Institute of Phytopathology and Applied Zoology

Head: Prof. Dr. Karl-Heinz Kogel

Molecular Analyses on the Mechanism of Nonhost Resistance of Barley (*Hordeum vulgare* L.) to the Wheat Powdery Mildew Fungus (*Blumeria graminis* f.sp. *tritici*)

Inaugural Dissertation for the Achievement of the Degree
Doktor der Agrarwissenschaften
at the Faculty of Agricultural and Nutritional Sciences, Home Economics and Environmental Management

Justus-Liebig-Universität Giessen

Submitted by
Dipl.-Ing. agr. Ruth Eichmann
from Usingen

Board of Examiners

Chairman of the Committee	Prof. Dr. Ernst-August Nuppenau
1. Referee	Prof. Dr. Karl-Heinz Kogel
2. Referee	Prof. Dr. Wolfgang Friedt
3. Referee	Prof. Dr. Sylvia Schnell
Examiner	Prof. Dr. Bernd Honermeier
Examiner	Prof. Dr. Wolfgang Köhler
Examiner	PD Dr. Ralph Hückelhoven

Date of oral examination: 22.12.2005

Parts of this work have already been published:

EICHMANN, R., SCHULTHEISS, H., KOGEL, K.-H. AND HÜCKELHOVEN, R. (2004) 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.

EICHMANN, R., BIEMELT, S., SCHÄFER, P., SCHOLZ, U., JANSEN, C., FELK, A., SCHÄFER, W., LANGEN, G., SONNEWALD, U., KOGEL, K.-H. AND HÜCKELHOVEN, R. (in press) Macroarray expression analysis of barley susceptibility and nonhost resistance to *Blumeria graminis*. J. Plant Physiol. doi:10.1016/j.jplph.2005.06.019.

1 Introduction

Crop plants are confronted with a huge array of potentially phytopathogenic viruses, bacteria and fungi. Considering the large number of possible combatants, it is quite astonishing that only very few 'specialists' eventually succeed in colonizing a plant. Under certain conditions however, these pathogens can cause severe damage with high yield losses and reduction in crop quality and monetary gain. Oerke and Dehne (1997) have estimated that about 17.5 % of the possible yield worldwide is lost due to pathogen infections. Taking into account that resources are limited and that more and more arable land is eroded, it will be even more difficult to supply a growing world population with adequate amounts of food. This goal demands the cultivation of highly productive crops in monocultures, which, through enormous selection pressure, leads to the emergence of fungicide resistant pathogen races or the breakdown of established genetic resistances. In order to continue to provide increasing crop quality and quantity it will be important to develop and realize new sophisticated resistance strategies. It is thus necessary to gain comprehensive information on both the pathogen's infection strategy and the processes that underlie the plant's defense reactions.

1.1 Host-pathogen relationship

Phytopathogenic agents like bacteria, viruses and fungi pursue various strategies in order to utilize plants for their own propagation. When a pathogen succeeds in colonizing a plant and accomplishes its lifecycle, the interaction is considered as being compatible and the host plant then is susceptible to the virulent microorganism. In case of successful plant defense prior to pathogen propagation, the interaction between the resistant host and the avirulent pathogen is referred to as incompatible (Schlösser 1997).

Fungal pathogens are the most prevalent agents, causing severe diseases of plants. They show high variability in terms of morphology, infection strategy, and evoked symptoms. According to their general lifestyle or their infection process, most fungal pathogens can be classified into two major categories: biotrophs and necrotrophs. Biotrophs derive their nutrients from the living host cell. They are mostly, though not always generating specialized feeding structures, called haustoria, and their infection

is often controlled on the level of race-specific resistance, frequently involving death of the infected host cell to destroy the pathogen's means of existence. Examples include rust fungi as well as powdery and downy mildews (Gould 2004; Oliver and Ipcho 2004). In contrast necrotrophs often produce toxins in order to kill their host's cells and thereupon feed on the dead tissue. In many cases restriction of such pathogens, e.g. *Fusarium* specs. or *Botrytis cinerea,* is dependent on the presence of genes that collectively contribute to quantitative resistance (see chapter 1.6.1). Some microorganisms do not fit into either class, since an initial biotrophic phase is followed by necrotrophic growth and pathogens of the kind are regarded as hemibiotrophs. The causing agent of spot blotch disease, *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) and the rice blast fungus (*Magnaporthe grisea;* anamorph: *Pyricularia grisea*) are exemplary representatives of this intermediate category (Schäfer *et al.* 2004; Czymmek *et al.* 2002).

1.2 The interaction of barley with cereal powdery mildew fungi

Barley (*Hordeum vulgare* L.) is a diploid, self-pollinating plant that belongs to the sweet grass family (*Poaceae*). It is one of the most ancient cultivated grains and was originally grown in the Fertile Crescent where it derived from its wild progenitor *H. spontaneum* (Harlan and Zohary 1966). With 160 million tons, barley ranks fourth among the major crops in world wide production. Barley is an annual grass and according to its requirement for cold temperatures one distinguishes winter and spring forms. Winter barley needs vernalization, i.e. exposure to a period of cold temperatures, which later ensures the normal development of heads and grains. Winter barley thus is usually sown in the fall and completes its development during the following spring and summer. Due to climatic needs, the growing region for winter barley is predominantly restricted to Europe. It is mainly used as livestock feed, since the kernels are rich in carbohydrates with moderate amounts of protein, calcium and phosphorus. In contrast, spring barley requires only short exposure to low temperatures and can thus be sown in spring. Globally, the spring form prevails. It is well suited for utilization in malting and alcohol production processes with malt houses making particular demands on the kernel's germination capacity and on protein content and quality to allow for consistent malting. A small amount of the produced barley is used for human food in form of pearl barley or flour.

Barley plants are quite undemanding in terms of climate conditions and soil quality. They are grown preferentially in semi-heavy soil under both dry and humid conditions but sensitively react to harsh chill and soil compaction.

Largely depending on the prevailing climate, there are big differences concerning the severity and frequency in appearance of diverse fungus-related diseases in barley worldwide. Among the most common diseases that particularly affect spring barley in central Europe one can find net blotch (caused by *Drechslera teres*), scald (caused by *Rhynchosporium secalis*), leaf rust (caused by *Puccinia hordei*) and powdery mildew disease (caused by *Blumeria graminis* f.sp*. hordei*). During strong epidemics, the latter provokes yield losses of up to 25 % with early infections adversely affecting crop density and number of kernels per ear, whereas infections at later times rather reduce the thousand-kernel weight. Powdery mildew fungi infect monocotyledonous as well as dicotyledonous plants, thereby causing the symptomatic white to gray powdery-surfaced pustules that can appear on all above ground parts of a diseased plant.

Only recently, intense electron microscopic and molecular studies led to certain changes in the taxonomic classification of powdery mildew fungi. They are currently attributed to the order of *Erysiphales* with the family of *Erysiphaceae*, which splits into five tribes (*Erysipheae, Golovinomycetinae, Cystotheceae, Phyllactinieae* and *Blumerieae*) and several subtribes with more than 10 genera (Braun *et al.* 2002). The taxonomic classification of cereal powdery mildew fungi thus is as follows: Kingdom: *Fungi* / Phylum: *Ascomycota* / Class: *Plectomycetes* / Order: *Erysiphales* / Family: *Erysiphaceae* / *Blumeria graminis*. Powdery mildew fungi of the genus *Blumeria* affect plants of the *Poaceae* family thereby showing high host-species specificity. Different *formae speciales* (f.sp.) of *B. graminis* are specialized to only one cereal species. The barley powdery mildew fungus (*B. graminis* f.sp. *hordei*, *Bgh*), for example, successfully accomplishes its lifecycle on barley plants but does not grow on wheat and, vice versa, wheat powdery mildew fungus (*B. graminis* f.sp. *tritici*, *Bgt*) can grow on wheat but is incompatible with barley. It should be noted that *forma specialis* resistance is regarded as one type of nonhost resistance because it is determined on the species level (Niks 1988; Heath 1991; see chapter 1.6.4). This study focuses on the interaction of barley with both the compatible *Bgh* and the incompatible *Bgt*.

1.3 The compatible interaction

Cereal powdery mildew fungi are obligate biotrophic ecto-parasites, which take up nutrients from epidermal tissue of its host plant. Starting in spring, asexual conidia of the fungus spread with the wind. Once a conidium gets into contact with its host plant, the spore germinates within two hours and attaches itself to the leaf surface by generating the primary germ tube, which is also used for surface recognition and early water uptake (Green *et al.* 2002) Temperature and moisture strongly influence germination: slightly cold and humid conditions promote development of cereal powdery mildew fungi. 4 to 8 hours after inoculation (HAI), the secondary or appressorial germ tube forms, from which the pathogen initiates the actual colonization. In order to penetrate a plant cell, the fungus needs to develop an appressorium that, by enzymatic and/or mechanical means, drives the so-called penetration peg through cuticle and epidermal cell wall 12 to 15 HAI (Green *et al.* 2002, Braun *et al.* 2002). Deriving from the appressorium, the fungus develops its feeding organ, the haustorium, which forms within the host cell around 16 HAI (Figure 1.1 C). In doing so, the fungus does not enter the symplast but instead invaginates the host plasma membrane, and the host cell remains intact. The so-called haustorial complex constitutes the host-parasite interface. It comprises the haustorium, the enclosing, though modified host plasma membrane, also termed extrahaustorial membrane, and the extrahaustorial matrix in-between (Green *et al.* 2002). *B. graminis* typically forms digitate haustoria. The multi lobed shape provides an extended surface area and facilitates the absorption of nutrients (Braun *et al.* 2002). When the fungus has successfully established the primary haustorium, it continues its growth by developing epiphytic elongated secondary hyphae. The branched mycelium spreads across a large area around the initial penetration site, thereby sinking secondary haustoria into further host cells. Finally, conidiophores arise from the superficial hyphae, each generating a perpendicular chain of about 8 asexual spores (Braun *et al.* 2002). At that time, fungal colonies become apparent as the typical velvety powdery mildew pustules (Figure 1.1 B). The spores are eventually detached by water or wind and start their way to a new infection cycle. During the summer, *B. graminis* forms brownish fruit bodies called cleistothecia, which mature sexual ascospores. Ascospores allow fungal hibernation.

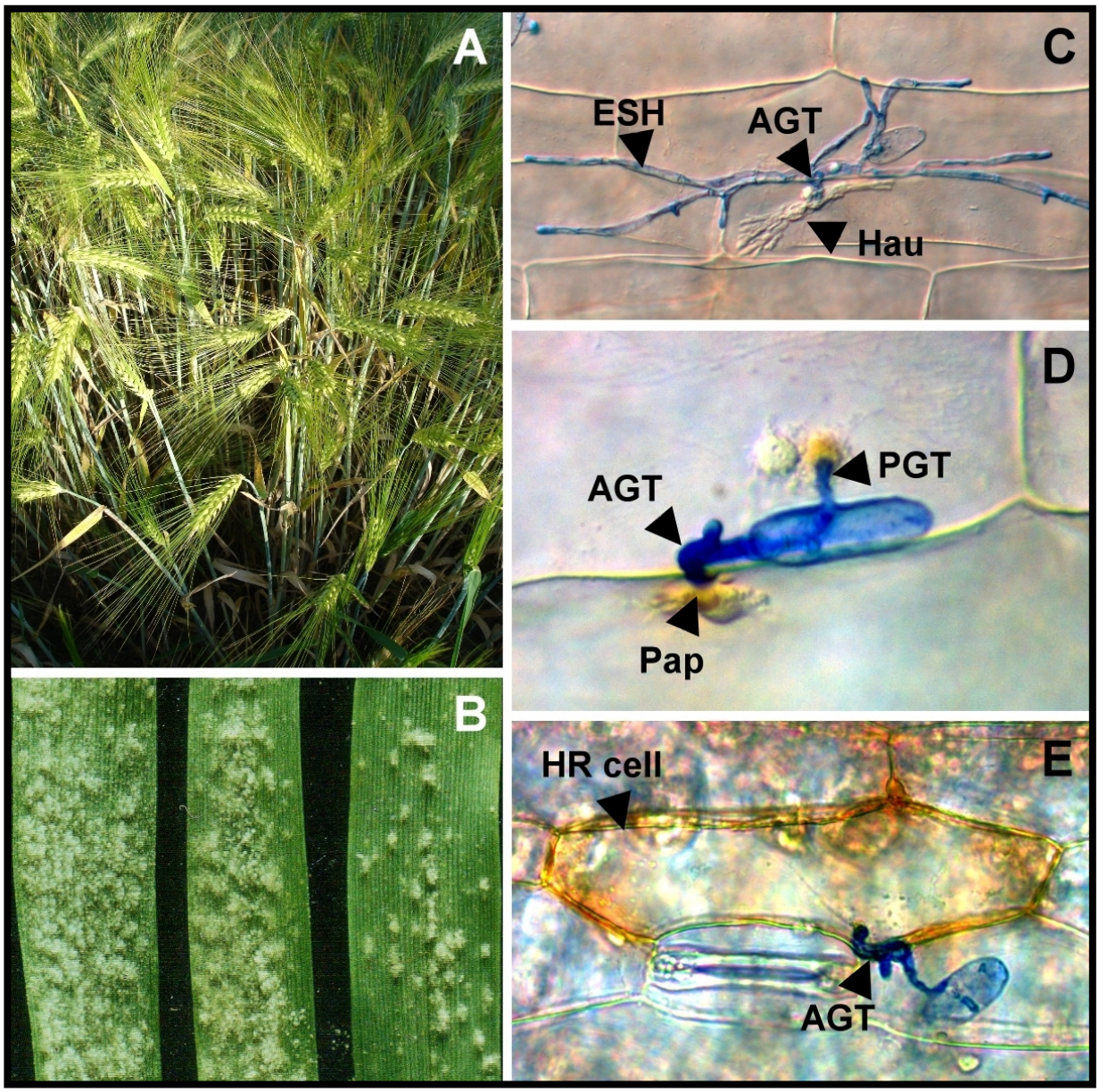


Figure 1.1: The interaction of barley with the barley powdery mildew fungus. A Barley (*Hordeum vulgare* L.) plants. **B** Barley leaf segments showing symptoms of the powdery mildew disease. Superficial mycelium and conidiophores form typical velvety pustules (picture: Birgit Jarosch). **C** Starting from the appressorial germ tube (AGT), *Bgh* successfully establishes its haustorium (HAU) within a barley epidermal cell and continues its extracellular growth by forming elongated secondary hyphae (ESH; picture: Ralph Hückelhoven). **D** A cell wall apposition, also called papilla (PAP), prevents *Bgh* from penetrating the host epidermal cell. Brownish diaminobenzidine-polymers indicate the presence of hydrogen peroxide in papillae beneath the primary germ tube (PGT) and the AGT (chapter 1.4.1). **E** Hypersensitive reaction (HR) of a barley epidermal cell to attack by *Bgh*. Upon staining with diaminobenzidine, the whole cell displays accumulation of brownish polymers indicating hydrogen peroxide production (chapter 1.4.2).

1.4 Defense mechanisms

Plants usually do not defenselessly encounter pathogen attacks but in contrast possess an array of physical, chemical or biochemical means to effectively withstand the attack of diverse intruders. It is comprehensible that depending on the type of pathogen and the mode of interaction, different defense strategies are required. Defense mechanisms can generally either be preformed or induced. Cutin containing cuticle and the epidermal cell wall represent a preformed barrier and effectively exclude the majority of potentially pathogenic microbes from entering plant tissue. However, pathogens may find a way to circumvent this first line of defense and to start establishing an infection. In response to that, induced defense mechanisms come into play, which are aimed at actively and more specifically combating the invader (Heath 1991). In case of a biotrophic pathogen that starts its infection from a single host cell, it appears reasonable on part of the plant to either prevent the parasite from entering the cell or, if this tactic fails, to deprive the invader of nutrients. Both strategies can be found in the barley-cereal powdery mildew fungus interaction.

1.4.1 Formation of cell wall appositions

Immediately after attaching to the leaf surface the powdery mildew fungus attempts to penetrate plant cuticle and cell wall. Plant epidermal cells try to resist the penetration attempt by local cell wall reinforcement underneath appressorium and penetration peg (Aist 1976). The formation of cell wall appositions, also termed papillae (Figure 1.1 D), involves active deposition of the polysaccharide callose (1,3-glucans) and phenolic compounds as well as protein cross-linking (von Röpenack *et al.* 1998; Zeyen *et al.* 2002; Jacobs *et al.* 2003). Upon contact with a germinating spore, a massive and directed reorganization of the cytoskeleton, especially the actin scaffold, towards the site of attempted penetration can be observed within the host cell (Kobayashi *et al.* 1993, 1997; Takemoto and Hardham 2004). This subcellular process is likely related to effective defense as in the compatible interaction polarized actin remodeling is markedly reduced (Opalski *et al.* 2005). Rearrangement of host cytoskeleton may be involved in the frequently observed rapid translocation of the nucleus to the contact area, presumably in order to facilitate gene transcription in response to fungal attack. Also site-directed cytoplasmic streaming and trafficking of Golgi-bodies and other small vesicle-like structures are known to be influenced by

actin microfilaments and may contribute to the accumulation of papilla material where required (Takemoto and Hardham 2004). During the interaction of barley with *B. graminis*, vesicles form visibly within 10 to 15 HAI and, by fusing with the plasma membrane, deposit papilla material onto the inner surface of the cell wall (McKeen and Rimmer 1973; Bushnell and Berquist 1975; Zeyen *et al.* 2002). Beyond that, vesicles can contain antimicrobial substances including hydrolytic enzymes, phenolic conjugated polyamines and hydrogen peroxide (H_2O_2, Hückelhoven *et al.* 1999, 2001a; Trujillo *et al.* 2004a; Collins *et al.* 2003). Local H_2O_2 accumulation accompanies attempted fungal penetration. This accumulation is followed by oxidative cross-linking of proteins as well as phenolic polymerization and correlates with penetration resistance, though it is also conceivable that H_2O_2 may be directly toxic to the fungus (Thordal-Christensen *et al.* 1997; Hückelhoven *et al.* 1999; Zeyen *et al.* 2002). Due to incorporation of phenolic, lignin-like compounds, papillae fluoresce intensely upon UV-light excitation (Kunoh *et al.* 1982; Koga *et al.* 1988). It should be noted, however, that also the primary, non-invasive germtube of *B. graminis* to some extend induces host cell responses of the kind described above (Thordal-Christensen *et al.* 1997; Hückelhoven *et al.* 2001a). The formation of cell wall appositions occurs irrespective of whether the interaction results in rejection of the pathogen or in its successful ingress. In the case of susceptibility, this barrier is ineffective and the fungus simply penetrates the papilla. Interestingly, H_2O_2 accumulation cannot be observed in penetrated papillae (Hückelhoven *et al.* 1999). But it still remains to be elucidated what kind of host or pathogen derived factors actually render the cell wall apposition effective or ineffective.

