (field isolate, Aachen, Germany, 1995, designated *Bgt*A95), was inoculated on to 7-day-old barley first leaves to give a density of 10–20 conidia/mm^2. *Bgt*A95 was maintained on wheat cv. Chancelor in the greenhouse.

Microscopy of infection structures and H_2O_2 cytochemistry

Leaf fixation [in 0.15% trichloroacetic acid (w/v) in ethyl-alcohol : chloroform (4 : 1; v/v)], bright-field microscopy and fluorescence microscopy was performed as described by Hückelhoven and Kogel (1998). Whole-cell autofluorescence and discontinuity of cytoplasmic strands were taken as a reliable measure of cell death (Görg *et al*., 1993; Hückelhoven *et al*., 2000b; Koga *et al*., 1988).

Because the defence reactions of short and long epidermal cells are different, only short cells directly adjacent to stomata (cell type A) and short cells not directly adjacent to stomata (type B) were evaluated, whereas long epidermal cells covering vascular tissue (type C) were excluded (for leaf epidermal cell distribution see Koga *et al*., 1990). Host cells with only one attempted penetration were evaluated exclusively to avoid misinterpretation due to induced effects.

H_2O_2 was detected using the DAB method as described previously (Hückelhoven *et al*., 2000a; Thordal-Christensen *et al*., 1997). DAB (0.5 mg/mL) was dissolved in water (pH 3.8, HCl) and injected into barley first leaves 2 h before fixation (Hagborg, 1970).

When stated, the statistical significance of results was analysed using a Student's *t*-test.

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Chapter 14

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu J-L, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P (2003) SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* 425: 973-977

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SNARE-protein-mediated disease resistance at the plant cell wall

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Failure of pathogenic fungi to breach the plant cell wall constitutes a major component of immunity of non-host plant species—species outside the pathogen host range—and accounts for a proportion of aborted infection attempts on 'susceptible' host plants (basal resistance)[1–4]. Neither form of penetration resistance is understood at the molecular level. We developed a screen for *penetration* (*pen*) mutants of *Arabidopsis*, which are disabled in non-host penetration resistance against barley powdery mildew, *Blumeria graminis* f. sp. *hordei*, and we isolated the *PEN1* gene. We also isolated barley *ROR2* (ref. 2), which is required for basal penetration resistance against *B. g. hordei*. The genes encode functionally homologous syntaxins, demonstrating a mechanistic link between non-host resistance and basal penetration resistance in monocotyledons and dicotyledons. We show that resistance in barley requires a SNAP-25 (synaptosome-associated protein, molecular mass 25 kDa) homologue capable of forming a binary SNAP receptor (SNARE) complex with ROR2. Genetic control of vesicle behaviour at penetration sites, and plasma membrane location of PEN1/ROR2, is consistent with a proposed involvement of SNARE-complex-mediated exocytosis and/or homotypic vesicle fusion events in resistance. Functions associated with SNARE-dependent penetration resistance are dispensable for immunity mediated by race-specific resistance (*R*) genes, highlighting fundamental differences between these two resistance forms.

Most types of plant pathogens fail to produce disease on the majority of plant species. Although 'non-host' resistance is the most common form of resistance, its basis is poorly understood owing to the dearth of tractable genetic systems. This contrasts with 'race-specific' resistance triggered by corresponding *AVIRULENCE (AVR)/R* genes in otherwise compatible host–pathogen interactions, for which many components have been identified[5]. Suicide of cells surrounding the infection site (often referred to as the hypersensitive response) typically accompanies *R*-gene-mediated resistance, and hypersensitive-response-like cell death can also be associated with non-host resistance. These drastic measures form secondary lines of defence that are normally triggered once a fungus has overcome active defences at the plant cell periphery[3,6].

We investigated whether the immunity of the model plant *Arabidopsis* to the barley powdery mildew *B. g. hordei* could be used to develop a system for dissecting non-host resistance. *Blumeria g. hordei* conidiospores germinated on *Arabidopsis* but most sporelings failed to enter the plant cells, accompanied by the formation of a cell wall deposition (papilla) by the plant cell directly beneath penetration attempts. About 10% of sites showed successful penetration as indicated by the presence of a fungal feeding structure (haustorium; Fig. 1a); however, most of the penetrated cells underwent hypersensitive-response-like cell death (Fig. 1b), manifested as whole-cell autofluorescence. Haustoria became encased in deposits containing callose, as revealed by aniline blue staining. Rarely, short hyphae were produced on the leaf surface (Fig. 1a), indicative of successful nutrient uptake through haustoria, before further fungal growth was invariably halted. Independent screens for *Arabidopsis* mutants allowing increased penetration by *B. g. hordei* (*pen* mutants) were performed, using either whole-cell autofluorescence or induced callose deposition as indicators of penetration. Mutants were identified for at least three genes (*PEN1*, *-2* and *-3*; data not shown). Mutant alleles of *PEN1* were recovered from each screen.

Map-based cloning of *PEN1*, supported by the sequencing of four mutant alleles (Fig. 1c), revealed that it encodes *A. thaliana* syntaxin (At)SYP121 (ref. 7). The *pen1-1* mutation results in a stop codon early in the open reading frame and presumably leads to complete loss of PEN1 function. *pen1-1* mutant plants allowed a sevenfold higher incidence of *B. g. hordei* penetration compared with wild-type plants, as well as a concomitant increase in the incidence of hypersensitive-response-like cell death induced by *B. g. hordei* (Fig. 1b). Further *B. g. hordei* growth was invariably arrested in *pen1-1* plants. Thus, although impairment of penetration resistance would be necessary for *Arabidopsis* to be an effective host for *B. g. hordei*, it is not sufficient. *Nicotiana tabacum* SYR1, a tobacco homologue of PEN1/AtSYP121 (AtSYR1), has been suggested to have roles in mediating abscisic acid signalling, stomatal closing and normal growth in tobacco[8]; however, *pen1* mutants showed no discernible defects in general growth, stomatal closing ability, or root development (data not shown).