1.4.2 The plant Hypersensitive Reaction and regulation of programmed cell death in animals

Once the fungus has overcome the induced physical, papilla-based barrier and started haustorium formation, it may face another line of defense, the plant's hypersensitive reaction (HR; Figure 1.1 E). This post-penetration defense mechanism aims at disturbing nutrient uptake by the invader by means of rapid and localized host cell suicide, and is targeted particularly to restrict biotrophic pathogens (Koga *et al.* 1990; Heath 2000a). HR in plant-pathogen systems occurs in incompatible interactions and is strongly determined by both host and parasite genotype. It

requires direct or indirect recognition of a pathogen-derived avirulence (*AVR*) gene product by the corresponding product of a plant resistance (*R*) gene and is a general characteristic of race-specific resistance (Jørgensen 1994; see chapter 1.6.2). Activation of this gene-for-gene relationship initiates signal transduction that first induces an oxidative burst, i.e. a rapid production of reactive oxygen intermediates (ROI) namely hydrogen peroxide (H_2O_2) and superoxide radical anions ($O_2^{\cdot-}$, see chapter 1.4.4), and eventually leads to the onset of plant cell death (Alvarez *et al.* 1998; Jabs *et al.* 1996; Lamb and Dixon 1997; Hückelhoven and Kogel 2003). HR does not necessarily involve death of only a single infected cell, but may comprise a few cells surrounding the point of attack. HR is clearly distinguishable from necrosis in that it is dependent on highly regulated signal transduction and *de novo* protein biosynthesis. Therefore it is rather considered to be a form of programmed cell death (PDC) similar to apoptosis in animals (Dangl *et al.* 1996). In animals, a certain apoptosis pathway is dependent on cytochrome *c* release from mitochondria, which subsequently initiates a cascade of so-called caspases prior to execution of cell death (Green and Reed 1998). Cytochrome *c* release itself is controlled by a number of pro- and anti-apoptotic proteins of the BCL-2 family of cell death regulators (e.g. BAX and BCL-X_L; Tsujimoto and Shimizu 2000; Green and Kroemer 2004). Furthermore, mitochondria are an important source of ROI and alteration of the cellular redox state is known to be an alternative mechanism to activate caspases during apoptosis (Green and Reed 1998). Compared to apoptosis in animals, only limited information is available concerning the mechanisms and regulation of plant PCD. However, animal and plant PCD share some common biochemical and morphological features such as nuclear condensation, DNA fragmentation, shrinking of the cytoplasm and membrane dysfunction (Hammond-Kosack and Jones 1996; Dangl *et al.* 1996; Heath 2000a; Grey 2002; Greenberg and Yao 2004). The mechanism by which PCD and in particular HR is executed in plants remains largely unclear. Since a number of fungal toxins operate by targeting at mitochondria, involvement of these organelles in plant PCD has been assumed (Walton 1996). Cytochrome *c* release from mitochondria could be observed in plant cells undergoing heat induced cell death (Balk *et al.* 1999). Although no established homologs of mammalian caspases have been identified in plants so far, indirect evidence points to the contribution of proteins with caspase-like protease function to the control of cell death activation in plants (Woltering *et al.* 2002; Watanabe and Lam 2004). In

contrast, critical regulators of mitochondrial induced apoptosis like BAX and BCL-X_L, are not present in plants at all (Watanabe and Lam 2004). The regulatory pathway of apoptosis per se, however, seems to be conserved evolutionally, since the ectopic expression of mammalian apoptosis regulators can promote or suppress cell collapse in plants as well (Hoeberichts and Woltering 2003). Other homologs of animal cell death suppressors that do not belong to the BCL-2 family of proteins have been identified in plants, among them DAD (DEFENDER AGAINST APOPTOTIC DEATH, Gallois *et al.* 1997; Orzáez and Granell 1997) and BAX INHIBITOR-1 (BI-1; Kawai *et al.* 1999; Sanchez *et al.* 2000). BI-1 has originally been identified during a screening for mammalian proteins that rescue yeast from BAX-induced cell death (Xu and Reed 1998). The participation of BI-1 in plant responses to certain stress stimuli has been assumed, as transcripts of this BAX antagonist accumulate during aging and in response to wounding, pathogen infection or H_2O_2 and salicylic acid (SA) treatment (Sanchez *et al.* 2000; Hückelhoven *et al.* 2001b). Besides being involved in pathogen restriction, PCD participates in a series of growth and developmental processes and aids in removing harmful or excess plant cells (Hoeberichts and Woltering 2003). In the barley-powdery mildew interaction, HR can easily be recognized upon UV-light excitation by whole cell autofluorescence due to cross-linking of phenolic compounds, or by visualization of H_2O_2 that accumulates throughout the cell (Koga *et al.* 1990; Hückelhoven *et al.* 1999). HR is usually accompanied by a strong production of phytoalexins and so called pathogenesis related proteins (Koga *et al.* 1990; Hammond-Kosack and Jones 1996).

1.4.3 Antimicrobial compounds and pathogenesis related proteins

Low molecular weight antimicrobial substances further weaken the attacking microorganism. Antimicrobial metabolites that are constitutively present in plants are called phytoanticipins. Saponins and alkaloids, which are stored in vacuoles, belong to this category. Furthermore, defense mechanisms are often also accompanied by an induced accumulation of antimicrobial chemicals directly at the site of pathogen development. By definition, phytoalexins are first synthesized by the plant in response to diverse forms of stress, including microbial invasion (VanEtten *et al.* 1994). There is a multitude of various organic molecules that act as phytoalexins in different plant species. Cereals mostly produce cyclic hydroxamic acids and diterpenoids. Since the presence of phytoalexins can be deleterious to plant cells as

well, an induced and transient accumulation seems to be appropriate (Pedras and Ahiahonu 2005).

Of course pathogen attack, its recognition and subsequent onset of defense mechanisms leads to a fundamental cellular reorganization on side of the plant. This is mirrored by considerable changes in the plant's transcriptome. A set of certain genes displays a strong transcript accumulation particularly in response to pathogen infection. The corresponding proteins are thus termed pathogenesis-related (PR) and can either be antimicrobial themselves or else participate in defense associated cellular processes (van Loon and van Strien 1999). There are 17 PR protein families, whose functions are largely acknowledged. Some of them can directly affect fungal cell wall components or membranes, e.g. a β-1,3-glucanase (PR-2; Davidson *et al.* 1987), several types of chitinases (PR-3, PR-4, PR-8, PR-11; Kragh *et al.* 1990; van Loon *et al.* 1994) and a thaumatin/osmotin-like protein that induces pore formation in fungal membranes (PR-5; Abad *et al.* 1996). Others are peptides with more general antimicrobial activity, like PR-12 (defensin, Broekaert *et al.* 1995) and PR-13 (thionin; Bohlmann and Apel 1987) or are involved in oxidative processes, e.g. PR-9, a peroxidase (Thordal-Christensen *et al.* 1992). In barley, expression of a gene of the PR-1 family, *PR-1b*, is frequently used as reliable marker for the attack of *B. graminis* and other pathogens. Though its actual biological function remains unknown, a certain antimicrobial impact of the protein on *Phytophthora infestans* and *Uromyces fabae* has been noted (Niderman *et al.* 1995; Rauscher *et al.* 1999). Actually, a certain contribution of PR-1b to penetration resistance of barley to the barley powdery mildew fungus could recently be demonstrated (Schultheiss *et al.* 2003a).

1.4.4 Generation and role of reactive oxygen intermediates in plant defense

Rapid generation of reactive oxygen intermediates (ROI) is an important component of the resistance response of plants to pathogen challenge. Reactive oxygen derivatives are produced during the stepwise reduction of molecular oxygen (O_2) to water (H_2O). The most important ROI are superoxide radical anions ($O_2^{\cdot-}$), hydroperoxyl radicals ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) and the extremely short-living hydroxyl radicals (·OH). ROI occur as toxic byproducts of the respiratory and photosynthetic electron transport in mitochondria and chloroplasts. Although they may emanate from one another, the generation of the divers ROI can also occur independently and from different biochemical sources. H_2O_2 for example is produced

in peroxisomes with the collaboration of glycolate oxidase and as side-product of the fatty acid *β*-oxidation, at the cell wall upon peroxidase or oxalate-oxidase activity or during spontaneous or enzymatic dismutation of $O_2^{\cdot-}$ wherever it occurs (Lamb and Dixon 1997; Zhou *et al.* 1998; Corpas *et al.* 2001). Plasma membrane integral NADPH-oxidase, also known as respiratory burst oxidase homolog (RBOH), is supposed to be one of the main sources for $O_2^{\cdot-}$-generation. A significant involvement of RBOH in the ROI generation during plant-pathogen interactions has been emphasized (Simon-Plas *et al.* 2002; Torres *et al.* 2002; Yoshioka *et al.* 2003; Sagi *et al.* 2004). The enzyme produces $O_2^{\cdot-}$ extracellularly by accomplishing the transfer of one electron from NADPH to oxygen (Groom *et al.* 1996; Sagi and Fluhr 2001). This process is then followed by immediate (enzymatic) dismutation of $O_2^{\cdot-}$ to H_2O_2, which is frequently accompanied by transient occurrence of ·OH (Lamb and Dixon 1997). Besides, superoxide anions result from excess activity of the electron transport chain in mitochondria during certain stress situations, from peroxidase activity by a complex reaction involving NADH oxidation, and are generated within chloroplasts by photosystem I and II (Lamb and Dixon 1997; Møller 2001; Corpas *et al.* 2001). Due to their high ambition to gain electrons, ROI and in particular ·OH can easily react with organic molecules such as phenols, fatty acids, proteins and nucleic acids and would cause oxidative damage to cellular compounds (especially membranes) if there were not efficient mechanisms to scavenge and detoxify them. Most forms of biotic or abiotic stress disturb cellular integrity and metabolic equilibrium, resulting in enhanced production of ROI. A variety of enzymes are known to be watching the cell's redox status. Superoxide dismutase, ascorbate peroxidase, catalase and glutathione peroxidase for example are involved in detoxification reactions and are thus preventing excess production of ROI. Many of the ROI detoxifying enzymes and certain antioxidative low-molecular-weight compounds can be found in leaf peroxisomes, which are quite important to maintain an uncritical cellular redox status (Corpas *et al.* 2001). Besides ascorbate and glutathione, α-tocopherol, phenols and flavonols assist the above mentioned enzymes by acting as ROI scavengers (Mittler *et al.* 2004). To maintain their antioxidant property, ascorbate and glutathione in turn need to be kept in a reduced state. This is accomplished by another set of enzymes, e.g. monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase, which are using NAD(P)H in order to regenerate reduced ascorbate and glutathione (Noctor and Foyer 1998). However, considering the vast number of ROI

producing and detoxifying enzymes in plants and their ubiquitous localization in almost every compartment together with the fact that certain ROI are obviously able to diffuse throughout the cell, the ROI network needs to be regarded as highly complex. It is thus astonishing that ROI even participate in the control and adjustment of rather specific cellular processes like PCD, hormonal regulation of plant development, and response to abiotic and biotic stress stimuli including pathogen attack. Besides being toxic to attacking microorganisms, ROI are involved in all defense reactions described above, i.e. cross-linking of cell wall components during papilla formation, execution of HR and in defense gene expression, and beyond that do also partake as signaling molecules in defense related signal transduction pathways (Lamb and Dixon 1997). This purpose requires tight modulation of ROI production and scavenging dynamics in terms of intensity, duration and localization (Mittler *et al.* 2004). Likewise, the regulation of the ROI network of course strongly depends on the respective pathosystem. While during infections with necrotrophs the plant may need to bear down pathogen triggered ROI production, the plant itself might induce a local ROI amplification in order to restrict the dispersion of a biotrophic pathogen through the onset of HR (Govrin and Levine 2000). In the barley-powdery mildew fungus interaction for example, $O_2^{\cdot-}$ and H_2O_2 typically accumulate in epidermal and mesophyll tissue close to the point of fungal attack (Thordal-Christensen 1997; Hückelhoven and Kogel 1998, Trujillo *et al.* 2004a). As indicated by histochemical staining, the accumulation pattern of the respective oxygen species can thereby clearly be distinguished, both spatially and temporally. While cellular inaccessibility, i.e. resistance, correlates with H_2O_2 accumulation either locally (around the site of papilla formation) or throughout the whole cell (during HR), occurrence of $O_2^{\cdot-}$ staining can be observed in connection with successful penetration, for example in close proximity to the haustorium (Hückelhoven and Kogel 1998; Trujillo *et al.* 2004a). Since $O_2^{\cdot-}$ does not accumulate within or around effective papillae, local H_2O_2 production seems to be independent from the production of superoxide radical anions. In contrast, one can find $O_2^{\cdot-}$ accumulation in living epidermal and mesophyll cells directly surrounding a cell that underwent HR, which points to an involvement of superoxide in containing cell death (Hückelhoven and Kogel 2003).

1.4.5 The role of Ca^{2+} in defense responses

Since Ca^{2+} ions play crucial roles in a multitude of signal transduction events, eukaryotic cells maintain cytoplasmic free Ca^{2+} levels to quite low and stable concentrations between 100 and 200 nM. The apoplast and certain cellular compartments such as the endoplasmic reticulum (ER) and the vacuole, in contrast, serve as Ca^{2+} stores and Ca^{2+} concentrations here can reach µM or even mM ranges (Bush 1995). Rapid changes in cytosolic free Ca^{2+} levels belong to the most common and earliest responses of plant cells to pathogen challenge. The perception of extracellular signals, e.g. through recognition of fungal elicitors, is followed by an immediate Ca^{2+} influx into the cytosol, which is supposed to control defense reactions (Blume *et al.* 2000). Initiation of the oxidative burst for example is partially influenced by increased Ca^{2+} levels since superoxide-producing NADPH oxidase becomes activated directly by these ions (Grant *et al.* 2000; Sagi and Fluhr 2001). The emerging H_2O_2 itself then triggers an array of physiological responses that can be associated to pathogen restriction: it can boost another Ca^{2+} influx by activating calcium channels, which is required for the onset of HR, but it is also involved in cell wall cross-linking during papilla formation (Price *et al.* 1994; Levine *et al.* 1996; Thordal-Christensen *et al.* 1997). Likewise, the plasma membrane-localized callose synthase enzyme requires Ca^{2+} for activity in callose deposition at sites of attempted fungal attack (Hammond-Kosack and Jones 1996). In animals, a critical contribution of Ca^{2+}-storing ER to apoptosis has been emphasized. Upon oxidative stress, mobilization of ER calcium stores can initiate the activation of cytoplasmic death pathways either directly or by inducing cytochrome *c* release from mitochondria whereby Ca^{2+} release seems to be regulated by pro- and anti-apoptotic proteins of the BCL-2 family (see chapter 1.4.2; Breckenridge *et al.* 2003). Whether a similar process does also take place in plant cells remains elusive. However, a general involvement of the ER in modulating cellular Ca^{2+} pools during plant stress responses, including pathogen attack, has been suggested. In this connection, the possible participation of ER resident calcium-binding proteins with chaperone function was mentioned (Persson *et al.* 2001; Wyatt *et al.* 2002).

The calcium-binding protein calmodulin functions as sensor of cytosolic Ca^{2+} concentrations and is involved in processes that regulate cell death and *PR* gene expression (Heo *et al.* 1999). Interestingly, calmodulin also binds to MLO, a modulator of defense and cell death mechanisms in a Ca^{2+} dependent manner. MLO

is discussed as being a target to the powdery mildew fungus to trigger defense suppression (see chapter 1.5 and 1.6.3). Increases in cytoplasmic free Ca^{2+} in some cases may therefore be intended by the pathogen as part of its infection strategy (Kim *et al.* 2002a, 2002b; Panstruga and Schulze-Lefert 2003).

1.5 Establishment of compatibility

It is now widely accepted that the relationship between a host and an attendant microorganism initially relies on basic incompatibility, which the potential pathogen needs to overcome in the first place (Heath 1981). Pathogen derived factors that add to this purpose are termed pathogenicity or compatibility factors. Compatibility factors contribute to the establishment of compatibility by i) breaking of preformed defense barriers, ii) suppressing induced defense mechanisms and/or iii) actively killing the host cell if it suits the lifestyle of the pathogen (Briggs and Johal 1994; Toyoda *et al.* 2002). During the early stages of its development, *B. graminis* is able to release lytic enzymes, among them cutinases, cellulases and possibly pectinases (Green *et al.* 2002). It remains unclear to what extend their production contributes to pathogenicity but it is obvious, that activity of such enzymes may considerably facilitate fungal penetration by weakening host cuticle and cell wall. During the compatible interaction of barley with the powdery mildew fungus, production of $O_2^{\cdot-}$ can be detected around the penetration site, the haustorium and adjacent anticlinal cell walls (Hückelhoven *et al.* 2000a; Trujillo 2004a). $O_2^{\cdot-}$ production is triggered very early, supposedly upon contact of the fungal penetration peg or haustorium initial with the plasma membrane, and might thus have a role in cellular accessibility to *B. graminis*. It is conceivable that $O_2^{\cdot-}$ itself or concomitant occurring hydroxyl radicals may facilitate fungal ingress by softening cell wall cohesion (Schopfer *et al.* 2002; Trujillo *et al.* 2004a). Whether fungus derived molecules exist that are aimed to induce $O_2^{\cdot-}$ production remains to be elucidated (Hückelhoven and Kogel 2003). Other experiments indicate that *B. graminis* may encounter elicitation of local H_2O_2 burst beneath the appressorial germtube through induction of an innate extracellular catalase, which could be able to scavenge plant derived H_2O_2 (Zhang *et al.* 2004). Defense suppression in the barley-powdery mildew interaction can be observed on the cellular level. The term “induced accessibility” refers to the phenomenon, that successful penetration of a single host cell by a compatible “inducer” isolate of *B. graminis* renders this cell

susceptible to a subsequently attacking, otherwise avirulent or nonhost “challenger” isolate, possibly by active down-regulation of host defense within the cell (Kunoh *et al.* 1985; Lyngkjær and Carver 1999; Olesen *et al.* 2003). Likewise, it could be demonstrated that in single penetrated barley cells, enhanced expression of certain *PR* genes was repressed (Gjetting *et al.* 2004).

Emerging evidence suggests that powdery mildew fungi may even target or utilize host proteins in order to support their own growth. Such factors are required for powdery mildew susceptibility (and are thus termed susceptibility factors) and conversely, mutation of the respective host genes results in resistance towards the fungus but not to other pathogens (Hückelhoven 2005). Race unspecific broad-spectrum resistance of barley to *Bgh* can for example be achieved by mutation of the *MLO* gene, which completely restricts fungal penetration of epidermal cells (Büschges *et al.* 1997). It is still unclear, however, how *Bgh* takes advantage of the functional MLO protein in terms of susceptibility (see chapter 1.6.3). In the Arabidopsis *powdery mildew resistant 6* (*pmr6*) mutant loss of a pectate lyase-like protein alters the composition of the plant cell wall, resulting in enhanced, though cell death independent, resistance towards the otherwise compatible Arabidopsis powdery mildew (*Erysiphe cichoracearum*, Vogel *et al.* 2002). Surprisingly, Arabidopsis plants impaired in the function of callose synthase (PMR4) and thus in pathogen-associated callose deposition at sites of fungal attack, show enhanced powdery mildew resistance all the same. This actually paradox phenomenon can be explained by an obvious hyper-activation of the SA defense pathway in *pmr4* mutant plants after the fungus has penetrated the host cell. Vice versa, one can assume that the powdery mildew fungus may utilize PMR4 in order to suppress SA-dependent defense (Nishimura *et al.* 2003). In contrast, disturbance of the SA-dependent defense by mutation in the *enhanced disease resistance 1* (*edr1*) gene (encoding a mitogen-activated protein kinase kinase kinase) in Arabidopsis leads to a generally more unspecific resistance not only to powdery mildew fungi (Frye *et al.* 2001). This supports the view that host susceptibility factors exist, which assist powdery mildew fungi in establishing basic compatibility (Hückelhoven 2005). The establishment of compatibility through the pathogen is always accompanied by the attempt of the plant to readjust its resistance status, which in turn leads to the development of more parasite specific host resistances (Heath 1981).

1.6 Genetics and molecular mechanisms of resistance to powdery mildew fungi

Complete host resistance towards powdery mildew fungi is mainly determined monogenically, and its inheritance follows either dominant or recessive transmission. A couple of race-specific and non-specific powdery mildew resistance genes have been isolated and are currently under intense investigation. In contrast, only limited information is available concerning quantitative powdery mildew resistance.

1.6.1 Quantitative resistance

Quantitative resistance (also termed partial or horizontal resistance) is generally expressed as a polygenic trait, i.e. several different genes contribute collectively to a resistance phenotype (Jørgensen *et al.* 1994; Schlösser 1997). The additive effects of these genes confer rather unspecific resistance to a multitude of different pathogen races. The number of participating genes and their individual effects, which may vary from one plant cultivar to another, as well as the aggressiveness of the pathogen genotype, mainly determine effectiveness and durability of quantitative resistance. Quantitative resistance may be sufficient to prevent high yield losses by lowering infection rates and pathogen dispersion but especially obligate biotrophs such as powdery mildew fungi are frequently able to overcome it. It is possible, however, that also little-effective race-specific resistance genes contribute to partial resistance, albeit to a minor degree. Thus, partial and qualitative race-specific resistance may share some overlap (Jørgensen *et al.* 1994).

1.6.2 Race-specific resistance

Race-specific resistance is conferred monogenically and depends on the meeting of corresponding host and pathogen genotypes. Following the gene-for-gene principle (Flor 1955, 1971), race-specific resistance only occurs when a plant possesses a dominant resistance (*R*) gene that matches the cognate avirulence (*AVR*) gene of the attacking pathogen. Upon interaction of the respective gene products a signaling cascade becomes activated that eventually mounts a set of effective defense mechanism, among them initiation of an oxidative burst followed by the onset of HR, formation of cell wall appositions and the production of phytoalexins and PR proteins

(Dangl and Jones 2001; Lamb and Dixon 1997; Kuć 1995). Conceptually, *R* gene products must be able to execute two important functions: sensing and recognition of the pathogen (signal perception) and initiation of defense responses (signal transduction). The vast majority of known resistance gene products do justice to these demands by carrying two functional protein domains: a variable leucin-rich repeat (LRR) domain, mediating protein-protein interactions in a ligand-receptor like manner and a nucleotide-binding site (NBS), responsible for signal transduction (Young 2000). NBS-LRR proteins can be divided into two major classes: NBS-LRR proteins of the one class carry an additional *N*-terminal coiled-coil (CC) effector domain (CC-NBS-LRR proteins). The others are classified according to a characteristic *N*-terminal Toll-Interleukin receptor (TIR) sequence (TIR-NBS-LRR proteins; Hammond-Kosack and Jones 1997; Ellis and Jones 1998). The Arabidopsis *RPM1* gene for bacterial resistance is an example for the CC-NBS-LRR class of R proteins (Grant *et al.* 1995); the tobacco *N* gene, which confers virus resistance, falls into the TIR-NBS-LRR category (Whitham *et al.* 1994). In addition, other types of plant R proteins have been described: The tomato *PTO* gene encodes a serine/threonine kinase, which confers resistance to *Pseudomonas syringae* (Martin *et al.* 1993). NBS-LRR proteins and PTO are predicted to reside within the cytoplasm. Pathogen recognition here is likely to be intracellular and might afford transport of AVR proteins into the cell (Nimchuk *et al.* 2001). Other R proteins have transmembrane domains and most likely exert their function on the cell surface: *XA21* from rice combines an extracellular LRR and a cytoplasmic serine/threonine kinase to confer resistance to *Xanthomonas oryzae* (Song *et al.* 1995). Cf-X proteins of tomato possess an extracellular LRR domain and a short cytoplasmic stretch with unknown function, and recognize secreted AVR proteins of *Cladosporium fulvum* (Piedras *et al.* 2000). Strikingly, *R* genes are often organized in complex clusters. The barley *MLA* cluster (powdery mildew resistance locus a) on barley chromosome 5, for example, comprises more than 30 resistance specificities that confer race-specific resistance to the barley powdery mildew fungus. At least 3 sub-families of CC-NBS-LRR protein genes have been characterized within this locus (Jørgensen 1994; Wei *et al.* 1999). The striking sequence similarity in the CC-NBS domain of different *MLA* specificities gave reason to speculations that they might be alleles of a single gene. Comparatively higher sequence diversity in the LRR region refers to its role in determining AVR recognition specificity (Schulze-Lefert and Panstruga 2003).

The molecular mechanisms behind regulation of plant disease resistance genes are largely unknown. Some downstream elements of *R* gene mediated signaling pathways have been identified. *RAR1* (required for *MLA*-specified resistance) for example encodes a protein with two zinc finger motifs called CHORD-I and CHORD-II (Cys- and His-rich domain) and is involved in defense signaling conferred by some but not all *MLA* specificities, although they are closely related (Freialdenhoven *et al.* 1994; Shirasu *et al.* 1999a). RAR1 itself putatively interacts with the SGT1 protein (suppressor of G-two allele of skp1), which is a subunit of the so-called SCF (SKP1-Cullin-F-box protein) ubiquitin ligase complex. This complex is supposed to be involved in the ubiquitin-mediated protein degradation pathway (Azevedo *et al.* 2002; Peart *et al.* 2002). The role and importance of proteolysis in plant defense remains elusive. Specific degradation of pathogen-targeted defense suppressor proteins has been assumed. A general involvement, however, is rather unlikely, since specific *R* gene mediated defense pathways have different and sometimes no requirements for RAR1 and SGT1 to activate downstream components (Tör *et al.* 2003). Alternatively, a cochaperone-like activity of RAR1 and SGT1 has been proposed. In this scenario, these proteins would assist (though not through direct physical interaction) in converting recognition inactive R proteins into forms competent for AVR effector protein recognition and activation of defense signaling (Shirasu and Schulze-Lefert 2003; Bierie *et al.* 2004). The fact, that apparently disadvantageous *AVR*-genes have not been erased by selection pressure from pathogen populations, suggests that these genes might exert some other, essential function in susceptible host plants (Kjemtrup *et al.* 2000; Abramovitch 2003; Jones and Takemoto 2004). This can most convincingly be observed in plant-virus interactions, where an *AVR* gene product can also be required for viral replication, encapsulation or movement (Nimchuk *et al.* 2001). Considering the possibility that some AVR proteins contribute to virulence of the pathogen through direct interaction with host proteins, it was assumed that R proteins might also function to ‘guard’ these pathogenicity targets either by direct interception of incoming pathogen-derived AVR effector proteins or by binding to complexes of such effector proteins with their pathogenicity target. Both recognition events would subsequently be followed by activation of defense responses (Lahaye and Bonas 2001). Race-specific resistance is quite effective and disease symptoms become almost completely suppressed. However, *R* gene mediated resistance, like most monogenically conferred resistances, can relatively easy be overcome by the

respective pathogen upon adequate selection pressure, and therefore often displays only minor durability during its agronomic usage.

1.6.3 *mlo*-mediated broad-spectrum resistance

Since its introgression in the 1970s the presence of homozygous recessive alleles of the *MLO* gene (*powdery-mildew-resistance gene o*) confers broad-spectrum resistance of European spring barley cultivars to all occurring isolates of the barley powdery mildew fungus (Jørgensen 1992). Broad-spectrum powdery mildew resistance turned out to be durable in the field ever since. It should be noted, however, that lack of MLO is also coming along with some pleiotropic effects such as enhanced susceptibility of barley to *Magnaporthe grisea* and toxins of *Bipolaris sorokiniana* (Jarosch *et al.* 1999; Kumar *et al.* 2001). Strikingly, *mlo*-mediated resistance to *Bgh* is expressed prior to fungal invasion, which appears to be based on early formation of effective cell wall appositions that is accompanied by a local H_2O_2 burst (Freialdenhoven *et al.* 1996; Hückelhoven *et al.* 1999; Piffanelli *et al.* 2002). *mlo*-mutant plants seem to possess an overall enhanced resistance status, which is mirrored by a more intense accumulation of *PR* gene transcripts in *mlo* compared to *Mlo* genotypes in response to pathogen challenge (Peterhänsel *et al.* 1997; Piffanelli *et al.* 2002) Under axenic conditions *mlo*-plants show spontaneous papillae deposition but also onset of mesophyll cell death that eventually results in spatially restricted necrotic spots. Thus, a dual role of the MLO protein in suppressing both leaf cell death and pathogen defense has been proposed. The *MLO* locus has been mapped to the long arm of barley chromosome 4H. The corresponding gene could be isolated and encodes a protein of about 60 kDa (Büschges *et al.* 1997). Analysis of the amino acid sequence of the functional MLO protein revealed the presence of 7 transmembrane helices with the *C*-terminus extending into the cytoplasm and the *N*-terminus into the apoplast. A reminiscence of animal and fungal G-protein-coupled receptors, which participate in transducing extracellular signals through the membrane, has been noted (Devoto *et al.* 1999). The protein does not show homology to any known resistance gene, and homologous sequences could only be traced in plant genomes. Until now, 32 *MLO*-like genes have been found in monocot and dicot plant species occurring in medium-sized gene families (Kim *et al.* 2002a; Devoto *et al.* 2003). Accumulation of *MLO* transcript can be observed during senescence and in response to biotic or abiotic stress stimuli mirroring a general

involvement of the protein in cell death protection and in processes related to defense and stress (Piffanelli *et al.* 2002, Kim *et al.* 2002a). The fact that the functional plant protein is required for defense suppression together with the specificity of *mlo*-mediated resistance to powdery mildew fungi indicate that MLO may serve as susceptibility factor or in other words that *Bgh* may target MLO in order to render plants accessible (Kim *et al.* 2002b; Hückelhoven 2005). The protein exerts its function in defense suppression at the plant plasma membrane and its activity is in part controlled by a calcium-dependent interaction with calmodulin (Devoto *et al.* 1999; Kim *et al.* 2002b). The *mlo*-mediated resistance response in barley is dependent on at least two additional genes, *ROR1* (*required for mlo specified resistance*) and *ROR2*. In the respective barley *mlo*/*ror* double mutant plants, partial restoration of disease susceptibility to *Bgh* comes along with reduced spontaneous leaf cell death (Freialdenhoven *et al.* 1996; Peterhänsel *et al.* 1997). Weakening of penetration resistance here seems to be related to a compromised ability to accumulate H_2O_2 at sites of fungal attack and is accompanied by a generally retarded and reduced onset of *PR* gene expression (Hückelhoven *et al.* 2000b; Piffanelli *et al.* 2002).

1.6.4 Nonhost resistance

Every plant pathogen has only a limited range of host species on which it can cause disease. The remaining plants are “nonhost plants” to this pathogen and can resist the attacker due to a multitude of different mechanisms that collectively contribute to nonhost resistance (Heath 2000b; Thordal-Christensen 2003). Since nonhost resistance is defined as the resistance presented by all cultivars of a plant species towards all races of a certain pathogen, the incompatible interactions of barley with inappropriate *Bgt* or of wheat with *Bgh* are considered to be nonhost interactions (Heath 1981). In general, plants possess diverse preformed and induced defense strategies, which are aimed at restricting pathogen establishment at different stages of its development, and of which some are commonly found in host resistance as well (Thordal-Christensen 2003). Plant surface topography and constitution can either prevent activation of pathogen development in the fist place or inhibit fungal ingress by representing insuperable physical barriers. Lack of special surface characteristics for example can simply impede host recognition and thus induction of appressoria formation by *B. graminis* (Green *et al.* 2002). Besides raising those rather unspecific

traits, plants can recognize pathogen surface-derived general elicitors (reminiscent of so called pathogen associated molecular patterns (PAMPs) in animal innate immunity), which thereupon trigger unspecific defense reactions (Nürnberger and Brunner 2002; Nürnberger and Lipka 2005). Among the most prominent examples of PAMP-like factors, which induce defense reactions in plants, one can find lipopolysaccarides and the peptide flagellin from bacteria, or constituents of fungal cell walls like chitin and glucan (Felix *et al.* 1999; Bedini *et al.* 2005). General elicitors can trigger the formation of cell wall appositions and the induction of *PR* genes (Schweizer *et al.* 2000a). In case a biotrophic pathogen is able to enter nonhost tissue anyway, the plant can stop it from acquiring nutrients by an HR of the affected cell. Until now, only limited knowledge is available on the genetic background of nonhost resistance. Segregation analysis with regard to the outcome of nonhost interactions between grasses and inappropriate races of *B. graminis* indicated the participation of major genes (Niks 1988; Tosa 1992; Matsumura and Tosa 1995). For plant-bacteria nonhost interactions the involvement of pathogen recognition based on the interaction of *AVR* with *R* gene products following the gene-for-gene principle has been demonstrated. In this scenario one or more pathogen-derived *avirulence* (*avr*) gene products mediate recognition by an *R* gene product, which is present in all genotypes of a certain plant. Consequently, those plants are nonhosts to the invader (Thordal-Christensen 2003).

The wheat powdery mildew fungus (*Bgt*) does not accomplish its infection cycle on barley plants. Its growth already becomes arrested while it is about to penetrate the epidermal host cell or soon after it has entered it. The defense mechanisms behind resistance to avirulent appropriate or inappropriate races of powdery mildew fungi in barley are barely distinguishable. Both rely on penetration resistance and/or the rapid onset of HR within attacked or already penetrated host cells (Kita *et al.* 1981; Tosa and Shishiyama 1984; Tosa *et al.* 1990; Hückelhoven *et al.* 2001a). Since penetration frequencies of *Bgt* into barley epidermal cells are usually rather low, penetration resistance seems to play a major role during this interaction, although there are considerable plant cultivar-specific differences (Trujillo *et al.* 2004a, b). Interestingly, single cell overexpression of the cell death suppressor gene *MLO* can modulate nonhost penetration resistance and allows *Bgt* to penetrate otherwise inaccessible barley cells. Thus, nonhost resistance in the cereal-powdery mildew fungus interaction is unlikely to rely on the lack of compatibility factors but rather

depends on active defense reactions (Elliott *et al.* 2002; Trujillo *et al.* 2004b). Only a few other specific plant factors are known, which significantly contribute to the establishment or maintenance of nonhost resistance. Arabidopsis is a nonhost plant to the wheat powdery mildew fungus (*Bgt*). During a screen for Arabidopsis mutants, which are impaired in penetration resistance towards *Bgt*, the *PEN1* (increased penetration by *Bgt*) gene could be isolated, which appears to be required for complete nonhost resistance. *PEN1* and its barley ortholog *ROR2*, encode functionally homologous syntaxins residing within the plasma membrane. Syntaxins are thought to be involved in membrane fusion events (e.g. fusion of vesicles to one another) or in facilitating exocytosis, emphasizing the conserved role of papilla-related vesicle trafficking in both host and nonhost resistance of monocotyledonous and dicotyledonous plants (Collins *et al.* 2003). Besides, Yun *et al.* (2003) could demonstrate that loss of *EDS1* function (*enhanced disease susceptibility 1*) together with inhibition of actin polymerization severely compromised nonhost resistance to *Bgt* in such a way that the fungus could even accomplish its infection cycle on Arabidopsis plants in some cases. Since the susceptibility could not be restored to the extend of a compatible interaction, it was speculated, that in addition, Arabidopsis may lack some specific compatibility factors crucial for *Bgt* or alternatively, additional EDS1 and actin independent defense mechanisms may contribute to this type of nonhost resistance. Due to its durable effectiveness, nonhost resistance has recently moved into the focus of scientific interest, since it promises to be of use for the generation of resistant crop plants.

1.7 Objectives

The high durability and completeness of nonhost resistance against a huge spectrum of potentially pathogenic microorganisms turns it into a valuable topic for basic research and modern plant production. Considerable effort has been made to elucidate signal transduction processes and regulatory elements of plant defense. In spite of a number of investigations, which examined the transcriptome of plants during the interaction with powdery mildew fungi, we are still far from understanding the nature of nonhost resistance especially in terms of its constancy. Hence, this study was aimed at contributing to the understanding of the mechanisms that underlie nonhost resistance and its counterpart, basic compatibility. For this purpose, the model system of barley interacting with appropriate or inappropriate *formae speciales* of *Blumeria graminis* was used.

The cDNA macroarray technique is a versatile tool to compare expression of a great many of genes that are potentially involved in the host or nonhost interaction. The macroarray membranes that were used in the present study mainly comprised cDNA fragments from plants pretreated with a chemical resistance activating compound, and were therefore expected to be enriched in defense related genes. Hybridization of the macroarray membranes with probes from primary leaves of barley plants inoculated with either appropriate *Bgh* or inappropriate *Bgt*, respectively, should help to identify common and divergent elements of compatibility and incompatibility.

The second part of this work, too, focused on the basic principles of defense responses in the nonhost interaction of barley with the inappropriate *forma specialis* of *B. graminis*. *BAX INHIBITOR-1* (*BI-1*) suppresses non-specific *mlo*-mediated and background penetration resistance of the host barley epidermal cells to *Bgh.* To discover a potential link between non-specific host and nonhost resistance, it was interesting to investigate, whether the BI-1 protein would also affect nonhost resistance of barley to inappropriate *Bgt*. Additionally, various molecular and cytological tools were applied to analyze BI-1 function in cell death and defense regulation.

2 Materials and methods

2.1 Plants, pathogens and inoculation

The barley (*Hordeum vulgare* L.) cultivar Ingrid and its corresponding backcross line BCIngrid-*mlo5* (I22), which does not possess the functional MLO protein, were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Jörn Pons-Kühnemann (Justus-Liebig Universität, Giessen, Germany) provided barley cultivar Manchuria. 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). After seven days of growth, inoculation experiments were conducted using either the appropriate pathogen barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*, *Bgh*) race A6, which was provided by Jörn Pons-Kühnemann (Justus-Liebig Universität, Giessen, Germany), or the inappropriate wheat powdery mildew fungus (*Blumeria graminis* f.sp. *tritici*, *Bgt*) field isolate A95, which was gained near Aachen by Ulrich Beckhove. For inoculation, the leaves were fixed with the abaxial side up and put under a tent-like frame. Spores of the respective powdery mildew fungus were evenly spread on barley leaves giving a density of 80-100 conidia mm^{-2} for gene expression studies and 150 conidia mm^{-2} for gene function assessment on transformed leaf segments. Control plants were mock inoculated, i.e. they were treated alike without applying any spores. *Bgh* was maintained on *Hordeum vulgare* cv. Golden Promise, *Bgt* on *Triticum aestivum* cv. Kanzler in climate chambers providing the above conditions.

2.2 Macroarray-based identification of differentially expressed genes

2.2.1 Macroarray generation

The cDNA macroarray used in this study comprised 1,536 cDNA fragments. Among the clones, 1,344 derived from the GAN library which was created from barley (cv. Ingrid) leaf epidermis from seven days old seedlings that were treated with acibenzolar-*S*-methyl (synonym: Benzo(1,2,3)thiadiazole-7-carbothioic acid [BTH],

Novartis, Basel, Switzerland) by soil drenching (40 mg l^{-1} soil volume). In total, the normalized GAN library contained 3,036 sequences that were 25.43 % unique when compared to the Affymetrix unigene dataset (http://barleybase.org/; Close *et al.* 2004; Sophia Biemelt, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany, personal communication). 192 clones were specifically selected for additional spotting, among them some known pathogen-responsive or chemically induced genes (Besser *et al.* 2000; Hückelhoven *et al.* 2001b; Eckey *et al.* 2004; Jansen *et al.* 2005). The cDNA was amplified by PCR using vector specific primers (forward 5'-gttttcccagtaacgacgacgtt-3'; reverse 5'-caggaaacagctatgaccatg-3') and spotted in duplicate onto nylon membranes (Hybond N+, Amersham Bioscience, Freiburg, Germany) in a 3x3 pattern using Microgrid II (Biorobotics, Cambrigde, UK) as described by Sreenivasulu *et al.* (2002). Macroarray membranes were provided by Patrick Schäfer (Justus-Liebig Universität, Giessen, Germany).

2.2.2 Synthesis of ^{33}P-cDNA and hybridization procedure

For comparative macroarray analysis barley (*Hordeum vulagare* L.) cultivar Ingrid 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). Inoculation of barley plants was accomplished after seven days by applying spores of either barley powdery mildew fungus *Blumeria graminis* (DC) Speer f.sp. *hordei* (*Bgh*) race A6 or wheat powdery mildew fungus *Blumeria graminis* (DC) Speer f.sp. *tritici* (*Bgt*) isolate A95, respectively, giving a density of 80-100 conidia mm^{-2}. Control plants were mock inoculated. First leaves of the plants were harvested 12 and 24 hours after inoculation (HAI) and crushed in liquid nitrogen.

2.2.2.1 Isolation of poly(A)$^+$-RNA

Poly(A)$^+$-RNA was isolated from 300 mg of crushed material using oligo(dT)-magnetic beads (Dynal, Hamburg, Germany) following the user manual. For this purpose, 125 µl of magnetic bead solution were separated magnetically and then washed twice with 200 µl lysis buffer. After that, 300 mg of crushed plant material were homogenized in 1.5 ml lysis buffer. After centrifugation at 4°C and 14,000 rpm for 5 min, the supernatant was added to the magnetic beads. After vortexing, the suspension was incubated at room temperature for 5 min upon constant inversion of the reaction tubes. After magnetic separation, the supernatant was discarded and the

remaining beads were washed 3 times with 500 µl washing buffer containing lithium dodecylsulphate.

- **Lysis buffer:**

100 mM	Tris
500 mM	LiCl (lithium chloride)
10 mM	EDTA (ethylen diamino tetraacetic acid)
1 %	LiDS (lithium dodecylsulphate)
	in A. bidest.$_{DEPC}$
	use HCl to adjust pH to 8.0
add 5 mM	DTT (dithio threitol) at the beginning of probe synthesis

- **Washing buffer with LiDS:**

10 mM	Tris
150 mM	LiCl
1 mM	EDTA
	in A. bidest.$_{DEPC}$
	use HCl to adjust pH to 8.0
add 0.1 %	LiDS at the beginning of probe synthesis

2.2.2.2 Synthesis of first strand cDNA

In order to prepare for first strand cDNA synthesis, beads were washed three times in 1x reverse transcription (RT) buffer. Transfer of the solution into new reaction tubes accompanied each washing step. After magnetic separation, 50 µl of freshly prepared reverse transcription mix were added to the beads. Upon occasional shaking, the reaction mix was incubated in a water bath at 42°C for 1 h. After that, the solution was separated magnetically and the supernatant was discarded. Beads were washed twice in 250 µl 1x reverse transcription buffer (Promega GmbH, Mannheim, Germany). Elution of mRNAs was accomplished by incubating the beads at 95°C for 2 min in 50 µl of elution buffer. The heating was followed by immediate magnetic separation of solution and beads. The supernatants (containing mRNAs) were collected into a new reaction tube. First strand cDNAs remained attached to the magnetic beads and served as template for synthesis of ^{33}P labeled cDNAs.