The barley–*B. g. hordei* combination also provides a useful system for the analysis of penetration resistance. Mutants of the barley *MLO* suppressor of resistance show highly effective penetration resistance against all tested *B. g. hordei* isolates. *ROR1* and *ROR2* were identified in a mutant search as genes required for full *mlo* resistance[2], but they also contribute to low-level basal penetration resistance expressed in 'susceptible' wild-type *MLO* backgrounds (Supplementary Fig. 1a). Combining mutations in *ROR1* and *ROR2* had an additive effect on susceptibility (Supplementary Fig. 1b). We isolated *ROR2* using a barley–rice syntenic-map-based cloning

approach (Supplementary Fig. 2a). A *ROR2* co-segregating syntaxin gene showed a 31-amino-acid in-frame deletion in the mutant *ror2-1* line (ROR2Δ31) (Fig. 1c; see also Supplementary Fig. 2b). Complementation of the *ror2-1* mutation by microprojectile-mediated introduction of a genomic clone driven by the native promoter into leaf epidermal cells confirmed that the gene is *ROR2* (Fig. 1d).

Transgenic *Arabidopsis* plants expressing a green fluorescent protein (GFP)–PEN1 fusion protein from the native *PEN1* promoter revealed a plasma membrane location for PEN1 (Supplementary Fig. 3a). ROR2 also showed a plasma membrane distribution in subcellular fractions analysed using a ROR2 antibody (Supplementary Fig. 3b). Of the 24 syntaxins in *Arabidopsis*[7], PEN1 has the closest resemblance to ROR2 (62% identity and 77% similarity in the cytosolic region; other syntaxins have 55% or less identity and 75% or less similarity; Fig. 1c). A construct containing the *PEN1* coding sequence driven by the barley *ROR2* promoter complemented the penetration phenotype in *ror2-1* mutant plants (Fig. 1d). These data indicate that PEN1 and ROR2 are functionally homologous syntaxin family members possessing a specialized resistance function conserved between monocotyledons and dicotyledons. PEN1 and ROR2 also provide a mechanistic link between non-host and basal penetration resistance.

Syntaxins are members of the SNARE superfamily of proteins that mediate membrane fusion events. SNARE proteins anchored on different membranes interact through their SNARE domains to form a four-helix SNARE bundle, thereby providing much of the energy required to drive membrane fusion[9]. Plasma membrane syntaxins (Qa-SNARE domain) typically combine with a SNAP-25 protein (Qb- and Qc-SNARE domains) and an R-SNARE protein anchored on exocytotic vesicles. Notably, the *pen1-3* substitution alters a glycine that is invariant among all nine *Arabidopsis* subgroup 1 syntaxins, at one of the 16 Qa-SNARE residues that contribute to stabilizing interactions with other SNARE proteins in membrane-fusing complexes[10] (Fig. 1c).

We used a candidate gene approach to identify other factors required for *B. g. hordei* penetration resistance in barley, by silencing homologues of other SNARE proteins or SNARE-associated proteins in leaf epidermal cells. A SNAP-25 homologue was shown to be required for full resistance (construct 1, Fig. 2a), identifying it as a potential binding partner for ROR2 in a resistance-mediating SNARE complex. The product of predicted molecular mass 33.7 kDa was named HvSNAP34 (Supplementary Fig. 4). Owing to the limited silencing often obtained using this system (data not shown), the contribution of HvSNAP34 to resistance may be greater than the 4–7% penetration failure accounted for here. Cells silenced for HvSNAP34 were tested for their ability to mount resistance triggered by the *R* gene *MLA1* (ref. 11), which encodes an intracellular protein containing a nucleotide-binding domain and leucine-rich repeats (Fig. 2b). Resistance against an isolate of *B. g. hordei* containing the corresponding AVRMLA1 determinant was conferred specifically by MLA1 and not by the closely related MLA6, indicating that HvSNAP34 is dispensable for *R*-gene-mediated resistance.

We used the cytosolic regions of wild-type ROR2 and mutant ROR2Δ31 proteins, as well as full-length HvSNAP34, in yeast two-