- **Reverse transcription Mix:**

10 µl	5x AMV (Avian Myeloblastosis Virus) RT buffer
0.5 µl	100 mM DTT
12.5 µl	2mM dNTPs (deoxyribonucleotide triphosphates)
1.5 µl	RNase inhibitor (30u/µl)
3 µl	AMV reverse transcriptase (24u/µl; Promega GmbH)
22.5 µl	A. bidest.$_{DEPC}$

- **5x RT buffer:**

250 mM	Tris
250 mM	KCl (potassium chloride)
50 mM	$MgCl_2$ (magnesium chloride) x 6 H_2O
	in A. bidest.$_{DEPC}$
	use HCl to adjust pH to 8.3

- **Elution buffer:**

2 mM	EDTA
	in A. bidest.$_{DEPC}$

2.2.2.3 Random prime labeling

Synthesis of ^{33}P-labeled cDNA and hybridization of macroarrays was performed following the instructions by Sreenivasulu *et al.* (2002) and Potokina *et al.* (2002). Magnetic beads were washed twice in 250 µl of A. bidest., added to 35 µl of distilled water and 5 µl of $(dN)_6$ random primer (1 mg/ml; Megaprime™ DNA labeling system, Amersham Biosciences Europe GmbH, Freiburg, Germany) and then incubated for 3 min at 95°C. Random prime labeling was accomplished by consecutively combining 10 µl of labeling buffer (Megaprime™ DNA labeling system, Amersham Biosciences Europe GmbH, Freiburg, Germany), 5 µl [α-33P] dCTPs (50 µCi/µl; Amersham Biosciences Europe GmbH, Freiburg, Germany) and 1 µl of Klenow fragment exo$^-$ (10 u/µl; Fermentas GmbH, St. Leon-Rot, Germany). Incubation for 60 min at 37°C followed this step. After magnetic separation, non-integrated nucleotides were removed with the supernatant. The final elution was preceded by a washing step with 150 µl elution buffer. Finally, magnetic beads were incubated in 150 µl elution buffer for 3 min at 95°C and immediately underwent magnetic separation. The supernatant was collected in a new reaction tube. After repetition of this step, eluates were combined and purified using a 0.2 µm Anapore™ spin column (Whatman, Kent, UK)

giving the final probe. Prior to hybridization, probe radiations were measured and adjusted, when necessary.

2.2.2.4 Pre-hybridization and hybridization of macroarray membranes

For pre-hybridization, macroarray membranes were transferred into glass tubes and incubated for 2 h at 65°C in 15 ml of hybridization buffer. After denaturation, probes were transferred into the glass tubes containing 10 ml of fresh hybridization buffer. After overnight hybridization, the arrays were washed stringently with 0.2x SSC / 0.1% SDS and 0.1x SSC / 0.1% SDS for 15 minutes each. The arrays were exposed for three days to a Fuji BAS-MS 2025 imaging plate (Fuji Photo Film, Tokyo, Japan) and then scanned with a Molecular Imager FX Phosphoimager (Bio-Rad, München, Germany).

- **100x Denhardt's solution**

1 g	Ficoll
1 g	PVP (polyvinylpyrolidone)
1 g	BSA (bovine serum albumin)
ad 50 ml	A. bidest.$_{DEPC}$

- **20x SSC (sodium salt citrate)**

3 M	NaCl (sodium chloride)
0.3 M	tri-sodium citrate
	in A. bidest.$_{DEPC}$
	use HCl to adjust pH to 7.0

- **Hybridization buffer**

20 ml	100x Denhardt's solution
133 ml	20x SSC (sodium salt citrate)
0.1 %	SDS (sodium dodecylsulphate)
ad 400 ml	A. bidest.$_{DEPC}$
100 µg/ml	Salmon sperm (Invitrogen GmbH, Karlsruhe, Germany)

2.2.3 Data analysis

Data acquisition was performed with the software package ArrayVision™ (Imaging Research Inc., St. Catharines, Canada), and normalization and statistical computing of signal intensities with the free software R (http://www.r-project.org). Genes with

signal intensities 5 fold above local background and which were more than 2.5 fold up- or down-regulated after inoculation compared to the control in 2 of 2 biological experiments at 12 HAI or 2-3 of 3 biological experiments at 24 HAI were considered as pathogenesis-regulated and some of them were selected for further analysis.

2.2.4 Confirmation of differential gene expression

2.2.4.1 Northern analysis

For independent confirmation of differential gene expression, inoculation experiments were performed as described in chapter 2.1. Leaves were harvested 0, 4, 8, 12, 24 and 48 h after inoculation. 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 Northern analysis, 15 µg of total RNA were separated in a 1.2 % agarose gel and blotted on Hybond™-N^+ nylon membranes (Amersham Biosciences Europe GmbH, Freiburg, Germany). Probe labeling of individual cDNA fragments was carried out with the random prime HexaLabel™ DNA Labeling Kit (Fermentas GmbH, St. Leon-Rot, Germany) following manufacturer's instructions using [α-^{32}P] dCTPs (50 µCi/µl; Amersham Biosciences Europe GmbH, Freiburg, Germany). PCR-amplified inserts of cDNA clones served as template. For pre-hybridization and over-night hybridization, a sodium phosphate hybridization buffer was applied. Membranes were first washed twice in 2x SSC, 0.1 % SDS for 5 minutes and then more stringently in 0.1x SSC, 0.1 % SDS for 10 to 15 min. Finally, signals were detected with a Molecular Imager FX Phosphoimager (Bio-Rad, München, Germany).

- **Hybridization buffer**

5 g/l	BSA
45 g/l	SDS
1000 µl/l	0.5 M EDTA
	in sodium phosphate (pH 7.2)

Barley *PR1-b* served as positive control for strong *Bgh*-induced gene expression (Bryngelsson *et al.* 1994). Non-radioactive Northern analysis was performed for detection of this gene. For this purpose, 5 µg of total RNA from each sample were separated in an agarose gel and blotted by capillary transfer to a positively charged

nylon membrane. Detection of mRNAs was performed according to the DIG-system user's guide with fluorescein labeled anti-sense RNA probes (Hückelhoven *et al.* 2001b). Before immunodetection, the blot was washed stringently for two times 20 min in 0.1x SSC, 0.1 % SDS at 68°C.

2.2.4.2 Semi-quantitative RT-PCR

To detect low-level transcripts, semi-quantitative two-step RT-PCR was used. 5 µg of total RNA from the above experiment were reverse transcribed to first strand cDNA. cDNA synthesis was primed with oligo (dT) using M-MuLV Reverse Transcriptase (Fermentas GmbH, St. Leon-Rot, Germany) in a total volume of 25 µl. 250 ng aliquots of the first strand cDNA were subsequently used as template for common PCR amplification with gene specific primers under stringent conditions. PCR cycle numbers were empirically optimized to be 25 or less to avoid over-cycling. As control for constitutive gene expression, a ubiquitin coding gene (GenBank accession number M60175) was used. Again, barley *PR1-b* served as a positive control for strong *Bgh*-induced gene expression (Bryngelsson *et al.* 1994). For primer sequences and specific PCR conditions see Table 2.1.

Table 2.1 Primer sequences

Clone ID	predicted protein	forward primer (5'→3')	reverse primer (5'→3')	annealing temperature	product size
GAN004A17F HvD00177	subtilase	CCAGCTCAATCTCAATCTTC	ATGTCAAGCTCCCAAAGG	55°C	257 bp
HvD00107	protein kinase (Pti1)	GCCCCAGAGTACGCAAT	TGGTCAATTCTGTGGATCA	57°C	389 bp
GAN003B22F	hypothetical protein	CGACGACTGCAGCAACT	CAAAGATGCCGAACAACA	53°C	552 bp
GAN003C21F	RLK19	GGCTGGTGGAGGTACAGA	GACCGCTGACCAGTGATT	56°C	171 bp
HvD00108	autophagy 8c	TGCTTCGTTGATGTCTGC	CAACACACGGGCACATAA	53°C	380 bp
HvD00126	no significant similarity	AACAGAGCAGGAGGTAACAA	TCCTTAAGCACCAGAACG	55°C	284 bp
GAN001L08F GAN001J13F GAN003B13F	cysteine proteinase	ATGAACGCTGTGGCAAA	AAAATGGATGGATCTGGAA	57°C	388 bp
GAN002N07F	luminal binding protein 3 (BiP3)	GAGGAGTTTGCCGAGGA	AAATTGGCTCCCCCTTC	57°C	378 bp
GAN002I20F GAN003M08F GAN001N06F GAN002O07F GAN003P08F	glycine-rich cell wall structural protein	TCAAACTGATCATCGCCAAT	TCCAACCATTGTGACATCTTG	56°C	651 bp
HvD00174	barley lipid transfer protein 4 (BLT4)	GGTGCCTCAAGAGTGTCG	TGCCCATGGTACAACGTA	54°C	369 bp

2.3. Structural and functional characterization of the cell-death suppressor BAX INHIBITOR-1 (BI-1)

2.3.1 Expression analysis of *BI-1*

BI-1 expression pattern was examined by using the OneStep RT-PCR kit (Qiagen, Hilden, Germany) for semi-quantitative reverse transcription polymerase chain reaction following manufacturer's instructions. Amplification of a 758 bp *ACTIN* cDNA fragment (GenBank accession AJ234400), which served as control for constitutive gene expression, was achieved with primers 5´-ctgtaggaaatggctgacgg-3´ (5´ primer) and 5´-tcggatcacctgacccat-3´ (3´ primer). Primers 5´-atggacgccttctactcgacctcg-3´ (5´ primer) and 5´-gccagagcaggatcgacgcc-3´ (3´ primer) were used to obtain a 478 bp *BAX INHIBITOR-1* (*BI-1*) cDNA fragment (GenBank accession AJ290421). To avoid over-cycling, 25 cycles were used for *ACTIN* and 30 cycles for *BI-1* during the exponential amplification phase. cDNAs were separated in agarose gels.

2.3.2 Construction of *pGFP-BI-1*

BI-1 ORF (Hückelhoven *et al.* 2003) was amplified by PCR using the primers 5'-ggatcccaacgcgagcgcaggacaagc-3' (5' primer, containing a *Bam*HI site) and 5'-gtcgacgcggtgacggtatctacatg-3' (3' primer, containing a *Sal*I site), and subsequently cloned into the pGEM-T vector (Promega, Mannheim, Germany). After sequence confirmation, the *Bam*HI-*Sal*I fragment was cloned into the expression vector pGY1 (Schweizer *et al.* 1999). The GFP coding fragment was amplified using the oligonucleotides 5'-ggatccatggtgagcaagggcgag-3' (5' primer, containing a *Bam*HI site) and 5'-ggatccttgtacagctcgtccat-3' (3' primer, containing a *Bam*HI site), which eliminates the stop codon, and was inserted in frame into pGY1-BI-1 using the internal *Bam*HI site of the BI-1 forward primer (i.e. at the N-terminal end of BI-1). The cloning was accomplished by Holger Schultheiss, Justus-Liebig Universität, Giessen, Germany.

2.3.3 Mutagenesis of barley *BI-1*

To characterize the connection between structure and function of the BI-1 protein in disease resistance suppression and cell death, respectively, the *BI-1* gene was modified in different conserved amino acids and truncated by the C-terminus. Subsequently, the altered proteins were examined concerning their ability to suppress resistance responses of barley to powdery mildew fungi.

In vitro mutagenesis of barley *pBI-1* (*BI-1* in pGY-1; Hückelhoven *et al.* 2003) was accomplished by means of the Transformer™ Site-Directed Mutagenesis Kit (Clontech, Heidelberg, Germany). By using different mutagenic primers, diverse specific base changes were introduced into the *BI-1* sequence, which resulted in amino acid exchanges in the expressed protein. Concomitant to the mutagenic primer, the method required employment of a selection primer that mutates a unique restriction site within the plasmid. In our case, selection primer caused mutation of the *Ssp*I recognition sequence into a *Eco*RV site.

For denaturation and primer annealing, the following compounds were put together and incubated for 5 min in a 100°C water bath.

6 µl	T4 polymerase buffer (Fermentas GmbH, St. Leon-Rot, Germany)
100-200 ng	Plasmid for mutagenesis
150-225 ng	Selection primer (phosphorylated)
150-225 ng	Mutagenesis primer (phosphorylated)
ad 20 µl	A. dest.

After immediate transfer of the reaction tubes into ice water, T4 DNA polymerase and T4 DNA ligase (Fermentas GmbH, St. Leon-Rot, Germany) were added. The mixture was subsequently incubated for 1-2 h at 37°C in order to allow DNA elongation and ligation:

20 µl	Reaction mix (see above)
2 µl	10 mM ATP
1 µl	T4 DNA polymerase (2-4 u/µl)
2 µl	T4 DNA ligase
5 µl	A. dest.

Incubation at 70°C stopped the reaction. Primary selection of mutated plasmid was accomplished by restriction enzyme digestion with *Ssp*I for 1-2 h at 37°C:

10 µl	Reaction mix (see above)
1 µl	*Ssp*I (Fermentas GmbH, St. Leon-Rot, Germany)
5 µl	Enzyme buffer (Fermentas GmbH, St. Leon-Rot, Germany)
34 µl	A. dest.

After inactivation of the enzyme through incubation at 70°C for 5 min, the mixture of mutated and unmutated plasmids was transferred into BMH 71-80 *mut*S *Escherichia coli* cells and used LB medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl; pH 7.0) containing 50 µg/ml ampicillin for multiplication of the transformed cells. After plasmid isolation, DNA was subjected to another selective restriction enzyme digestion with *Ssp*I for 2 h at 37°C. Since the mutated DNA lacks enzyme recognition sequence, it is resistant to digestion. The parental, unmodified plasmid, however, becomes linearized and is thus much less efficient in transformation of bacterial cells.

1 µl	Mixed plasmid DNA (max. 0.1 µg)
1 µl	*Ssp*I (Fermentas GmbH, St. Leon-Rot, Germany)
5 µl	Enzyme buffer (Fermentas GmbH, St. Leon-Rot, Germany)
43 µl	A. dest.

After addition of another µl of *Ssp*I enzyme, the reaction mix was again incubated at 37°C for 1 h to ensure thorough restriction digestion. Finally, the selectively digested plasmid was transferred into competent Dh5α *E. coli* cells and distributed on LB agar plates containing 100 µg/ml ampicillin. Single colonies were amplified and DNA was isolated. After sequencing, clones were selected, which were positive for specific base change. For sequences of the respective mutagenic primers and resulting amino acid exchanges see Table 2.2.

Table 2.2 BI-1 mutants and primers used for site directed mutagenesis

BI-1 mutant	primer sequence	aa exchange	aa position
BI-1P28A	GCCAGATCTCC**G**CCGCCGTGCAGT	Pro → Ala	28
BI-1H33L	CGTGCAGTCCC**T**CCTCAAGCTCGT	His → Leu	33
BI-1P104A	GCTTCGGTTGGA**G**CTCTGATTGAG	Pro → Ala	104
BI-1G123R	AGGGTTTGTC**A**GAACCGCCATCGC	Gly → Arg	123
BI-1F128L	CGCCATCGCCTT**G**GGGTGCTTCTC	Phe → Leu	128
BI-1S152F	CCTGCTCTCGT**T**TGGCCTGTCGAT	Ser → Phe	152
BI-1S155P	CGTCTGGCCTG**C**CGATCCTGCTCT	Ser → Pro	155
BI-1D192A	ACATGGTGTACG**C**CACGCAGGAGA	Asp → Ala	192
BI-1R224L	CCGTCCTCGTCC**T**AGTCCTCATCA	Arg → Leu	224

BI-1 with truncated C-terminus was amplified by PCR using primers 5'-ggatcccaacgcgagcgcaggacaagc-3' (5' primer, containing a *Bam*HI site) and 5'-tcagagcatgatgatgaggac-3' (3' primer) and subsequently cloned into the pGEM-T vector (Promega, Mannheim, Germany). After sequence confirmation, the *Bam*HI-*Sph*I fragment was cloned into the expression vector pGY1 (Schweizer *et al.* 1999). Thus, a stop codon was inserted into the nucleotide sequence at position 689, and the terminal 17 amino acids of the wild type protein were removed, giving *pBI-1ΔC*. All *BI-1* variants were verified by sequencing prior to ballistic delivery into barley epidermal cells (see chapter 2.3.4).

2.3.4 Transient transformation and evaluation of penetration efficiency

Barley leaves were transformed via ballistic delivery of expression vectors into single epidermal cells of barley leaf segments according to a transient transformation protocol originally developed for wheat (Schweizer *et al.* 1999). Each shot delivered 312 µg 1.1 µm tungsten particles coated with 0.5 µg of either *pGFP* (*GFP* under control of CaMV 35S promoter) or *pUbiGUS* (*uida*, *β*-glucuronidase gene from *Escherichia coli* under control of the maize ubiquitin promoter) as reporter gene together with the expression construct of the gene of interest or empty pGY-1 vector as control. All genes were subcloned into pGY-1 to be controlled by the constitutive CaMV 35S promoter (Hückelhoven *et al.* 2003; Schweizer *et al.* 1999). Table 2.3 gives an overview of all experiments employing transient transformation, providing information on barley cultivar and *forma specialis* of *B. graminis* used, on

concentration and identity of the expression constructs as well as the marker gene employed for the respective experiment. Progression of overexpression was allowed for 4 hours prior to inoculation with powdery mildew spores. Inoculation with 150 conidia mm^{-2} led to attack of approximately 50 % of transformed cells. Two days after inoculation, the penetration frequencies of germinated *Bgh* or *Bgt* conidia on transformed epidermal cells, were evaluated using fluorescence and brightfield microscopy as described previously (Schultheiss *et al.* 2002). For *β*-glucuronidase activity (GUS) staining, leaves were vacuum-infiltrated with a solution of the substrate for GUS, X-gluc, and incubated over night at 37 °C as described by Schweizer *et al.* (1999). For each individual variant, a minimum of 50 interaction sites were evaluated. The penetration efficiency, as a measure for resistance of bombarded cells, was calculated as number of penetrated cells divided by number of attacked cells multiplied by 100.

2.3.5 Localization of BI-1 fusion constructs

Barley leaf segments were transiently transformed via particle bombardment as described above with each shot delivering 0.5 µg *DsRED* plasmid (pe35AscloptRed; DsRed-C1 under control of CaMV 35S promoter, obtained from Edgar Maiss, University of Hannover, Germany) together with 0.8 µg *pGFP-BI-1 or pGFP-BI-1ΔC* fusion construct (Table 2.3). DsRED was taken as control for protein localization in the cytosol and nucleus (Dietrich and Maiss 2002).

Leaf segments were analyzed 3 days after transformation. Fluorescence of DsRED and GFP-BI-1 was detected by confocal laser scanning microscopy (CLSM, Leica TCS SP2, Leica Microsystems, Bensheim, Germany) by 24 to 72 h after transformation. GFP was excitated 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.

To examine subcellular accumulation of the GFP-BI-1 fusion protein in connection with fungal attack, barley leaf segments were inoculated 4 h after ballistic transformation with spores of either *Bgh* or *Bgt*. Confocal laser scanning microscopy followed 48 to 72 h after inoculation.

Table 2.3 Transient transformation experiments

experiment	plant genotype	*B. graminis* f.sp.	plasmid I	conc./shot	plasmid II	conc./shot	(additional) marker gene
Functional characterization of BI-1 and GFP-BI-1	Manchuria	*Bgt*	*pBI-1* or *pGFP-BI-1*	0.8 µg	-	-	*GFP (UbiGUS)*
Functional characterization of BI-1	Ingrid	*Bgt*	*pBI-1*	0.8 µg	-	-	*UbiGUS*
Functional characterization of BI-1	BCIngrid-*mlo5* (I22)	*Bgt*	*pBI-1*	0.8 µg	-	-	*UbiGUS*
Simultaneous overexpression of *BI-1* and *MLO*	Manchuria	*Bgt*	*pBI-1*	0.5 µg	*pMLO*	0.5 µg	*UbiGUS*
BI-1 overexpr. in combination with H_2O_2 staining	BCIngrid-*mlo5* (I22)	*Bgh*	*pBI-1*	0.8 µg	-	-	*GFP*
Functional characterization of BI-1 mutants	Manchuria	*Bgt*	*pBI-1P28A* *pBI-1H33L* *pBI-1P104A* *pBI-1G123R* *pBI-1F128L* *pBI-1S152F* *pBI-1S155P* *pBI-1D192A* *pBI-1R224L* or *pBI-1ΔC*	0.8 µg	-	-	*GFP*
Localization of BI-1 fusion proteins	Manchuria	(*Bgh* or *Bgt*)	*pGFP-BI-1* or *pGFP-BI-1ΔC*	0.8 µg	-	-	*DsRED*
Suppression of BAX induced single-cell death	Manchuria	-	*pBI-1* *pBI-1ΔC* *pAPX* or *pBCL-X_L*	1.6 µg	*pBAX*	0.05 µg	*GFP*
Suppression of BAX induced single-cell death	GFP-BI-1 stable transformants	-	*pBAX*	0.05 µg	-	-	*sGFPHDEL*

2.3.6 H_2O_2 staining of transiently transformed leaf segments

Transient overexpression of *BI-1* and *MLO* in the *Bgh* resistant barley line BCIngrid-*mlo5* was carried out with detached leaves on agar plates as described above. 4 hours after ballistic transformation, leaves were inoculated with *Bgh* and fungal development was allowed during 14 hours prior to histochemical staining. The histochemical detection of H_2O_2 with 3,3'-diaminobenzidine (DAB) was basically performed as described previously (Thordal-Christensen *et al.* 1997, Hückelhoven *et al.* 1999). 1 mg/mL DAB was dissolved in water (pH 3.8, HCl). After cutting the edges of the transiently transformed leaves, the segments were immediately transferred into reaction tubes with 300 µl of DAB-solution and incubated for 4 to 6 hours at room temperature. Leaves were fixed in 7.4% formaldehyde in 25 mM piperazine-*N*, *N*'-bis (2-ethanesulfonic acid, PIPES, pH 6.8) buffer with 2 mM EGTA, 2 mM $MgCl_2$ and 0.05% Tween 20 (w/v) at room temperature for at least 3 hours and then discoloured in 70 % ethanol for 12 hours. Using fluorescence microscopy GFP-expressing cells were identified and evaluated as to whether they were attacked by *Bgh* and if so, whether the attempted penetration was successful or could be rejected by the plant cell. Attacked cells were further examined concerning whether there was localized DAB staining underneath the appressorial germ tube or not.