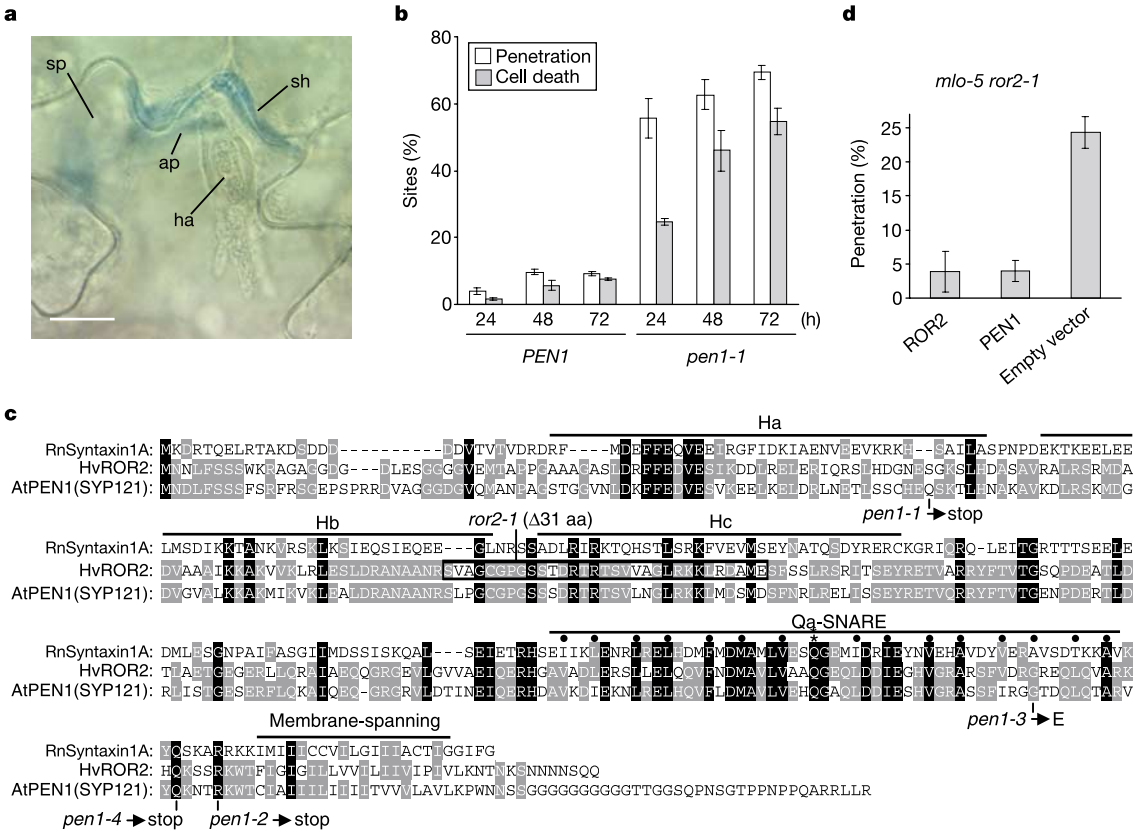

Figure 1 PEN1 and ROR2 are functionally homologous syntaxins. **a**, Multidigitate haustorium (ha) formed by *B. g. hordei* in a wild-type *PEN1 Arabidopsis* epidermal cell after successful cell wall penetration. External fungal structures are stained blue. ap, appressorium; sh, secondary hypha; sp, conidiospore. Scale bar, 10 μm. **b**, Frequency of penetration and cell death at *B. g. hordei–Arabidopsis* interaction sites. The times indicated are times after inoculation. **c**, ROR2 and PEN1 mutations. Rat neuronal syntaxin 1A is included to show positions in the Qa-type SNARE domain that contribute to stabilizing ionic (asterisk) or hydrophobic (black circle) interactions with other SNARE proteins[10,24], and to show locations of Ha, Hb and Hc helices[12]. **d**, Complementation of the *ror2-1* mutation in barley by *ROR2* and *PEN1*. Expression constructs were introduced into leaf epidermal cells of the *mlo-5 ror2-1* partially susceptible genotype.

hybrid protein interaction assays (Fig. 3a). Both forms of ROR2 interacted with HvSNAP34; however, the Δ31 deletion strongly enhanced binding to HvSNAP34 in addition to allowing formation of ROR2Δ31 homomultimers (Fig. 3a). In other plasma membrane syntaxins, the amino terminus forms an autonomously folded bundle comprising helices Ha, Hb and Hc (Fig. 1c), which binds reversibly with the Qa-SNARE domain, suppressing interactions with other SNARE proteins and the formation of high-order homomultimers *in vitro*[12–14]. The Δ31 deletion covers most of the predicted Hc helix (Fig. 1c). Therefore, the altered SNARE binding of ROR2Δ31 is probably due to disruption of similar intramolecular interactions within ROR2, leading to a constitutively open state.

The ROR2Δ31 protein produced by the endogenous *ror2-1* allele is unaltered in membrane location, and is only slightly reduced in abundance (Supplementary Fig. 3b), suggesting that its inability to confer resistance is due to disruption of a critical biochemical function requiring the region deleted in this protein. Notably, overexpressed ROR2Δ31 acted as a potent inhibitor of resistance in a wild-type ROR2 background (Fig. 3b), probably by sequestering interacting partner(s) of ROR2 (for example, HvSNAP34) into non-functional complexes. Overexpression of ROR2Δ31 also increased susceptibility in a mutant *ror2-1* background (Fig. 3c), suggesting that either the *ror2-1* mutant retains partial ROR2 activity, or that another syntaxin sharing interacting partner(s) with ROR2 contributes to the resistance. Cells overexpressing ROR2Δ31 were able to mount resistance triggered by the *R* gene *MLA1* (Fig. 3d), reinforcing the notion that SNARE functions related to penetration resistance are not critical for *R*-gene-mediated resistance.

Because the requirement for SNARE proteins implies a role for membrane fusion in resistance, we examined whether the incidence of *B. g. hordei*-associated vesicles detectable by light microscopy (>1 μm) was altered by mutations in the *MLO*, *ROR1* and *ROR2* genes. Consistent with previous observations[15], large (2–3 μm) vesicles containing H_2O_2 could be observed in the host cells beneath appressoria (Fig. 4a). Vesicles appeared to aggregate and coalesce with time, and disappeared by 72 h at sites of primary penetration attempts (not shown). The appearance of vesicles was significantly influenced by mutations in each of the *MLO*, *ROR1* and *ROR2* genes, with vesicle incidence being positively associated with levels of resistance to *B. g. hordei* penetration (Fig. 4b).