- **10x PBS buffer**

80 g/l	NaCl
2 g/l	KCl
7.65 g/l	Na_2HPO_4 x 2 H_2O (di-sodium hydrogen phosphate dihydrate)
2 g/l	KH_2PO_4 (potassium dihydrogen phosphate anhydrate)
	in A. dest

2.3.7 Cell death assay in barley

The *Ssp*I/*Xba*I fragments of murine BAX out of pSD10.a-Bax and mouse BCL-X_L out of pL009 (see below and Ligr *et al.* 1998), respectively, were cloned blunt/sticky into the plant expression vector pGY-1 (Schweizer *et al.* 1999) under the control of the constitutive CaMV 35S promoter after restriction digestion with *Sma*I and *Xba*I, resulting in *pBAX* and *pBCL-X_L*.

In order to check whether mammalian BAX was able to induce cell death in barley, *pBAX* was ballistically transferred into barley epidermal cells together with 0.5 µg per shot *pGFP* as marker gene plasmid. *pBAX* Concentrations ranged from 0.05 to 0.8 µg per shot. In the control experiment, empty vector pGY-1 instead of *pBAX* was delivered in the respective amounts.

To further assess whether barley BI-1 was functional in preventing BAX-induced cell death in barley, similar experiments were carried out, in which overexpression of murine *BAX* was accompanied by overexpression of either barley *BI-1*, barley *ascorbate peroxidase* (*APX*, accession number AJ006358; Hess and Börner 1998) or mouse *BCL-X_L* that served as positive control for BAX-antagonism. To assess, whether the C-terminal 17 amino acid were important for cell death suppression, the truncated barley BI-1 variant, BI-1ΔC was co-expressed in the same way. Each shot delivered 0.05 µg *pBAX* together with 1.6 µg of *pBI-1*, *pBI-1ΔC*, *pAPX*, *pBCL-X_L* or empty pGY-1 into barley epidermal cells. Using fluorescence microscopy, the *GFP* co-expressing cells were evaluated in terms of their viability. For this purpose, the following categories were applied: i) intact cytoplasmic strands, cytoplasmic movement clearly visible; ii) cytoplasmic strands apparently intact, but no cytoplasmic movement; and iii) strong vacuolization, no intact cytoplasmic strands, no cytoplasmic movement. Microscopic evaluation was conducted 10 to 14 h after transformation.

2.3.8 DAPI staining of transiently transformed barley leaf segments

To visualize possible BAX-induced changes in the morphology of nuclei, ballistically transformed barley leaf segments were fixed over night in a 3.5 % formaldehyde solution. In order to render the plant cell-wall diffusible for DAPI, the leaf segments were transferred into enzyme solution [0.5 % lysing enzyme from *Trichoderma hazianum* (Sigma-Aldrich Chemie GmbH, München, Germany) prepared in osmotic stabilizer (0.1 M sodium citrate buffer; 0.6 M manitol) pH 6.0] for 10 min. After a washing step in 1x PBS buffer (phosphate buffered saline, pH 7.2), leaf segments were incubated in 0.01 mg/mL 4',6-diamino-2-phenylindole dihydrochloride (DAPI) in 1x PBS buffer for 30 min, washed three times with 1x PBS buffer, and examined under the fluorescence microscope.

2.3.9 Assessment of BAX suppression in stably transformed, *GFP-BI-1* expressing barley plants

2.3.9.1 Construction of sGFPHDEL as marker for cytoplasmic movement

The *CALRETICULIN 3* (*CRT3*) gene from barley is supposed to encode an ER resident calcium storage protein (Kaufman 1999). Barley *CRT3* was amplified using primers 5'-gtcgacgccaccacctactcttcgtc-3' (5' primer, containing a *Sal*I site) and 5'-ctgcagtgtcaaatcccagcttctcc-3' (3' primer, containing a *Pst*I site) and subsequently cloned into the pGEM-T vector (Promega, Mannheim, Germany). Due to two internal *Bam*HI sites, restriction digestion of the coding DNA with this enzyme resulted in the excision of a 501 bp fragment (nucleotides 212 to 713 and amino acids 73 to 238, respectively), leaving the coding sequences for the CRT3 signal peptide and the C-terminal HDEL ER retention signal (Supplementary figure 1; Persson *et al.* 2003). The GFP coding fragment was amplified using the oligonucleotides 5'-ggatcccatggtgagcaagggcgag-3' (5' primer, containing a *Bam*HI site) and 5'-ggatccttgtacagctcgtccat-3' (3' primer, containing a *Bam*HI site), and then inserted in frame into the *Bam*HI digested, truncated *CRT3* fragment, i.e. between signal peptide and ER retention signal. After sequence confirmation, the *Sal*I-*Pst*I fragment was cloned into the expression vector pGY1 (Schweizer *et al.* 1999), giving *psGFPHDEL*. Fluorescence microscopy of *sGFPHDEL* expressing barley epidermal cells revealed that the resulting GFP fusion protein accumulated in small bodies or particles that quickly traversed the cells following cytoplasmic streaming.

2.3.9.2 *BAX* expression and assessment of cell viability

0.05 µg of *pBAX* together with 0.5 µg *psGFPHDEL* per shot as reporter were ballistically transferred into barley plants stably transformed with the *GFP-BI-1* fusion construct under the control of CaMV 35S promoter (provided by Jafar Imani and Valiollah Babaeizad, Justus-Liebig Universität, Giessen, Germany; unpublished). Single plants of 6 lines (#6(1)E1L2, #6(1)E4L3, #6(1)E4L4, #6(1)E5L10, #6(1)E82L1, #6(1)E14L1) of the T0 generation were chosen. All plants were positively tested to contain *GFP-BI-1* fusion plasmid. Lines derived from barley cv. Golden Promise, which served as *GFP-BI-1* negative control. According to PCR check, line #6(2)E12L2 did not carry *GFP-BI-1* plasmid and was taken as additional negative control (Jafar Imani and Valiollah Babaeizad, Justus-Liebig Universität, Giessen,

Germany, personal communication). 10 h after transformation cell viability was assessed by means of movement of sGFPHDEL-particles. Cells were considered as being alive, when there was vivid particle movement across the whole cytoplasm.

2.3.10 Yeast transformation and yeast viability assay

To determine whether the barley BI-1 protein is functional in rescuing yeast from *BAX*-induced lethality, *BI-1* was cloned into a yeast expression vector and co-transformed into *BAX*-expressing yeast cells. Frank Madeo (Eberhard-Karls Universität, Tübingen, Germany) provided yeast plasmids (pSD10.a-Bax, pL009, pRS315, pRS316) and wild type *Saccharomyces cerevisiae* strain WCG4. Plasmids pSD10.a-Bax and pL009 (slightly modified pSD10.a-Bcl-X_L, Ligr *et al.* 1998) contain cDNA of the respective mouse homologue under the control of a hybrid galactose-inducible (GAL1-10/CYC1) promoter and URA3 (pSD10.a-Bax) or LEU2 (pL009) as selectable marker. pRS315 and pRS316 were used as empty control plasmids. Barley *BI-1* was amplified by PCR using the primers 5'-ggatcccaacgcgagcgcaggacaagc-3' (5'-primer) and 5'-gtcgacgcggtgacggtatctacatg-3' (3'-primer), and subsequently cloned into the pGEM-T vector (Promega, Mannheim, Germany). After sequence confirmation, the *Sph*I/*Spe*I fragment was inserted into pL009, after *BCL-X_L* had been cut out with the same restriction enzymes, resulting in pΔL009-HvBI-1. Subsequently, the constructs were transformed into yeast cells in the following combinations: pRS315 + pRS316 (empty control), pRS315 + pSD10.a-Bax, pL009 + pRS316, pΔL009-HvBI-1 + pRS316, pSD10.a-Bax + pΔL009-HvBI-1 and pSD10.a-Bax + pL009 (positive control). The transformation procedure principally followed the lithium acetate method for small-scale yeast transformation as described in the Yeast Protocol Handbook (Clontech, Palo Alto, USA). *Saccharomyces cerevisiae* strain WCG4 was pre-cultured at 30°C in complete medium (YPDA, containing 1% yeast extract, 2% peptone, 2% glucose and 30mg/L adenine) to give a density of OD_{600} = 0.2 - 0.3. After centrifugation, competent cells were washed with sterile water and re-suspended in 1x TE/1x LiAc solution, after an additional centrifugation step. For simultaneous co-transformation, 0.1 µg of each plasmid (in the respective combinations) and 0.1 mg of solmon testes carrier DNA (Sigma-Aldrich Chemie GmbH, München, Germany) were added to 100 µl of yeast competent cells. Incubation at 30°C for 30 min followed addition of 600 µl of PEG/LiAc solution (40 % polyethylene glycol in 1x TE/1x LiAc solution). After adding

70µl dimethyl sulfoxide (DMSO) cells were transferred into a 42°C water bath and then put on ice for brief chilling. The suspensions were centrifuged and the cells re-suspended in sterile 1x TE buffer after the supernatant had been removed. Finally, cells were transferred onto agar plates with selective medium (synthetic dropout (SD) medium containing 0.17% nitrogen base without amino acids, amino acids and nucleotide bases) lacking leucine and uracil and containing 2% glucose as carbon source. After about 5 days of cultivation at 30°C, single colonies of each co-transformation event were picked and diluted in sterile water. For yeast drop assay (Chae *et al.* 2003), yeast suspensions were adjusted to a concentration of 400 cells/µL and then serial 10-fold diluted. 5 µl of each dilution were dropped onto plates with selective medium that provided either glucose (SD-Leu/-Ura/glucose) or galactose (SD-Leu/-Ura/galactose) as carbon source. The cells were incubated at 30°C for about 5 days before they were photographed.

- **YPDA medium**

2 %	Peptone
1 %	Yeast Extract
0.003 %	Adenin
2 %	Glucose
2 %	Agar (for plates only)
	in A. dest.
	use HCl to adjust pH to 6.5 and autoclave

- **Synthetic dropout (SD) medium**

0.17 %	Yeast nitrogen base without amino acids
0.5 %	$(NH_4)_2SO_4$
0.059 %	amino acids from 10x Dropout stock
2 %	Glucose or galactose
2 %	Agar (for plates only)
	in A. dest.
	use HCl to adjust pH to 5.8 and autoclave

- **10x Dropout stock** (without leucine and uracil)

200 mg	L-Adenine hemisulfate salt
200 mg	L-Arginine HCl
200 mg	L-Histidine HCl monohydrate
300 mg	L-Isoleucine
300 mg	L-Lysine HCl
200 mg	L-Methionine
500 mg	L-Phenylalanine
2000 mg	L-Threonine
200 mg	L-Tryptophan
300 mg	L-Tyrosine
1500 mg	L-Valine

- **10x TE buffer**

0.1 M	Tris-HCl
10 mM	EDTA
	in A. dest.
	adjust pH to 7.5 and autoclave

- **10x LiAc solution**

1 M	Lithium acetate
	in A. dest.
	use acetic acid to adjust pH to 7.5 and autoclave

2.3.11 Protein extraction from yeast and immunoblot analysis

Yeast cells harboring pRS315 + pRS316 (empty control), pΔL009-HvBI-1 + pRS316 and pSD10.a-Bax + pΔL009-HvBI-1 were grown in 50 ml of selective SD-Leu/-Ura/glucose medium over night to an OD_{600} of 1.0 to 2.0. In order to induce BAX and BI-1 protein synthesis, cells were collected by centrifugation and transferred to SD-Leu/-Ura/galactose medium for incubation for 15 h at 30°C. Yeast cells were collected by spinning-down the culture in pre-cooled centrifugation vessels for 5 min at 4°C and 1000 g. For storage, pellets were quick-frozen in liquid nitrogen. Preparation of yeast protein extract principally followed the Urea/SDS method as described in the Yeast Protocol Handbook (Clontech, Palo Alto, USA). For protein extraction, cells were thawed and re-suspended in pre-warmed cracking buffer containing protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) and

then transferred into a reaction tube containing glass beads (Ø 0.4-0.6 mm). In order to free membrane-associated proteins, the samples were incubated at 70°C for 10 min prior to vigorous vortexing. After 5 min of centrifugation at 14,000 rpm, the supernatant was collected and the remaining pellet was re-incubated together with 100 µl of cracking buffer for 3-5 min at 100°C. Vigorous vortexing and a centrifugation step was performed prior to combination of the two supernatants. For Western blot analysis equal amounts of total yeast protein extract (approximately 20 mg) were separated on a 12 % SDS-polyacrylamide gel and then transferred onto a cellulosenitrate membrane (Protran BA 85; Schleicher & Schuell Bioscience, Dassel, Germany). Antisera from rabbits raised against barley BI-1 protein (dilution 1:1000 in 1x PBS + 0.05 % Tween 20) were used for immunodetection. ImmunoPure® Peroxidase conjugated goat anti-rabbit IgG (Pierce Biotechnology Inc., Rockford, USA) was taken as secondary antibody (dilution 1:5000 in 1x PBS + 0.3% bovine serum albumin), since this allowed chemiluminescence detection after addition of adequate substrate (SuperSignal® West Pico Chemilumenescent Substrate, Biotechnology Inc., Rockford, USA).

- **Cracking buffer**

8 M	Urea
5 %	SDS
40 mM	Tris-HCl (pH 6.8)
0.1 mM	EDTA
0.04 %	Bromphenol blue
	in A. dest.

3 Results

3.1 Macroarray-based expression analysis of barley host susceptibility and nonhost resistance to *Blumeria graminis*

In order to contribute to the understanding of gene regulation during host susceptibility and nonhost resistance, a macroarray-based approach was pursued to compare gene expression in host and nonhost interactions of barley with powdery mildew fungi.

3.1.1 Macroarray construction and differential hybridization

Soil drench treatment of barley with BTH systemically protects leaves from infection by *Bgh* (Besser *et al.* 2000). Therefore, a barley cDNA library (GAN) constructed from barley epidermal peels after soil drench BTH treatment was expected to be enriched with cDNAs that might be involved in fungal defense. A cDNA macroarray was constructed by spotting 1,344 GAN cDNAs together with 192 specifically selected cDNAs on nylon membranes for differential hybridization (provided by Patrick Schäfer, Justus-Liebig Universität, Giessen, Germany; http://www.uni-giessen.de/fbr09/ipaz/ipaz/EichmannetalJPP.htm). According to EST sequencing and sequence cluster analysis, the 1,344 GAN clones represent 1,162 high-quality sequences corresponding to 919 singletons and 81 clusters resulting in exactly 1000 unigenes (Sophia Biemelt, Uwe Scholz and Uwe Sonnewald, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany, personal communication). Among them there are 300 that do not match any sequence contig represented on the commercially available Barley1 GeneChip (Affymetrix, Inc., Santa Clara, USA; Close *et al.* 2004), when applying a BLASTx2 e-value exclusion limit $>10^{-5}$. This further supported the view that both the libraries and corresponding cDNA arrays are valuable for barley transcriptome analyses. Some of the 192 additional cDNAs represented well-known positive or negative controls for defense-related gene expression. Others derive from differential screenings for potentially BTH- or *Bgh*-induced genes (Besser *et al.* 2000; Eckey *et al.* 2004; Hückelhoven *et al.* 2001b; Jansen *et al.* 2005).

For probe preparation, barley cv. Ingrid first leaves were inoculated with spores of *Bgh* or *Bgt* respectively. Additionally, mock inoculation was performed as control treatment. mRNAs from inoculated and control leaves were used for probe synthesis. Gene expression patterns were examined 12 and 24 hours after inoculation (HAI). At 12 HAI fungi have developed a mature appressorium but have not penetrated yet. At 24 HAI *Bgh* has established immature haustoria at about 60 % of interaction sites (Hückelhoven *et al.* 2000b) while at 24 HAI *Bgt* failed to penetrate the majority of attacked cells or was stopped by HR at 10-20 % of sites (Hückelhoven *et al.* 2001a; Trujillo *et al.* 2004a). Thus, relevant differences in transcript abundance during the compatible and incompatible interaction, respectively, should be visible at these times. Two independent biological experiments were conducted to analyze the 12 h time point after inoculation and both experiments were repeated technically by re-using the probe for hybridization to a different set of array membranes. Three independent biological samples without technical repetition were used for gene expression assessment at 24 HAI. Computational analysis included background subtraction as well as quantification and normalization of signal intensities. Genes, whose expression level was five fold above background and altered more than 2.5 fold in inoculated compared to control leaves, were considered as differentially expressed. Differential expression was only taken into account when the 2.5 fold difference appeared in two of two biological repetitions at 12 HAI or in at least two of the three biological repetitions at 24 HAI, respectively.

After differential hybridization and data analyses, the resulting data sets were juxtaposed. Application of selection criteria sorted 102 cDNA fragments corresponding to 94 unigenes that were pathogen-responsive at 12 or 24 HAI or both. An arbitrary selection of them was used for independent expression analysis (chapter 3.1.2). Out of 94 candidates 40 were grouped to be potentially induced upon pathogen infection whereas 54 genes appeared down-regulated. The major part (about 65 %) of the pathogen-responsive genes showed differential expression only at 24 HAI, while about 10 % of the clones were solely differentially expressed at 12 HAI. 25 % showed elevated or repressed transcript abundance at both times (Supplementary table 1). Importantly, only few differences in gene expression were detected upon attack of either *Bgh* or nonhost fungus *Bgt*, meaning that both fungi activated or repressed expression of a similar set of genes. According to the macroarray results, ten of 94 *B. graminis*-responsive genes were differentially

expressed only in the compatible interaction with *Bgh* (Supplementary table 1). Independent testing of three genes with differential expression between *Bgh* and *Bgt* confirmed two (autophagy, *HvD00108* and hypothetical protein, *GAN003B22F*; see below). 6 of 94 differentially expressed genes are not represented on the commercially available Barley1 microarray (*GAN001C14F, GAN002K18F, GAN003L01F, GAN003L02F, GAN001P03F, GAN003L21F,* Supplementary table 1). This appears relatively few when compared to the number of about 300 unique clones on the macroarray. However, the large number of 300 unique sequences on the array might appear due to over-representation of low transcript level genes in the normalized GAN library; and weakly expressed genes might not give sufficient signals over background to be accessible. Anyway, the macroarrays proved to be suitable to identify defense- and pathogenesis-related genes.

3.1.2 Differentially expressed genes

In order to check the reliability of the array results, Northern blot analyses and semi-quantitative two-step reverse-transcription polymerase chain reactions (RT-PCR) were conducted with 19 arbitrarily selected candidates. RNA samples of an independent biological experiment were used for this purpose. Analyses of the expression patterns revealed similar results compared to the macroarray experiments. Northern blot analyses were suitable to examine the expression pattern of candidates that showed high signal intensities on the cDNA arrays. RT-PCRs were conducted with low-level expression candidates.

Expression patterns of six candidates and one positive control (*PR1-b*) were checked by Northern blot analyses. In agreement with the macroarray results, a putative *PROTEIN DISULFIDE ISOMERASE* gene (*PDI*, *GAN002I14F*), a gene coding for a putative CALRETICULIN 3 protein (*CRT3*, *GAN002O14F*) and a putative *PEROXIREDOXIN* gene (*GAN001L10F*) showed elevated *B. graminis*-responsive expression. Upon inoculation with powdery mildew fungus, a putative *REMORIN* gene (*GAN003M18F*), a gene with no significant homology (*GAN003H11F*) and a putative *PHOSPHOGLYCOLATE PHOSPHATASE* (*PGP*, *GAN001L22F*) were down-regulated. Clone *GAN001L22F* was chimeric and additionally contained an *EARLY NODULIN* like EST. However, according to RT-PCR the *EARLY NODULIN* gene was not differentially expressed in connection with fungal infection. Expression of all genes was checked at 0, 4, 8, 12, 24 and 48 HAI. Most genes showed differential

expression not only at 12 and/or 24 HAI as indicated by the arrays but additionally at earlier and later times (Figures 3.1 and 3.2).

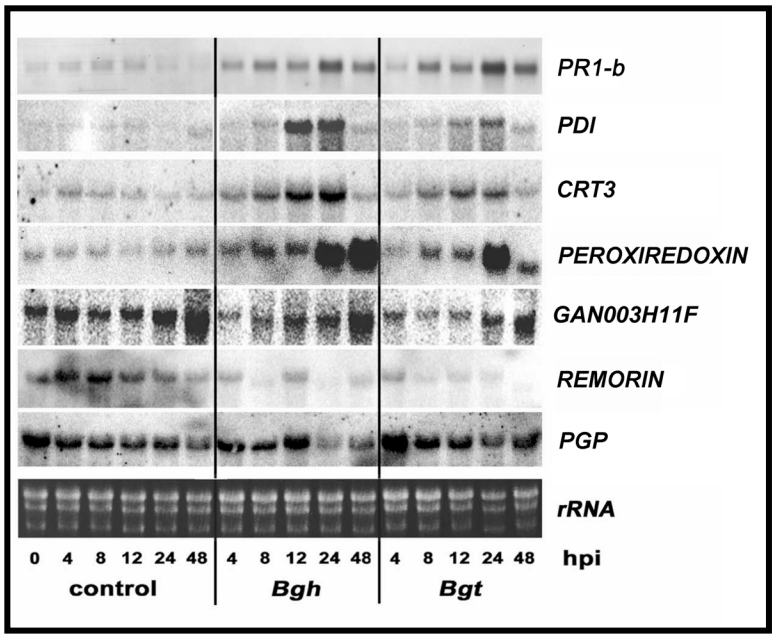


Figure 3.1: Independent confirmation of *B. graminis*-responsive gene expression by Northern analysis.
Genes with strong expression signals on the cDNA-macroarrays were selected for independent gene expression analysis. At the times indicated, RNA was extracted from ten barley first leaves inoculated with either virulent *Bgh* or the nonhost pathogen *Bgt*. Control plants were mock inoculated. *PR1-b* served as a positive control for *B. graminis*-responsive gene expression. Ethidium bromide staining of ribosomal RNA in a representative gel before blotting proves equal loading of total RNA.

Two-step RT-PCR was used to examine the expression of additional 13 genes. Genes coding for a putative *PTI1-LIKE PROTEIN KINASE* (*HvD00107*), a putative *LUMINAL BINDING PROTEIN 3* gene (*BIP3, GAN002N07F*), a gene with similarity to a *RECEPTOR-LIKE KINASE 19* (*RLK19*; *GAN003C21F*), a putative *CYSTEINE PROTEASE* (*GAN001L08F, GAN001J13F, GAN003B13F*) a putative *SUBTILASE* (*GAN004A17F, HvD00177*), a hypothetical protein (*GAN003B22F*), as well as an unknown protein (*HvD00126*) showed induced transcript accumulation as expected from the array results. *GAN003B22F* showed particularly high expression 24 h after inoculation with *Bgh*, as already indicated by the macroarrays (Table 3.1). Accordingly, a putative *AUTOPHAGY 8c* gene (*ATG8c*, *HvD00108*) was specifically up-regulated after inoculation with *Bgh.* A putative *GLYCINE-RICH CELL WALL STRUCTURAL PROTEIN* gene (*GRP*, *GAN002I20F, GAN003M08F*, *GAN001N06F, GAN002O07F, GAN003P08F*) and a putative *BARLEY LIPID TRANSFER PROTEIN 4* (*BLT4*) gene (*HvD00174*) were down-regulated. Again, most effects could be detected in a timeframe expanding the one originally comprised by the macroarray analyses. According to the array analyses, a putative *SOUL-LIKE PROTEIN* gene (*GAN001A10F, not shown*) was down-regulated 12 and 24 HAI with *Bgt* in one biological experiment. However, this repression could not be confirmed by RT-PCR,

emphasizing the importance of independent biological reproductions to gain reliable array results. Anyway, a *BIP3*-like gene (*GAN002N07F*), which was macroarray-indicated in only one biological repetition, though upon both *Bgh* and *Bgt* inoculation, was also induced according to RT-PCR analyses. Genes with similarity to an *HSP ASSOCIATED PROTEIN* (*GAN001O18F*) and to a *CLATHRIN COAT ASSEMBLY LIKE PROTEIN* (*GAN001O08F*) appeared induced upon inoculation, although this was not indicated by the macroarray results.

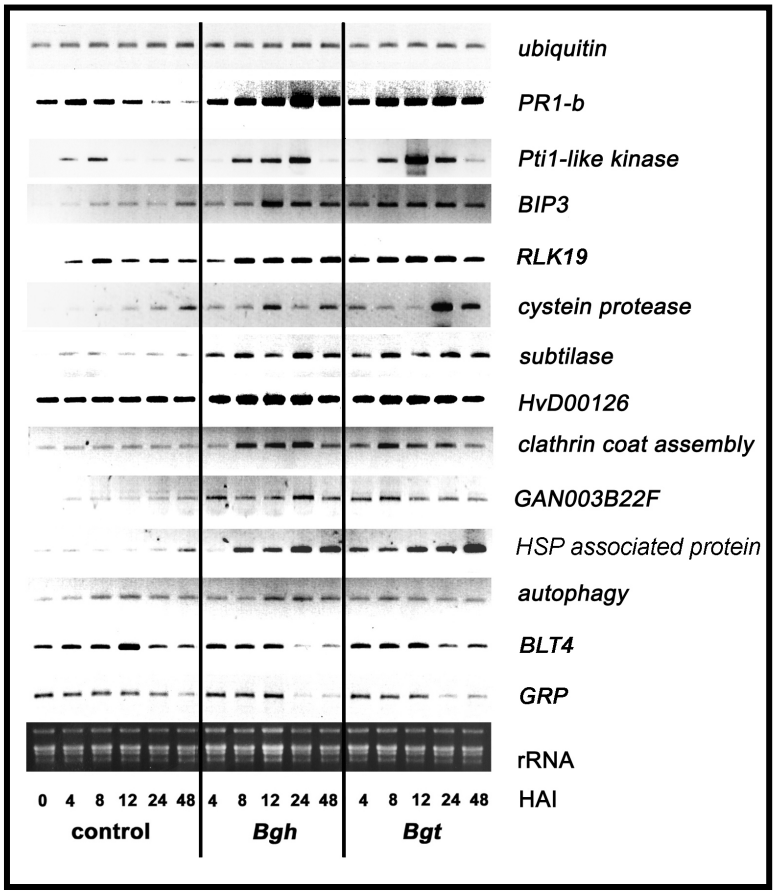


Figure 3.2: Independent confirmation of *B. graminis*-responsive gene expression by RT-PCR analysis.
Genes with low expression signals on the cDNA-macroarrays were selected for independent gene expression analysis by RT-PCR. At the times indicated, RNA was extracted from 10 barley first leaves inoculated with either virulent *Bgh* or nonhost pathogen *Bgt*. Control plants were mock inoculated. After reverse transcription, 250 ng aliquots of first strand cDNA were amplified during 20-25 cycles according to individual signal strength. The figure presents inverted gel photographs of ethidium bromide stained PCR products. *PR1-b* served as positive control for *B. graminis*-responsive gene expression. rRNA staining in a representative gel before first strand cDNA synthesis and *UBIQUITIN* RT-PCR as *B. graminis* non-responsive negative control display equal RNA loading.

3.1.3 Reliability of macroarray data

Our macroarray contained selected barley and wheat defense-related positive controls. Wheat *WIR2* and *WIR5*, wheat *PR1-1*, a wheat *PEROXIDASE* as well as *BARLEY BASIC PR1* and *PR5* displayed transcript accumulation upon inoculation with powdery mildew fungi, as was reported previously for wheat or barley

(Bryngelsson and Green 1989; Bryngelsson *et al.* 1994; Dudler *et al.* 1991; Rebmann *et al.* 1991a, b; Gregersen *et al.* 1997; Molina *et al.* 1999).
Additionally, several spots represented candidates for *Bgh*-induced gene expression because they had been identified before by differential cDNA-AFLP or subtractive hybridization for *Bgh*-induced genes (Eckey *et al.* 2004; Jansen *et al.* 2005). Several of them gave differential signals on the macroarrays, but others did not, since their expression was too weak to meet selection criteria. Together, internal controls as well as the reproducibility of gene expression patterns by biological repetitions and by independent semi-quantitative methods argue for the reliability of the macroarray results. Table 3.1 lists genes, which could be confirmed independently as differentially expressed.

3.1.4 Functional classification

All 94 differentially expressed genes were classified according to their presumptive function given by BLASTX results. About 25 % of the sequences belonged to a group of proteins with unknown function or showed no homology to genes in the database. 27 % of the genes encoded proteins involved in photosynthesis, metabolism, energy supply, translation or protein synthesis. Interestingly, most of them showed potential down-regulation. Twenty-three genes appeared to be involved in defense, stress or cell death, and most of them were up-regulated. Ten likely participate in cellular communication, signal transduction or in membrane trafficking and ten genes may be involved in protein fate and the secretory pathway since they are deemed to act as chaperones or in protein processing.

Table 3.1: Selection of barley genes[a] differentially expressed during interaction with *B. graminis*

Clone ID (accession number)	homology to TIGR[b] entry	identity (in %) to TIGR entry	E-value to TIGR entry	predicted protein	putative function	average fold changes *Bgh* 12h	*Bgh* 24h	*Bgt* 12h	*Bgt* 24h
GAN002I14F (CX631775)	TC146674	98	2.9e-43	protein disulfide isomerase (PDI)	protein processing	**4.7**	**8.5**	**5.9**	**7.4**
GAN002O14F (CX631311)	TC131592	96	3.3e-38	calreticulin 3 (CRT3)	protein processing	**1.9**	**2.2**	**3.3**	**2.3**
GAN001L10F (DN154953)	TC146841	99	5.5e-25	peroxiredoxin	defense, stress	**4.9**	**8.8**	**4.9**	**5.5**
HvD00107 (DN764060)	TC142181	97	1.4e-112	PTI1-like protein kinase	protein processing	**4.0**	**4.7**	**2.0**	**1.6**
GAN002N07F (CX631351)	TC139411	95	1.0e-59	luminal binding protein 3 (BIP3)	protein processing	**1.3**	**1.9**	**2.1**	**2.1**
GAN003C21F (DN764056)	TC140801	72	8.3e-39	receptor-like kinase 19 (RLK19)	defense, stress	**4.6**	**6.3**	**4.1**	**4.5**
GAN001L08F (DN154951) GAN001J13F (DN154922) GAN003B13F (CX631722)	TC148309 TC148309 TC148309	100 100 99	2.1e-43 2.4e-34 1.4e-37	cysteine proteinase	protein processing	**2.2**	**3.5**	**2.5**	**2.6**
GAN004A17F (CX631938) HvD00177 (CX631938)	TC134442 TC134442	99 99	1.5e-69 1.5e-69	subtilase	protein processing	**2.8**	**9.0**	**2.8**	**5.2**
GAN003B22F (CX631280)	TC147949	99	1.6e-59	hypothetical protein		**2.9**	**5.7**	**3.3**	**3.2**
HvD00126 (DN764070)	TC132885	99	8.2e-42	unknown protein		**2.6**	**2.5**	**2.9**	**1.6**
HvD00108 (DN764061)	TC147148	99	2.1e-92	autophagy	membrane trafficking	**1.9**	**2.4**	**1.6**	**1.5**
GAN001O18F (DN154856)	TC139611	94	1.4e-50	HSP associated protein	protein processing	**1.5**	**2.6**	**1.8**	**1.7**
GAN001O08F (DN154846)	TC133627	100	7.8e-37	Clathrin coat assembly like protein	membrane trafficking	**1.4**	**3.5**	**1.5**	**2.0**
GAN002I20F (CX631398) GAN003M08F (CX631846) GAN001N06F (DN154885) GAN002O07F (CX631334) GAN003P08F (CX631913)	TC132933 TC132933 TC132933 TC132933 TC132933	97 98 99 97 98	1.1e-55 4.1e-50 5.9e-39 3.9e-54 4.0e-55	glycine-rich cell wall structural protein (GRP)	defense, stress	1.1	4.5	1.5	2.6
HvD00174 (DN764083)	TC146796	100	2.5e-34	barley lipid transfer protein (BLT4)	defense, stress	1.2	2.4	1.1	1.7
GAN003M18F (CX631855)	TC110399	99	7.0e-79	remorin	signal transduction	1.3	1.7	3.3	2.5
GAN003H11F (CX631673)	BE411670	99	4.5e-63	no significant similarity		1.2	2.9	1.5	2.4
GAN001L22F (DN154897)	TC146506	98	5.1e-57	putative phosphoglycolate phosphatase	Carbohydrate metabolism	1.1	3.3	1.3	2.0

[a]Differential expression of genes listed was confirmed by macroarray-independent experimental reproduction (see Figures 3.1 and 3.2).

[b]TIGR barley database accessible under http://www.tigr.org. Please note that some clones matched the same TIGR entry and thus represent the same unigene.

[c]average fold induction (in bold) or repression at 12 HAI (4 repetitions) and at 24HAI (3 repetitions). We averaged normalized signal intensities from all experiments irrespective of selection criteria used to identify candidates.

3.2 Structural and functional characterization of the potential cell death suppressor BAX INHIBITOR-1

The interaction of cereals and cereal powdery mildew fungi represents a suitable model system for understanding biotrophic host-parasite relationships. The most important advantage of this interaction lies within the predominantly cell-autonomous control of successful infection or resistance. The transient transformation assay allows for easy and fast assessment of the contribution of putative defense or susceptibility factors to the outcome of the interaction on the single cell level and thus for manipulating different types of resistance (reviewed by Panstruga 2004). By delivering DNA expression constructs or double stranded RNA on microprojectiles, it is possible to examine both, the effects of gene overexpression (Nielsen *et al.* 1999; Schweizer *et al.* 1999) or conversely, upon taking advantage of the phenomenon of RNA interference (RNAi), the impact of gene function omission (Schweizer *et al.* 2000b). Besides, single cell expression of fusion constructs with fluorescing marker proteins in combination with confocal laser-scanning microscopy offers a versatile tool for examining changes in protein localization or protein-protein interactions during attack of the powdery mildew fungus (Panstruga 2004). Through this approach, elucidation of defense mechanisms and pre-selection of candidate genes for stable transformation of crop plants is facilitated. Recent reports provide evidence for the transferability of single cell transient transformation results to whole plant systems. Schultheiss and co-workers (2003b; in press) could demonstrate the contribution of a constitutively activated barley RAC/ROP protein ($HvRACB^{V15}$) to susceptibility of barley to the barley powdery mildew fungus in both, transiently transformed barley epidermal cells and stably transformed, $RACB^{V15}$ expressing barley plants. Likewise, transient and stable overexpression of a wheat *PEROXIDASE* conferred resistance to *Bgh* in single barley epidermal cells and transgenic barley plants, respectively (Schweizer *et al.* 1999; Altpeter *et al.* 2005).

The BAX INHIBITOR-1 (BI-1) protein is an acknowledged suppressor of BAX-induced cell death in mammalian and yeast cells. Furthermore, overexpression of the barley homolog of *BI-1* enhances susceptibility of barley epidermal cells to *Bgh* (Hückelhoven *et al.* 2003). This connection is quite reminiscent of the MLO protein, which is a proposed negative regulator of cell death and penetration resistance in the barley-powdery mildew interaction, too (Schulze-Lefert and Panstruga 2003). Here,

different cytological and molecular tools were applied to examine BI-1 function in cell death and nonhost resistance and to further characterize the protein in terms of its subcellular localization and the contribution of conserved protein domains to its function.

3.2.1 Yeast transformation and cell viability assay

To test if the barley BI-1 protein actually does exert the proposed function in repressing cell death, the protein was co-expressed together with murine BAX in yeast and single barley epidermal cells and its ability to protect these cells from BAX-induced lethality was evaluated.

In a colony formation assay, WCG4 yeast cells were co-transformed with murine *BAX* (pSD10.a-Bax) and either its mammalian antagonist *BCL-X_L* (pL009), or barley *BI-1* (pΔL009-HvBI-1). Each gene was under the control of a galactose-inducible promoter. As expected, considerable colony formation could be observed when yeast cells harboring the empty control plasmids pRS315 and pRS316 were transferred to either glucose or galactose containing selective medium. Similarly, co-transformation combinations without BAX, i.e. Bcl-X_L + pRS316 and BI-1 + pRS316, allowed for colony growth on either SD-Leu/-Ura/glucose or SD-Leu/-Ura/galactose plates. Due to BAX-induction, colony formation was abolished, when yeast cells, harboring BAX and the empty control vector pRS315, were transferred to galactose containing medium. Co-transformation of yeast cells with *BAX* and *BCL-X_L* was conducted as positive control for cell death rescuing. As expected, co-expression of *BCL-X_L* restored colony growth on galactose medium. In contrast, co-transformation with barley BI-1 did not support development of BAX-expressing yeast cells on SD-Leu/-Ura/galactose plates (Supplementary figure 2).

To confirm expression of barley *BI-1* in yeast cells, BI-1 protein accumulation was analyzed by Western blotting. Protein was extracted from yeast cells transformed with pRS315 + pRS316 (empty vector control), barley BI-1 + pRS316 or BAX + BI-1 after galactose induction. After separation by gel electrophoresis, proteins were transferred to nylon membranes. For immunodetection of BI-1 an antibody raised against the barley BI-1 C-terminus was used in a 1:1,000 dilution. BI-1 accumulation (expected protein band of around 26 kDa) could not be detected in protein extracts from yeast cells harboring BI-1 + pRS316 or BAX + BI-1 compared to extract from yeast cells co-transformed with the empty control plasmids pRS315 + pRS316.

3.2.2 BAX-induced collapse of single barley epidermal cells

Following the possibilities, that barley BI-1 was not expressed in yeast or that barley BI-1 requires its native plant system to exert a cytoprotective function, the transient transformation assay, which originally aimed to examine the participation of candidate genes in the interaction of cereals with cereal powdery mildew fungi (Schweizer *et al.* 1999) was adjusted. Despite of being a pro-apoptotic factor in animals, the BAX protein turned out to be a potent inducer of cell death in whole plant systems or plant protoplasts (Lacomme and Santa Cruz 1999; Kawai-Yamada *et al.* 2001; Baek *et al.* 2004; Yoshinaga *et al.* 2005). By ballistic transfer of the mammalian *BAX* gene together with the marker gene *GFP* (both under the control of the constitutive CaMV35S promoter) into single epidermal cells, a cell death phenotype could be induced, which was reminiscent of that observed in animal cells undergoing apoptosis. Surprisingly, the GFP protein turned out to be quite resistant to proteolytic activity that could be expected from processes within a collapsing cell. Hence, no significant BAX effect on the number of GFP cells was observable (data not shown). However, there were remarkable phenotypic differences in *BAX*-expressing compared to control cells concerning the GFP distribution. Within control cells solely expressing GFP, an intact scaffold of cytoplasmic strands was visible, and cytoplasmic streaming could easily be observed upon microscopic magnification, indicative of unaffected cell viability (Figure 3.3 A, C). In contrast, overexpression of mammalian *BAX* induced striking morphological changes in transformed cells already at very low concentrations (0.05 µg BAX plasmid per shot). By the time GFP accumulation became visible (examined 10 h after transformation), most of the cytoplasmic strands had disappeared and almost no cytoplasmic movement occurred. Instead, a “brindled” GFP accumulation pattern became prevalent, which likely derived from condensation and aggregation of the cytoplasm, indicating its collapse (Figure 3.3 B, C). Intriguingly, upon inoculation with spores of *Bgt* haustorium formation, i.e. invagination of the plasma membrane could be observed frequently in *BAX*-expressing GFP cells (data not shown). This finding suggested that plasma membrane and tonoplast remained intact. In a large number of *BAX*-expressing cells, cellular disorganization and large to medium-sized, round vesicle-like structures (reminiscent of apoptotic-bodies from animal cells) that were often present close to the nucleus (data not shown) became apparent. However, chromatin staining with diaminophenylindole (DAPI), which is commonly used as cytological

marker of apoptosis in yeast and mammalian cells, did not reveal any abnormal chromatin morphology, i.e. fragmentation or swelling of the nucleus in *BAX*-expressing compared to control cells (data not shown).

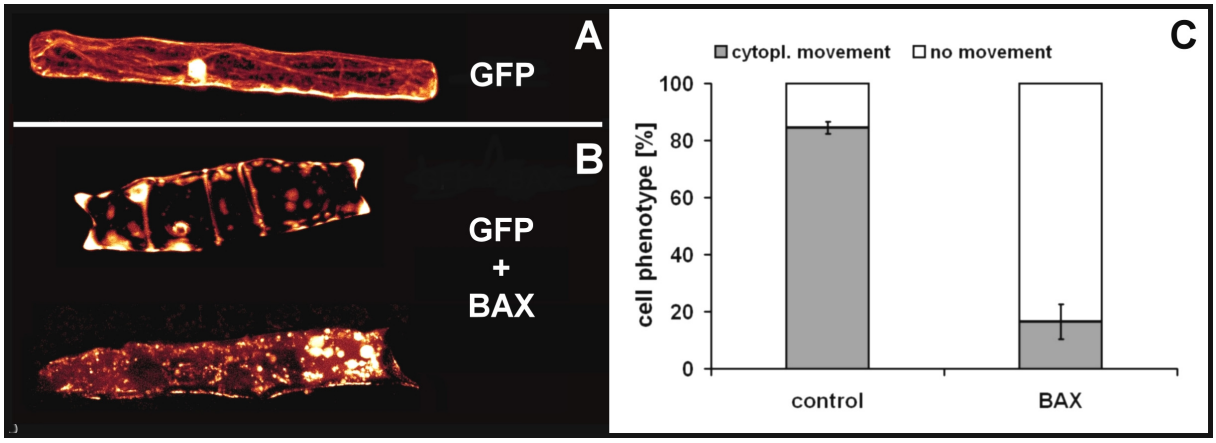


Figure 3.3: Single cell expression of a *BAX* gene from mouse induces cell death in barley. Confocal laser scanning whole cell projection of barley epidermal cells transiently expressing *pGFP* together with empty vector (**A**) or *pGFP* together with *pBAX* (**B**). Plasmids were coated on tungsten micro-projectiles and ballistically delivered into leaf epidermal cells. GFP distribution was examined 10 h after transformation. **C** *BAX* expression arrests cytoplasmic movement. Single barley epidermal cells were transiently transformed with *pGFP* together with empty vector (control) or *pGFP* together with *pBAX.* The cells were examined microscopically in terms of visible cytoplasmic movement along cytoplasmic strands. Microscopic evaluation took place 10 h after transformation. Columns represent mean values from five independent experiments.