Our findings obtained here (Figs 1b, 2b and 3d) and previously[6] show that components of penetration resistance, including SNARE-related functions, are not critical for *R*-gene-mediated, race-specific resistance or for secondary lines of non-host resistance. Moreover, *PEN1*, *-2* and *-3* differ from genes uncovered by searches for

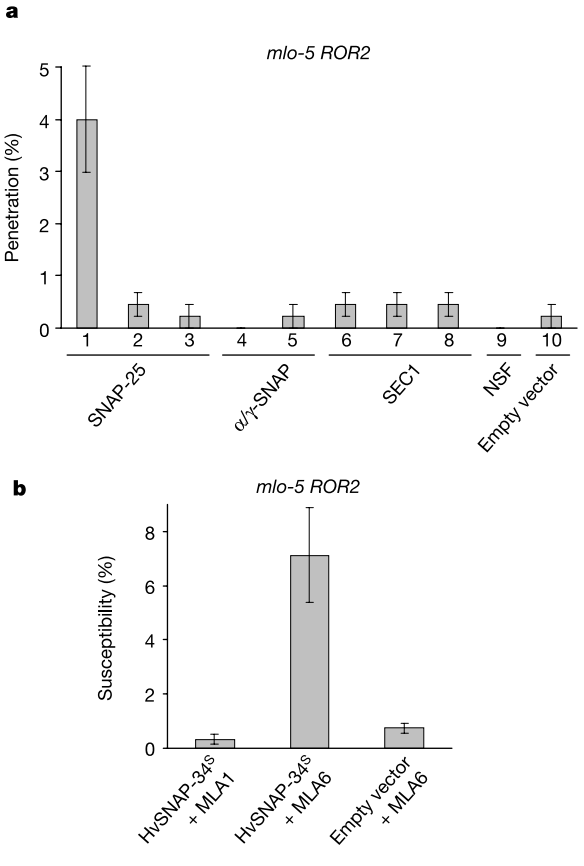

Figure 2 The barley SNAP-25 homologue HvSNAP34 is required for penetration resistance. **a**, Barley homologues of SNARE and SNARE-associated proteins were silenced in the highly resistant *mlo-5 ROR2* genotype. Representative mammalian homologues are used to indicate protein classes. Construct 1 targets HvSNAP34. GenBank accession numbers of targeted genes are listed in Methods. **b**, HvSNAP34-silenced cells retain MLA1-mediated race-specific resistance. The HvSNAP34 silencing (superscript s) construct was co-introduced with MLA1 or MLA6 *R*-gene constructs before challenge with the *B. g. hordei* isolate K1, which is recognized by MLA1 but not MLA6. Susceptibility of HvSNAP34^S plus MLA6 cells provided a control for both impairment of penetration resistance and *R*-gene-mediated resistance specificity.

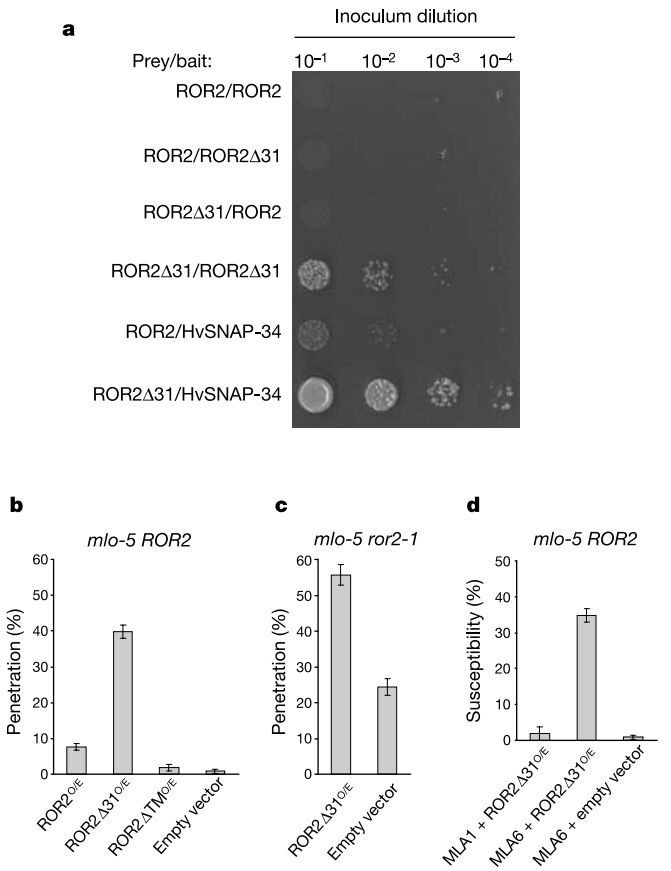

Figure 3 ROR2 interactions and overexpression. **a**, Yeast two-hybrid protein–protein interaction assays. Yeast cells were spotted on to a medium lacking histidine, upon which subsequent growth depends on interaction between bait and prey. **b**, **c**, Overexpression (O/E) constructs were introduced into barley leaves of the genotypes indicated. **d**, Cells overexpressing ROR2Δ31 retain MLA1-mediated race-specific resistance. The overexpression construct was co-introduced with MLA1 or MLA6 *R*-gene constructs before challenge with a *B. g. hordei* strain that is recognized by MLA1 but not MLA6. Susceptibility of ROR2Δ31$^{O/E}$ plus MLA6 cells provided a control for both impairment of penetration resistance and *R*-gene-mediated resistance specificity.

enhanced disease susceptibility mutants performed in contexts of high penetration incidence in compatible host–pathogen interactions (ref. 5 and our own unpublished data). Thus, basal/non-host resistance processes responsible for halting early stages of fungal ingress seem to act independently of other resistance types.

Three lines of evidence suggest that PEN1 and ROR2 mediate resistance by participating in SNARE complexes. First, resistance also requires the Qa- and Qb-SNARE-containing protein HvSNAP34. Second, the *pen1-3* substitution alters one of the Qa-SNARE positions that contribute to stabilizing interactions with other SNARE proteins. Third, the potent resistance inhibition observed upon ROR2Δ31 overexpression, together with the enhanced binding of ROR2Δ31 to HvSNAP34, is consistent with deregulated formation of binary SNARE complexes, which normally serve as transient intermediates in assembly of ternary complexes containing the additional R-SNARE.

The plasma membrane location of PEN1 and ROR2 may facilitate exocytosis; however, the reduced incidence of *B. g. hordei*-induced vesicles in the *ror2-1* mutant defies this simple interpretation. One possibility is that, in addition to facilitating exocytosis, ROR2 may also mediate homotypic fusion of vesicles to one another, in a manner similar to KNOLLE syntaxin-dependent homotypic vesicle fusion at the growing cell plate[16]. Homotypic fusion could allow the vesicles to achieve a size visible by light microscopy, and might account for the fact that the vesicles are relatively large compared with most exocytotic vesicles described in animals and plants[17,18]. One constituent of the vesicles is H_2O_2, a plant defence compound that can perform antimicrobial, cell-wall crosslinking and signalling functions[19]. Interestingly, the *B. g. hordei*-induced vesicles resemble, both in size and behaviour, coloured antimicrobial-compound-containing vesicles that coalesce in sorghum leaf epidermal cells beneath sites of attempted penetration by the fungus *Colletotrichum graminicola*[20]. Vesicles destined for exocytosis contain R-SNAREs, which join with binary syntaxin–SNAP-25 complexes on the plasma membrane to drive membrane fusion[9]. If vesicle-anchored R-SNARE partners of ROR2 and PEN1 can be identified they may allow isolation of vesicles critical for resistance, and their cargo. □

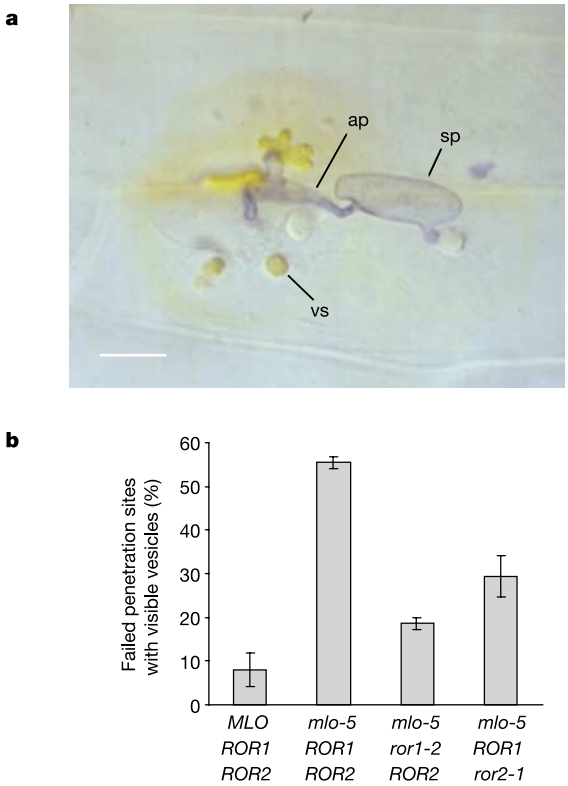

Figure 4 *Blumeria graminis hordei*-induced vesicles. **a**, A failed *B. g. hordei* penetration attempt with vesicles in barley cells. Fungal structures are stained blue. A vesicle (vs) is indicated. Brown DAB staining indicates the presence of H_2O_2. Scale bar: 10 μm. **b**, The incidence of *B. g. hordei*-induced vesicles is under genetic control. Interaction sites were classified as positive or negative for the presence of vesicles visible at ×400 magnification (approximately 1.0 μm or greater).

Methods

Arabidopsis pen1 mutant screen

M_2 populations were derived by ethylmethane sulphonate treatment of *Arabidopsis* Columbia (Col-0 or Col-3 *gl1*). In the two screens yielding *pen-1*, *-2* and *-4*, M_2 plants were inoculated with *B. g. hordei* isolate CR3, and after 48 h detached leaves were subjected to aniline blue epifluorescence staining to monitor callose[21] deposited in response to penetration. In the screen yielding *pen1-3*, M_2 plants were inoculated with *B. g. hordei* isolate K1, and 72 h later examined with ultraviolet light (excitation filter 365/12 nm; dichroic mirror 400LP) to monitor the autofluorescence resulting from the hypersensitive-response-like cell death triggered by penetration. The *pen* mutants were deposited in the *Arabidopsis* Stock Centre.