3.2.3 Overexpression of barley *BI-1* delays BAX-induced collapse of the cytoplasm

To assess whether concomitant overexpression of barley *BI-1* would prevent BAX-induced cell collapse, the respective expression constructs together with GFP as marker were simultaneously delivered into barley epidermal cells by means of ballistic transformation. Similarly, BAX-inhibiting properties of a truncated barley BI-1 variant (BI-1ΔC), the mammalian BCL-X_L (Ligr *et al.* 1998) and an ASCORBATE PEROXIDASE (APX) from barley (Hess and Börner 1998) were examined. By means of fluorescence microscopy, GFP co-expressing cells were evaluated in terms of their viability, i.e. status of cytoplasmic strands and occurrence of cytoplasmic movement. Assessment revealed that in control cells that were not expressing *BAX*, around 90 % of the cells were obviously intact, as indicated by a uniform scaffold of cytoplasmic strands and clear cytoplasmic movement by 10 h after ballistic transformation. The

brindled cell phenotype was typical for BAX-expression and could rarely be seen in control cells (Figure 3.4 A). In contrast, 46 % of the *BAX*-overexpressing cells were totally collapsed and displayed shrunken and aggregated cytoplasm but no cytoplasmic strands. Around 16 % of the cells were unscathed, while 37 % maintained at least some parts of the cytoplasmic strands. Co-expression of the mammalian BAX-antagonist *BCL-X_L* largely prevented barley epidermal cells from damage induced by the BAX protein. Only around 7 % of the cells collapsed while about 80 % were saved. The barley BI-1 protein had less protective effect on cellular integrity. Similar to solely *BAX*-expressing cells, cytoplasmic movement was visible in only about 14 % of the *BAX* and *BI-1* expressing cells. However, compared to the former only half as much cells collapsed completely and this effect proved statistically significant ($P < 0.05$). Truncation of the BI-1 C-terminus by 17 amino acids (BI-1ΔC) did not markedly impair BI-1 function. Overexpression of BI-1ΔC also significantly reduced BAX-induced collapse of the cytoplasm but only around 6 % of the GFP cells showed cytoplasmic movement. In the majority of the cells some cytoplasmic strands remained. In barley *APX* co-expressing cells, slightly but not significantly more cytoplasmic movement was observed (around 30 %) than in those, which were only expressing *BAX*. The collapse of the cytoplasm was also not significantly different from *BAX*-expressing cells (Figure 3.4 A, B).

In summary, ectopic expression of murine *BAX* induces collapse of barley epidermal cells while its mammalian counterpart BCL-X_L can almost completely prevent this effect. In contrast, the cell protective properties of barley BI-1 in this system are less strong, but overexpression of the protein obviously delays collapse of the cytoplasm and this effect seems to be independent from integrity of the protein's C-terminus.

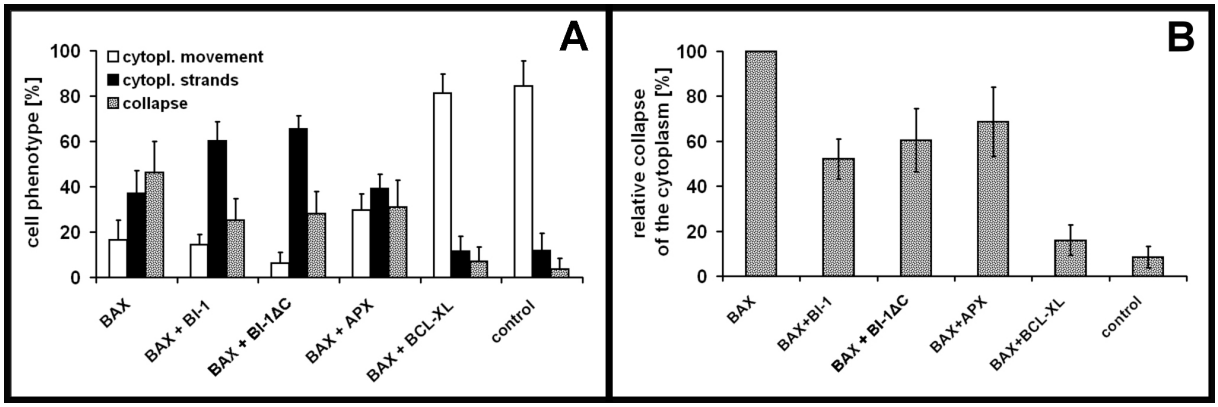


Figure 3.4: Effect of several putative BAX antagonistic proteins on the integrity of *BAX*-expressing barley epidermal cells. Barley cv. Manchuria primary leaves were transiently transformed with *pGFP* and *pBAX*. To assess their potential to alleviate BAX-induced lethality, *BI-1*, *BI-1* with truncated C-terminus (*BI-1ΔC*), a barley *ASCORBATE PEROXIDASE* (*APX*) gene or the mammalian BAX antagonist *BCL-X_L* were additionally transferred into barley cells. Control cells were transformed with empty vector only. **A** GFP-cells exhibited either fragmented cytoplasm (collapse), possessed intact cytoplasmic strands without any vital cytoplasmic movement (cytopl. strands) or were displaying intact strands together with vital movement of the cytoplasm (cytopl. movement). BCL-X_L and to a minor degree APX were able to elevate the number of cells with vital cytoplasmic movement, while BI-1 and BI-1ΔC did not. **B** Amount of collapse in cells expressing *BAX* together with a potential BAX antagonist (*BI-1*, *BI-1 ΔC*, *APX*, *BCL-X_L*) in relation to cells expressing BAX together with empty vector (BAX). Co-expression of *BI-1* or *BI-1ΔC* significantly reduced BAX-induced collapse of the cytoplasm (Student's *t* test $P < 0.05$).

3.2.4 Analysis of BAX-dependent cell death in stably transformed barley plants expressing a GFP-BI-1 fusion protein

To examine whether precedent BI-1 protein accumulation would enhance BI-1 suppression of BAX induced cell death, barley cv. Golden Promise plants, which stably express a GFP-BI-1 fusion construct under control of CaMV 35S were ballistically transformed with BAX-coated tungsten particles. Transgenic plants from different lines were used for this experiment. Transgenic lines were generated using *Agrobacterium tumefaciens*-mediated transfer of expression constructs into immature embryos isolated from seeds of barley cv. Golden Promise (Jafar Imani and Valiollah Babaeizad, Justus-Liebig Universität, Giessen, Germany, unpublished). The transgenic plants used for this experiment, came from the T0 generation, i.e. represented shoots regenerated from transformed barley embryos. All plants were checked for presence of the expression plasmid by means of PCR amplification of a

DNA fragment spanning the 35S::GFP transition. Except for line #6(2)E12L2 and parental cultivar Golden Promise, all plants used here proved positive for the expression plasmid. Immunodetection of GFP revealed considerable differences in expression level of the fusion construct between the lines (Jafar Imani, Justus-Liebig Universität, Giessen, Germany, personal communication). Beyond that, barley *GFP-BI-1* transgenic lines were examined by confocal laser scanning microscopy concerning GFP expression. In single, transiently transformed barley epidermal cells, GFP-BI-1 fusion protein usually accumulates within the continuous endomembrane system of ER and nuclear envelope (see below). GFP-BI-1 specific accumulation around the nucleus could be observed in lines #6(1)E4L3, #6(1)E4L4, #6(1)E8L1, #6(1)E14L1 and #5(1)E5L1. Line #6(1)E1L2 showed some, but rather weak expression of *GFP-BI-1*. Expression of the fusion construct was not determined for lines #6(1)E5L10 and #6(2)E12L2 (Ralph Hückelhoven, Justus-Liebig Universität, Giessen, Germany, personal communication).

To support easy detection of cytoplasmic movement in *BAX*-transformed cells, the cell organelle marker construct sGFPHDEL was employed. This fusion construct sGFPHDEL derives from the barley *CRT3* gene, a supposed ER resident calcium binding protein (Chen *et al.* 1994). The marker protein was constructed by transcriptionally inserting the GFP sequence into CRT3, thereby replacing amino acids 73 to 238 of CRT3 but maintaining the presumptive N-terminal signal peptide and the C-terminal HDEL sequence (Supplementary figure 1), which are supposed to be required for protein import into the lumen of the ER and for its retention there (Haseloff *et al.* 1997). Thus, sGFPHDEL was originally developed to be a marker for localization to the ER. In single epidermal cells expressing sGFPHDEL, however, the protein did not visibly localize to the ER but accumulated in a considerable number of smaller bodies or particles instead (Figure 3.5). These brightly fluorescing organelles turned out to be located in the immediate vicinity of cytoplasmic strands and were quite mobile. They quickly traversed the cells following cytoplasmic streaming. In cells inoculated with spores of the barley powdery mildew fungus, cellular organization and cytoplasmic streaming focus on the site of attempted penetration (Kobayashi *et al.* 1993). Consistent with that, in inoculated barley epidermal cells, sGFPHDEL-bodies were directed to the point of fungal attack (Figure 3.5 B). Since the fluorescence picture strongly resembled that obtained with GFP labeled Golgi-markers (Nebenführ *et al.* 1999; Satiat-Jeunemaitre *et al.* 1999; Saint-Jore *et al.*

2002; Brandizzi *et al.* 2004; Zheng *et al.* 2004), sGFPHDEL was considered to localize prevalently to the Golgi apparatus (Golgi stacks). Due to its accumulation in mobile bodies, sGFPHDEL represents a versatile tool to visualize cytoplasmic streaming, which is an indicator of cell viability.

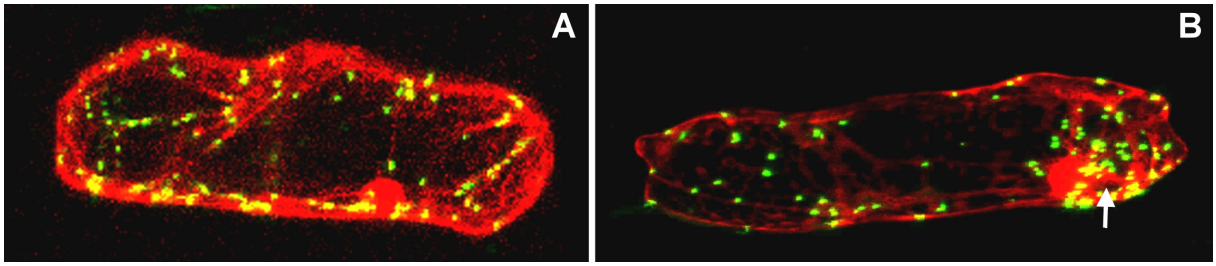


Figure 3.5: Localization of sGFPHDEL in barley epidermal cells. Confocal laser scanning whole cell projection of barley epidermal cells transiently expressing sGFPHDEL (green fluorescence) together with DsRED (red fluorescence). The GFP coding sequence was inserted in-between the N-terminal signal sequence and a sequence fragment encoding the C-terminal HDEL ER retention signal of the *CRT3* gene. The resulting sGFPHDEL plasmid was coated on tungsten particles and ballistically delivered into barley epidermal cells together with DsRED as cytoplasmic and nucleoplasmic marker. **A** sGFPHDEL accumulates in small bodies that move along cytoplasmic strands. **B** Transformed cell under attack of the barley powdery mildew fungus. sGFPHDEL-bodies gather around the site of attempted fungal penetration (arrow).

To address whether stably transformed plants expressing *GFP-BI-1* would be more resistant to BAX-induced cell death, sGFPHDEL was used to determine cytoplasmic movement and thus cell integrity. Leaf segments of control plants (i.e. a PCR negative transformant and the parent Golden Promise) and of GFP-BI-1 expressing T0 plants deriving from different lines were bombarded with *pBAX* and *psGFPHDEL*-coated tungsten particles. 10 h later, sGFPHDEL expressing epidermal cells were identified by means of brightly green fluorescing particles. For every cell, it was assessed whether these particles were moving vividly with cytoplasmic streaming (indicating living cells) or whether movement was clearly slowed down and locally restricted or had even stopped entirely (indicating dying or dead cells). In untransformed Golden Promise control plants only around 20 % of the marker gene-expressing cells displayed vivid cytoplasmic movement 10 h after co-transformation with *BAX*. In the following, this value provided the basis for the calculation of relative vital cytoplasmic movement in the transgenic lines. For the GFP-BI-1 negative line #6(2)E12L2 and the PCR-positive lines #6(1)E1L2 and #6(1)E4L4 equal or slightly lower percentages of living cells were acquired. By the same time, 20 % more cells resisted BAX-induced lethality in line #6(1)E5L10. In lines #6(1)E14L1,

#6(1)E8L1and #6(1)E4L3, however, cell survival was dramatically increased. Compared to the control, more than twice as much of the cells could withstand early cellular demise (Figure 3.6). Since significant survival of *BAX*-expression could never be observed in any wild-type barley cells, it appeared that GFP-BI-1 really does provide cell protection.

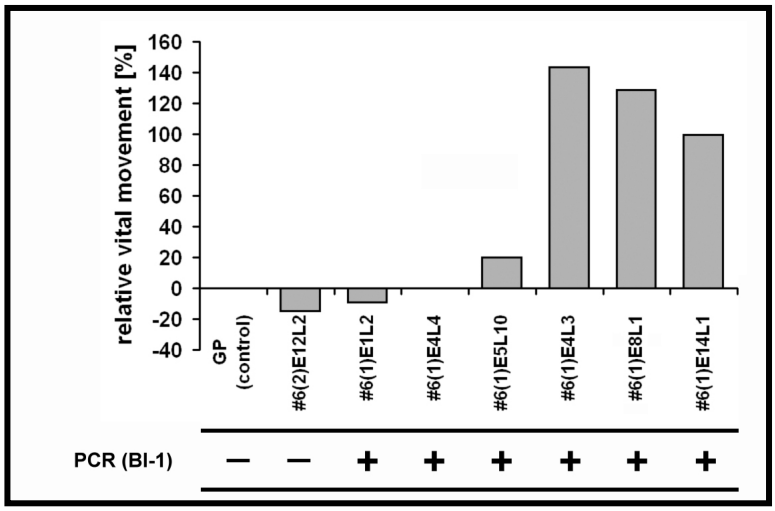


Figure 3.6: A stably over-expressed GFP-BI-1 fusion protein protects epidermal cells of transgenic barley lines from *BAX*-induced cell death. Segments of youngest leaves of T0 transgenic barley plants were ballistically transformed with a marker protein for cytoplasmic streaming (*sGFPHDEL*) together with the *BAX* gene from mouse. Transgenic plants were tested PCR positive (+) or negative (-) for the presence of the GFP-BI-1 fusion construct. Parental line Golden Promise (GP) and PCR negative line #6(2)E12L2 served as controls. *sGFPHDEL*-expressing cells could be identified by means of small green fluorescing bodies that moved along the cell with cytoplasmic streaming. Movement was taken as indicator of cellular viability. The proportion of living cells on the transformed cells gave the measure "vital cytoplasmic movement". The figure presents resulting values as relative values compared to the GFP-BI-1 negative control line Golden Promise. Data derive from a single biological experiment.

3.2.5 Expression of barley *BI-1* in response to *Bgt*

In previous studies, it had been demonstrated that a barley *BI-1* homolog was differentially expressed in barley leaves upon attack by *Bgh* in both susceptible *MLO* and penetration resistant *mlo5* lines (Hückelhoven *et al.* 2001b; 2003). Here, *BI-1* expression in the incompatible interaction of barley cv. Manchuria with the *Bgt* was examined at early interaction stages. Expression was studied in first leaves densely inoculated with conidia of powdery mildew isolate *Bgt*A95 during two days after inoculation. As a positive control for defense related gene expression, transcript accumulation of the *PATHOGENESIS-RELATED PROTEIN 1-b* (*PR1-b*) was examined (Bryngelsson *et al.* 1994). *PR-1b* showed enhanced expression upon inoculation with *Bgt* starting from 12 HAI onward (Figure 3.7 A). The same RNA was

used for one-step reverse-transcription (RT-) PCR to analyze *BI-1* expression. As demonstrated previously (Hückelhoven *et al.* 2003), a constitutive *BI-1* expression was detected that slightly increased with leaf age. After inoculation with *Bgt,* transcript accumulation of *BI-1* slightly increased (Figure 3.7 B).

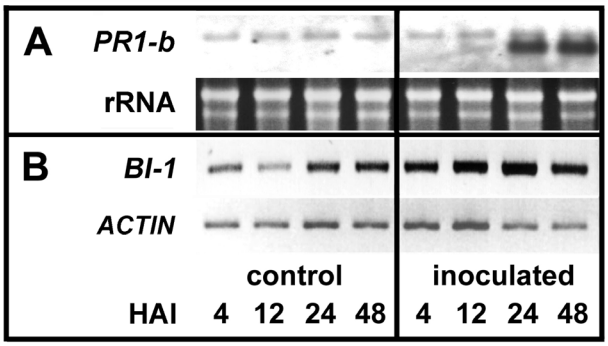


Figure 3.7: *BI-1* expression in the nonhost-interaction of barley with *Bgt*. A Gel blot analysis of *PATHOGENESIS-RELATED PROTEIN 1-b* (*PR1-b*) gene transcript as control for defense related gene expression. Total RNA was isolated from barley (cv. Manchuria) first leaves inoculated with conidia of *Bgt* and mock-inoculated control leaves 4, 12, 24 and 48 hours after inoculation (HAI). Equal loading of RNA (5 µg) was checked by ethidium bromide staining of rRNAs. **B** The same RNA was used for one-step RT-PCR analysis. As constitutive control, an *ACTIN-LIKE* cDNA fragment was amplified under specific conditions during 25 PCR cycles. For *BI-1*, RT-PCR with 30 cycles was carried out by applying specific conditions. Inverted pictures from ethidium bromide stained gels are shown.

3.2.6 *BI-1* overexpression compromises penetration resistance of barley to *Bgt*

To examine whether BI-1 is involved in defense mechanisms in the nonhost-system barley-*Bgt*, transient *BI-1* overexpression was performed in barley cv. Manchuria by ballistic transformation and subsequent microscopic evaluation of the interaction of *Bgt* with epidermal cells co-transformed with *BI-1* and *GFP* reporter gene. The cauliflower mosaic 35S promoter controlled expression of both genes. In five independent experiments, overexpression of *BI-1* led to significantly enhanced penetration efficiencies (PE) of *Bgt* in transformed cells ($P < 0.001$, Student's *t* test). The average PE increased from 2 % in control cells transformed with *GFP* and empty vector to 24 % in *GFP* and *BI-1* transformed cells (Figure 3.8 A). This effect was less strong when barley cv. Ingrid was used instead of Manchuria. Since Ingrid shows early cell death in nearly all cells penetrated by *Bgt* (Peterhänsel *et al.* 1997), the maize ubiquitin promoter controlled *uida*-reporter gene and *β*-glucuronidase (GUS) activity staining were used instead of the vital *GFP*-reporter to identify transformed cells. In three independent experiments, *BI-1* overexpression shifted *Bgt* PE from less than 1 to 5 %. A similar level of *BI-1*-induced accessibility was achieved, when

the backcross line BCIngrid-*mlo5* was used (Figure 3.8 B), which is completely resistant to penetration by appropriate *Bgh* (Jørgensen 1994; Hückelhoven *et al.* 1999; Peterhänsel *et al.* 1997). Interestingly, non-transformed Manchuria is slightly accessible to penetration by *Bgt*A95 whereas Ingrid is almost completely resistant (Hückelhoven *et al.* 2001a, Trujillo *et al.* 2004a).