Quantification of *pen1-1* mutant phenotype

Individual *Arabidopsis*–*B. g. hordei* interaction sites were characterized for penetration success using aniline blue and for the hypersensitive-response-like cell death using ultraviolet autofluorescence. Three repetitions, scoring 100 sites per time point and genotype, were performed.

PEN1 cloning

PEN1 was mapped using a Columbia *pen1-1* × Landsberg *erecta* F_2 population of 474 individuals using standard polymerase chain reaction (PCR)-based marker techniques.

ROR2 syntenic mapping and *ROR2* sequencing

See Supplementary Information. Full-length *ROR2* messenger RNA (AY246907) and genomic (AY246906) sequences were derived by rapid amplification of cloned ends and adaptor-mediated PCR methods.

Barley expression and silencing constructs

The BAC clone HvMBa693F23 was identified from the genomic DNA library of wild-type *ROR2* barley cv. Morex[22] by screening with a *ROR2* probe. Complementation with *ROR2* was performed using a HvMBa693F23 subclone containing the *ROR2* open reading frame flanked by 881 base pairs (bp) of 5′ sequence and 81 bp of 3′ sequence. The *PEN1* complementation construct contained the *PEN1* genomic coding sequence and terminator inserted behind 3.4 kilobases of *ROR2* 5′ untranslated region sequence. The fusion junction followed the ATG, resulting in a D to N substitution at the third position of the encoded PEN1 protein, which is otherwise identical to PEN1.

Overexpression constructs were made using the pUBI-Adaptor-NOS vector[11] containing the strong constitutive maize polyubiquitin (UBI) promoter. The ROR2ΔTM construct encoding the ROR2 cytosolic region was made by introducing a T285stop mutation. We confirmed PCR-derived clones by sequencing.

Homologues of SNARE or SNARE-associated proteins were identified in tBLASTn searches of the Syngenta TMRI rice genomic sequence database (http://portal.tmri.org/rice/RiceDescription.html) and the Triticeae expressed sequence tag and rice genomic sequence databases (NR and HTGS) at NCBI. Silencing fragments for HvSNAP34 spanned nucleotide positions 639–982 (coding) or 1007–1275 (3′ untranslated region) of the complementary DNA (AY247208). Other genes (GenBank accession numbers AY247209 to AY247214, AJ466709 and AV833528) were targeted for silencing using fragments of 115–351 bp. The pUAMBN silencing vector contains the UBI promoter and *MLA1* intron 3 located between two *att*L1–*ccd*B–*att*L2 cassettes for cloning inserts in inverted orientation using Gateway technology (Life Technologies).

R gene and GUS reporter constructs have been described[11].

Single-cell gene expression and silencing

Gene expression and silencing in barley leaf epidermal cells was performed essentially as described[11]. Gold microprojectiles (1.0 μm) were coated with a total of 12 μg plasmid DNA mixture per shot, using 8 μg of double-stranded RNAi construct, 0.6 μg of complementation construct but otherwise equal amounts of other constructs. Bombarded leaves were inoculated with *B. g. hordei* isolate K1 after 96 h (silencing) or 4 h (expression), and penetration frequencies were determined 48 h after inoculation. Generally, 150 interaction sites were assessed from each of three to four independent 'shootings' per construct combination. *MLA1* and *MLA6 R* genes confer pre-haustorial resistance in this system due to an overexpression effect[11]. Hence, in tests involving both penetration resistance and *R*-gene-mediated resistance, susceptibility was scored on the basis of haustorium formation.

Yeast two-hybrid analysis

Yeast two-hybrid tests were performed using the GAL4 system with the *HIS* reporter in yeast strain AH109 essentially as recommended by the suppliers (Clontech). Vectors (supplied by J. Uhrig) were made by adapting pACT2 and pAS2-1 (Clontech) to accept inserts using Gateway cloning technology (Life Technologies). PCR-derived cDNA clones were verified by sequencing, and the prey and bait constructs were co-transformed into

yeast. Liquid culture densities were equalized using absorption at 600 nm, and 10 µl of each dilution was spotted on to histidine minus medium before incubation.

Vesicle analysis

Vesicle analysis was performed on leaf segments stained with DAB to detect H_2O_2 as described[15]. Leaves of 7-day-old seedlings were inoculated with *B. g. hordei*, and 24 h later they were assessed by differential interference contrast microscopy for vesicles in the short cells of the adaxial epidermis[23]. Per genotype, 100 sites were scored from each of three leaves. Only sites at which penetration had failed were scored.

See Supplementary Information for barley genotype analysis with *B. g. hordei*, and PEN1 and ROR2 localization.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

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Correspondence and requests for materials should be addressed to P.S.-L. (schlef@mpiz-koeln.mpg.de). Sequences are deposited in GenBank under accession numbers AY246893 to AY246907 and AY247208 to AY247214.

Bone recognition mechanism of porcine osteocalcin from crystal structure

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Osteocalcin is the most abundant noncollagenous protein in bone[1], and its concentration in serum is closely linked to bone metabolism and serves as a biological marker for the clinical assessment of bone disease[2]. Although its precise mechanism of action is unclear, osteocalcin influences bone mineralization[3,4], in part through its ability to bind with high affinity to the mineral component of bone, hydroxyapatite[5]. In addition to binding to hydroxyapatite, osteocalcin functions in cell signalling and the recruitment of osteoclasts[6] and osteoblasts[7], which have active roles in bone resorption and deposition, respectively. Here we present the X-ray crystal structure of porcine osteocalcin at 2.0 Å resolution, which reveals a negatively charged protein surface that coordinates five calcium ions in a spatial orientation that is complementary to calcium ions in a hydroxyapatite crystal lattice. On the basis of our findings, we propose a model of osteocalcin binding to hydroxyapatite and draw parallels with other proteins that engage crystal lattices.

The primary structure of osteocalcin (OC) is highly conserved among vertebrates and contains three vitamin-K-dependent γ-carboxylated glutamic acid (Gla) residues at positions 17, 21 and 24 in porcine OC (pOC; Fig. 1a and Supplementary Fig. 1). Solution studies have shown that mature OC is largely unstructured in the absence of calcium and undergoes a transition to a folded state on the addition of physiological concentrations of calcium[8]. NMR analysis has shown that OC is a globular protein consisting of α-helical secondary structure in its folded state[8,9], but the detailed three-dimensional structure of OC has not been forthcoming.

To gain further insight into the structure of OC and its ability to recognize the hydroxyapatite (HA) mineral component of bone, we have determined the crystal structure of pOC at 2.0 Å using the Iterative Single Anomalous Scattering method[10]. Bijvoet difference Patterson map analysis detected the presence of three tightly bound Ca^{2+} ions and two S atoms corresponding to a disulphide bridge between Cys 23 and Cys 29, which together were used to phase the pOC structure. An atomic model corresponding to residues Pro 13 to Ala 49 was built into well-defined electron density (Supplementary Fig. 2) and refined to an R_{work} and R_{free} of 25.5% and 28.3%, respectively. Data collection and structure refinement statistics are summarized in Supplementary Table 1.

pOC forms a tight globular structure comprising a previously unknown fold (no matches in the DALI database[11]) with a topology consisting, from its amino terminus, of three α-helices (denoted α1–α3) and a short extended strand (denoted Ex1; Fig. 1b). Helix α1 and helix α2 are connected by a type III turn structure from Asn 26 to Cys 29 and form a V-shaped arrangement that is stabilized

4. Further publications in context to this work

Felle HH, Herrmann A, Hanstein S, **Hückelhoven R**, Kogel K-H (2004) Apoplastic pH- signalling in barley leaves attacked by the powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*). *Mol Plant-Microbe Interact* 17: 118-123.

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Schäfer P, **Hückelhoven R**, Kogel K-H (2004) The barley albino mutant Albostrians is supersusceptible to the hemibiotrophic fungus *Bipolaris sorokiniana* (Teleomorph: *Cochliobolus sativus*). *Mol Plant-Microbe Interact* 17: 366-373

Jain SK, Langen G, Börner T, Hess W, **Hückelhoven R**, Kogel K-H (2004) The barley albino mutant Albostrians shows enhanced resistance to *Blumeria graminis* f.sp. *hordei*. *Mol Plant-Microbe Interact* 17: 374-382

Kumar J, **Hückelhoven R**, Beckhove U, Nagarajan S, Kogel K-H (2001) A compromised *Mlo* pathway affects the response of barley to the necrotrophic fungus *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) and its toxins. *Phytopathol* 91: 127-133

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5. Previous publications in context to this work

Hückelhoven R, Fodor J, Preis, C, Kogel K-H (1999) Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with H_2O_2 but not with salicylic acid accumulation. *Plant Physiol* 119: 1251-1260

Kogel K-H, **Hückelhoven R** (1999): Superoxide generation in chemically activated resistance of barley in response to powdery mildew inoculation. *J Phytopathol* 147: 1-4

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7. Summary

In context to a growing world population and loss of arable land it is challenging to make food production more safe, environmentally compatible and healthy for the consumer as well as for the producer. Since plant diseases are still threatening these aims, it is of outstanding importance to understand the molecular and physiological basis of crop diseases and resistance. Therefore, this work focussed on the analysis of these aspects in the interaction of barley and wheat with powdery mildew fungi.

The first part of this work deals the role of reactive oxygen intermediates in the interaction of barley and wheat with inappropriate and appropriate *formae speciales* of *Blumeria graminis.* The use of susceptible barley mutants as well as the comparison of host with non-host resistance allowed to link H_2O_2 accumulation with host and non-host resistance whereas the role of $O_2^{\bullet-}$ appears to be rather diverse.

In the second part the isolation and characterisation of candidate genes with a potentially essential role in the interaction of barley with *Blumeria graminis* f.sp. *hordei* (*Bgh*) is described. The candidate approach followed a reverse genetics strategy starting with mRNA sequences in public databases. This led to the isolation of a set of copy DNA sequences. Some of the corresponding genes were strongly expressed in response to attack from *Bgh*. Functional analysis based on microprojectile mediated single-cell transformation with subsequent challenge of transformed cells by *Bgh* led to characterisation of some genes as potential resistance or susceptibility factors. The barley family of RAC/ROP-type small GTPases was characterised in detail. The role of the gene products is discussed with respect to penetration resistance and in particular with respect to their role in production of reactive oxygen intermediates and in cytoskeleton rearrangement.

The third part deals with the role of the cell death regulator protein BAX Inhibitor 1 (BAX: BCL-2 associated X protein; BCL-2: B-cell lymphoma protein-2) in barley susceptibility to *B. graminis*. The corresponding barley mRNA was isolated from leaf tissue and found to accumulate after powdery mildew attack and to transiently disappear after root treatment with a synthetic resistance activator. Importantly, over-expression of barley BAX Inhibitor 1 weakened background resistance of barley to *Bgh*, broke *mlo*-mediated penetration resistance and additionally broke non-host resistance to the wheat pathogen *B. graminis* f.sp. *tritici* (*Bgt*). BAX Inhibitor 1 was further analysed based on its amino acid sequence and literature data. This tagged

BAX Inhibitor 1 as an ancient cell death suppressor protein conserved in all higher eukaryotes, and traces its origin back to possibly prokaryotic ancestor proteins.

The last part of this work deals with non-host resistance and its mechanistic overlap with host resistance to *B. graminis*. In this regard, the studies concentrated mainly on the role of the genes *mlo*, *ror1* and *ror2,* whose role in resistance to *Bgh* is well established, in response to *Bgt*. Besides this, a set of different barley accessions was compared for their non-host resistance phenotypes in response to *Bgt*. Taken together, a similar role for *ror1* and *ror2* in host and non-host resistance to *B. graminis* was observed. Additionally, the multiplicity of different non-host defence reactions observed provoked the assumption that non-host resistance to *Bgt* is based on diverse defence pathways in barley.

8. Zusammenfassung

Vor dem Hintergrund einer wachsenden Weltbevölkerung und dem andauernden Verlust kulturfähiger Flächen ist es eine große Herausforderung, Pflanzenproduktion sicherer, umweltweltfreundlicher und sowohl für den Konsumenten als auch für den Produzenten gesünder zu gestalten. Weil Pflanzenkrankheiten diesen Zielen heute immer noch im Wege stehen, ist es von außerordentlicher Bedeutung, die physiologischen und molekularen Grundlagen von Krankheit beziehungsweise Resistenz zu verstehen. Aus diesem Grund konzentriert sich diese Arbeit auf die Analyse solcher Aspekte in der Interaktion von Getreidepflanzen mit Echten Mehltaupilzen.

Im ersten Teil dieser Arbeit wird die Rolle Reaktiver Sauerstoffintermediate in der Interaktion von Gerste und Weizen mit passenden und unpassenden *formae speziales* von *Blumeria graminis* beleuchtet. Die Verwendung anfälliger Mutanten der Gerste und der Vergleich von Wirt- und Nichtwirtresistenz zeigte eine enge Verknüpfung von H_2O_2 Akkumulation und effektiver Pathogenabwehr, wohingegen $O_2^{\bullet-}$ eine ambivalente Rolle in Resistenz und Anfälligkeit zu haben schien.

Im zweiten Teil ist die Isolierung und Charakterisierung von Kandidatengenen mit möglicherweise regulatorischer Funktion in der Interaktion von Gerste und *Blumeria graminis* f.sp. *hordei* (*Bgh*) beschrieben. Der Kandidatengenansatz ging von öffentlich zugänglichen Gersten-Sequenzinformationen aus und führte zur Isolierung

verschiedener mRNA-DNS-Kopien. Einiger der entsprechenden Gene waren stark durch Mehltaubefall induziert. Eine funktionelle Analyse durch Partikelbeschuss-vermittelte Einzelzelltransformation und anschließende Inokulation mit *Bgh* führte zur Charakterisierung einiger Gene als Resistenz- beziehungsweise Anfälligkeits-faktoren. Die Gersten-RAC/ROP Familie kleiner GTPasen wurde im Detail analysiert. Die Rolle der Genprodukte wird in Bezug auf die Produktion reaktiver Sauerstoffintermediate und auf die Zytoskelettorganisation diskutiert.

Der dritte Teil der Arbeit befasst sich mit der Rolle des Zelltodregulatorproteins BAX Inhibitor 1 (BAX: BCL-2 associated X protein; BCL-2: B-cell lymphoma protein-2) in der Anfälligkeit der Gerste gegen *B. graminis.* Die entsprechende mRNA wurde aus Blattgewebe isoliert und zeigte erhöhte Abundanz nach Mehltauinfektion. Die BAX Inhibitor 1 Expression wurde außerdem durch einen synthetischen Resistenzinduktor supprimiert. Interessanterweise, führte die Einzelzellüberexpression des BAX Inhibitor 1 Gens in Gerste zum Bruch sowohl *mlo*-vermittelter Penetrationsresistenz gegen *Bgh* als auch der Nichtwirtresistenz gegen *Blumeria graminis* f.sp. *tritici* (*Bgt*). Das BAX Inhibitor 1 Protein wurde außerdem aufgrund seiner Aminosäuresequenz und anhand von Literaturdaten als entwicklungsgeschichtlich alter Zelltodsuppressor mit möglicherweise prokaryotischem Ursprung beschrieben.

Im letzten Teil der Arbeit wird die Nichtwirtresistenz gegen unpassende Mehltauvarianten genauer beleuchtet. Diesbezüglich konzentrierten sich die Arbeiten hauptsächlich auf die Rolle der Gene *mlo*, *ror1*, und *ror2,* deren Rolle in der nicht spezifischen Wirtresistenz gut beschrieben ist. Darüber hinaus wurde eine Reihe von Gerstenkultivaren auf ihren Nichtwirtresistenzphänotyp untersucht. Insgesamt zeigte sich, dass *ror1* und *ror2* in Wirt- und Nichtwirtpenetrationsresistenz gegen *B. graminis* eine ähnliche Rolle spielen. Zusätzlich zeigte die Vielzahl unterschiedlicher Abwehrmechanismen in verschieden Gerstenkultivaren, dass die Nichtwirtresistenz gegen *Bgt* vermutlich über eine Vielzahl von Signalwegen reguliert ist.