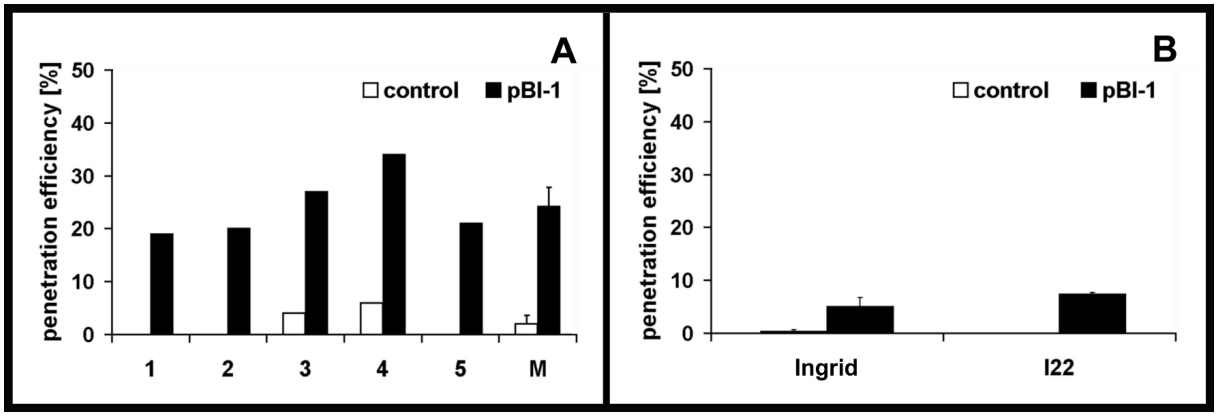


Figure 3.8: Overexpression of *BI-1* abolishes nonhost penetration resistance. A Penetration efficiency (PE) of *Bgt* to barley (cv. Manchuria) epidermal cells was evaluated in five independent experiments. M represents average values of independent experiments. PE of *Bgt* was significantly enhanced in cells that were bombarded with *pBI-1* compared to cells that were transformed with empty control pGY-1. Bars represent standard errors. *GFP* served as co-transformed reporter to identify transformed cells. **B** Average PE of *Bgt* on barley cv. Ingrid and BCIngrid-*mlo5* (I22). PE of *Bgt* was significantly enhanced when epidermal cells were bombarded with *pBI-1* compared with cells that were bombarded with empty control pGY-1. Columns represent average values from three independent experiments. Bars represent standard errors. The *uida*-gene served as a reporter to identify transformed cells by GUS-staining.

3.2.7 Simultaneous overexpression of *BI-1* and *MLO*

To investigate potential synergistic effects of BI-1 and MLO on the penetration rate of *Bgt*, *pBI-1* and *pMlo* were simultaneously delivered into barley epidermal cells through ballistic transformation. The GUS activity staining enabled identification of transformed cells in Manchuria. In these experiments, a general tendency for higher penetration rates in MLO or BI-1 overexpressing cells was observed, when compared to experiments with a *GFP*-reporter construct. This might reflect a better co-expression rate when different promoters controlled expression of transgene and reporter. It is also conceivable that the GUS assay supports detection of penetrated dead cells that might escape the vital marker GFP. To estimate individual effects of *BI-1* and *MLO,* barley first leaves were bombarded with the respective construct together with empty pGY-1 vector. Both MLO

and BI-1 promoted fungal entry into barley epidermal cells, but co-expression of both genes did not further enhance accessibility achieved by individual expression (Figure 3.9 A). 7 d after inoculation, however, fungal development improved in cells simultaneously expressing *MLO* and *BI-1* compared to cells expressing either of the genes. More *Bgt* germlings were able to establish haustoria and to develop elongated secondary hyphae (ESH) when *BI-1* and *MLO* were overexpressed together (38 % of transformed cells) compared to single gene overexpression (approximately 25 %; Figure 3.9 B). Although BI-1 performed similar to MLO in inducing initial accessibility to *Bgt*, overexpression of *BI-1* and *MLO* together appeared to allow development of longer ESH than overexpression of *MLO* or *BI-1* alone. Occasionally, formation of conidiophores and new conidia became visible on cells overexpressing *BI-1* and/or *MLO*. However, this occurred in less than 1 % of transformed cells.

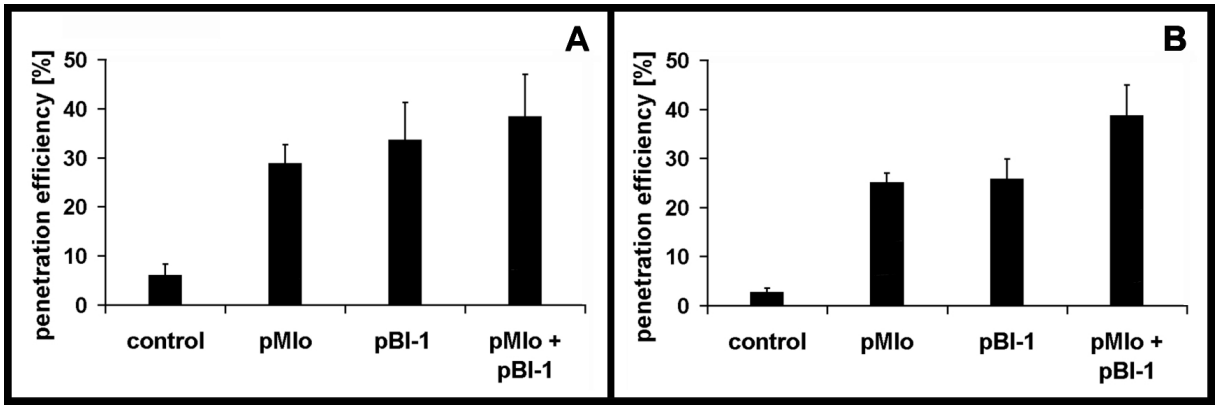


Figure 3.9: Separate and simultaneous overexpression of *MLO* and *BI-1* confer similar induction of defense suppression. Average PE of *Bgt* in three independent experiments. Barley cv. Manchuria was bombarded with *pMLO* and *pBI-1* both separately and simultaneously, or with empty control vector. **A** Overexpression of *pMLO* and *pBI-1*, respectively, induced enhanced susceptibility to *Bgt*. Simultaneous overexpression of both genes further enhanced PE slightly but not significantly when evaluated at 48 HAI. **B** Overexpression of *pMLO* and *pBI-1*, respectively, induces enhanced susceptibility to *Bgt*. Simultaneous overexpression of both genes further enhanced PE when evaluated at 7 days after inoculation. Columns represent average values. Bars represent standard errors.

3.2.8 Localization of a GFP-BI-1 fusion protein

To monitor the intracellular localization of the barley BI-1 protein, an expression construct was made that allowed translational fusion of GFP to the N-terminus of BI-1, giving GFP-BI-1 (provided by Holger Schultheiss, Justus-Liebig Universität, Giessen, Germany). Subsequently, this construct was introduced into barley epidermal cells via

particle bombardment. Co-expressed *DsRED* served as a marker for protein localization in the cytosol and nucleoplasm (Dietrich and Maiss 2002). GFP-BI-1 fluorescence was definitely distinguishable from that of DsRED. It appeared in a fine, net-shaped structure within the cytoplasm, indicative of endoplasmic reticulum (ER; Figure 3.10 A-C). In single confocal sections, GFP-BI-1 was detectable in the nuclear envelope, but did not enter the nucleus (Figure 3.10 D-F), which corresponds with findings of Kawai-Yamada *et al.* (2001) in *Arabidopsis* and yeast cells and Bolduc *et al.* (2003) in tobacco BY-2 cells. To confirm proper localization, the transient transformation assay was used to control whether the GFP-BI-1 fusion construct was functional in defense regulation of Manchuria against *Bgt* in. Since GFP-BI-1 fluorescence was comparably weak and difficult to observe with standard fluorescence microscopy it was necessary to perform transformation of barley epidermal cells with GFP-BI-1 together with either GFP or GUS as additional reporters. Subsequent evaluation of interactions of transformed cells with germinated conidia of *Bgt* revealed that, similar to the results obtained for overexpression of *BI-1*, the penetration efficiency of the fungus was clearly enhanced in *GFP-BI-1* expressing cells compared to control cells. In two independent experiments, penetration efficiency increased from 7 % to 23 % in the GUS experiment and from 11 % to 27 % when GFP served as reporter (Figure 3.10 G).

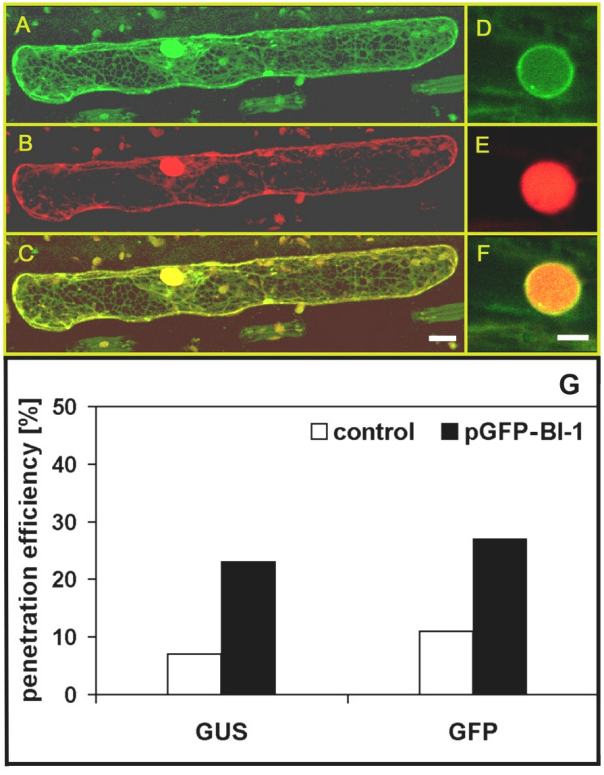


Figure 3.10: Localization and functionality of a GFP-BI-1 fusion protein. A-C Confocal laser scanning whole cell projection of a barley epidermal cell transiently expressing GFP-BI-1 together with DsRED. The plasmids were coated onto micro-projectiles and delivered into barley leaf epidermal cells. Scale bar = 12 µm. By 72 h after transformation, distribution of GFP-BI-1 (**A, D**) and DsRED fluorescence (**B, E**) was detected. **D-F** Single confocal section of the nuclear region of a transformed cell. Scale bar = 6 µm. **C+F** represent the respective merged images. **G** The GFP-BI-1 fusion protein is functional in defense suppression. Epidermal cells of barley cv. Manchuria were bombarded with *pGFP-BI-1* or empty control pGY-1 plus either *pGUS* or *pGFP* as reporter gene constructs, respectively. Penetration efficiency of *Bgt* was evaluated in one experiment each.

Additionally, subcellular localization of BI-1 was examined upon attack by the powdery mildew fungus. 4 h after ballistic delivery of the fusion construct into barley epidermal cells, the leaf segments were inoculated with spores of *Bgh* or *Bgt* and GFP fluorescence was inspected at sites of attempted or successful fungal penetration 24 to 48 h later. Upon fungal attack, the fusion protein was still detectable around the nucleus. The evenly distributed GFP-BI-1 accumulation in a mesh-like pattern, however, largely vanished upon fungal attack. Instead, there was a translocation of the protein. GFP-BI-1 often accumulated beneath the appressorial germ tube or around papillae. In case of successful penetration, one could occasionally detect GFP-BI-1 accumulation around the haustorium (Figure 3.11).

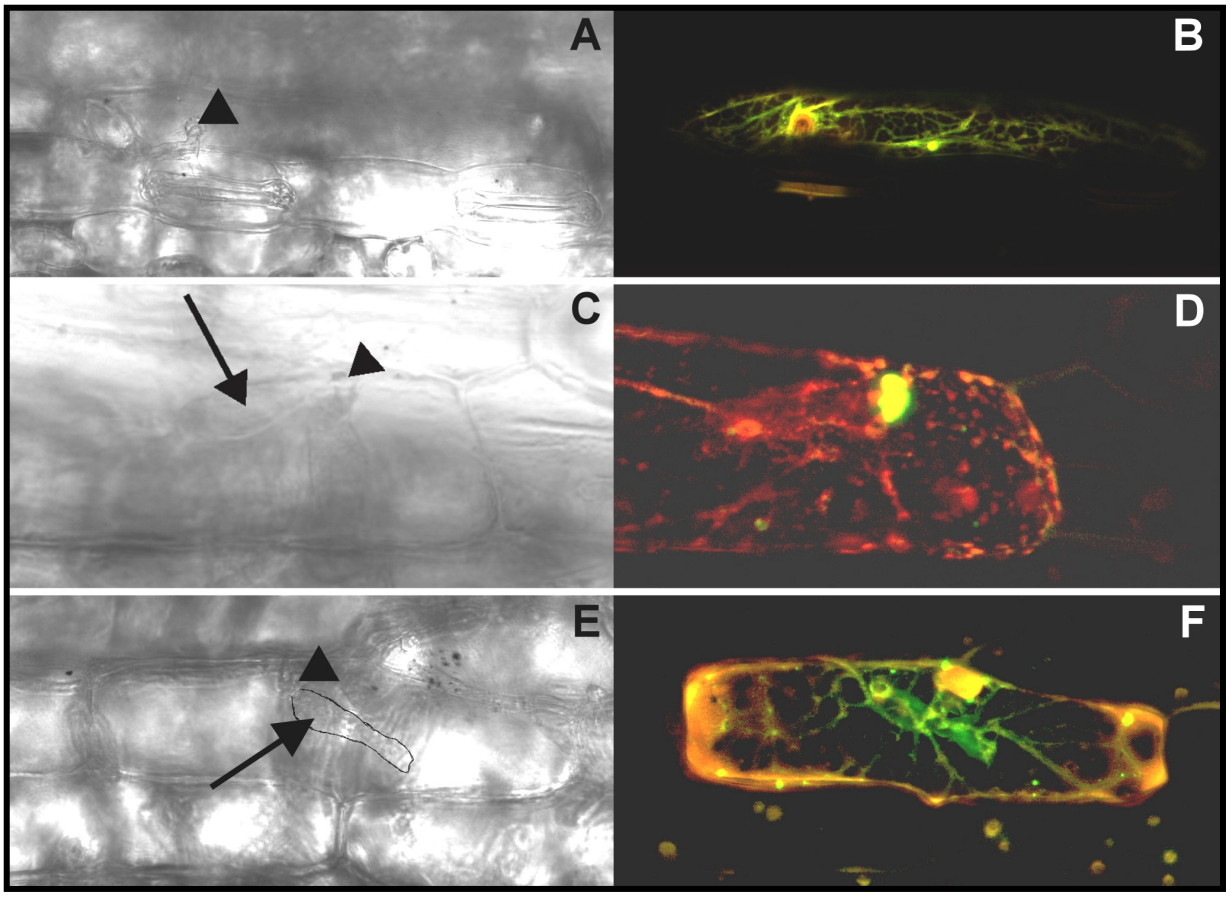


Figure 3.11: Subcellular distribution of a GFP-BI-1 fusion protein after inoculation with spores of the powdery mildew fungus. Barley leaf epidermal cells were co-bombarded with GFP-B-1 (green fluorescence) and DsRED (red fluorescence), inoculated with spores of *Bgt* (A, B) or *Bgh* (C-F) and analyzed by confocal microscopy 48 HAI. **A, C and E** Transmission pictures, visualizing points of fungal attack (►) and intracellular fungal structures (arrows). **B, D and F** Merged images of GFP and DsRED fluorescence. **A, B** GFP-BI-1 accumulation around papilla (Pap) in the incompatible interaction of barley with *Bgt*. **C-F** GFP-BI-1 accumulation at the penetration site (**D**) and around an immature haustorium (**F**).

3.2.9 H_2O_2 staining in *BI-1* overexpressing barley epidermal cells during the interaction with *B. graminis*

A confined apoplastic accumulation of hydrogen peroxide (H_2O_2) accompanies penetration resistance of barley to powdery mildew fungi (Hückelhoven *et al.* 1999). In barley, *mlo*-mediated broad-spectrum resistance to *Bgh* is determined by penetration resistance and this resistance can be reduced by single-cell overexpression of *BI-1* or *MLO* (Shirasu *et al.* 1999b; Hückelhoven *et al.* 2003). To assess whether BI-1 influences H_2O_2 accumulation during resistance modulation, H_2O_2-specific diaminobenzidine (DAB) staining (Thordal-Christensen *et al.* 1997) was performed in transiently transformed, *BI-1* overexpressing epidermal cells of resistant barley line BCIngrid-*mlo5* upon attack by *Bgh*. Similarly, DAB staining pattern was examined in *MLO* overexpressing and control cells inoculated with *Bgh*. Microscopic evaluation took place around 14 HAI, since this time is considered to be pivotal for success or failure of the attempted attack. In fixed and decolorized leaves, accumulation of marker protein GFP was still detectable in transiently transformed cells. Brown DAB polymers indicating the presence of H_2O_2 could also easily be observed. The interaction of transiently transformed, GFP-expressing cells with spores of *Bgh* was evaluated in two ways. On the one hand, the frequency of *Bgh* penetration was counted, recognized by the occurrence of immature haustoria within the cells. On the other hand it was quantified whether there was localized DAB staining underneath the attacking appressorial germ tubes (AGT) or not. As expected, penetration frequencies in control cells were quite low (about 5 %), while overexpression of *BI-1* allowed early *Bgh* penetration in about 20 % of the cases. Transfer of *MLO* expression plasmid into BCIngrid-*mlo5* epidermal cells complemented *mlo*-alleles and restored susceptibility. Average penetration frequency was about 60 % (Figure 3.12 A). Effects on penetration efficiency were significant ($P < 0.00005$ for BI-1 and $P < 0.05$ for MLO, Student's *t* test). When the share of interaction sites with DAB staining underneath the AGT in all interaction sites was calculated, it became apparent that in both *BI-1* and *MLO* overexpressing cells, frequency of DAB staining underneath the appressorium was significantly reduced (Figure 3.12 B). In six independent experiments, H_2O_2 accumulation was detected in about 53 % of cell wall appositions in control cells, while the proportion was significantly smaller in *BI-1*-expressing cells (28 %, $P < 0.005$). The difference was

even more pronounced in the 4 *MLO* experiments: on average, in 48 % of the interaction sites in the control, DAB-staining was visible beneath the appressorial germ tube, but in only about 15 % in the *MLO*-expressing variant ($P < 0.05$).

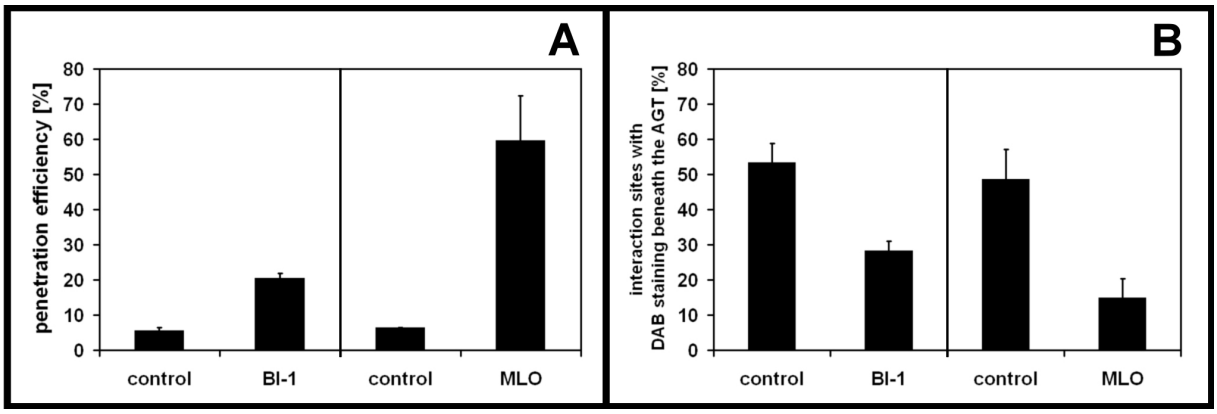


Figure 3.12: Overexpression of *BI-1* and *MLO* affect local accumulation of defense-associated hydrogen peroxide (H_2O_2) during suppression of penetration resistance. Primary leaves of *mlo5*-mutant barley plants were transiently transformed with the marker gene *GFP* together with either *pBI-1* or *pMLO*. Controls were transformed with the marker gene together with empty vector. 4 h after ballistic transformation, leaf segments were inoculated with spores of *Bgh* and fungal growth was allowed for 14 h. 3,3'-diaminobenzidine (DAB) was used for histochemical staining of H_2O_2. Columns represent mean values of six independent BI-1 experiments and five independent MLO experiments. Bars show standard errors. **A** Overexpression of *BI-1* and *MLO* significantly enhanced penetration efficiency of *Bgh* (Student's *t* test $P < 0.00005$ and $P < 0.05$, respectively). **B** Overexpression of *BI-1* and *MLO* significantly reduced the frequency of DAB staining underneath the appressorial germ tube (AGT; Student's *t* test $P < 0.005$ and $P < 0.05$, respectively).

3.2.10 Site-directed mutagenesis of barley *BI-1* cDNAs

Site-directed mutagenesis in combination with the transient transformation assay represents a versatile tool to assess the contribution of specific amino acids and/or domains to the function of a certain protein (Schultheiss *et al.* 2003b; Elliott *et al.* 2005). Alignment of several BI-1 proteins from plants and animals (see Hückelhoven *et al.* 2003) revealed a number of highly conserved amino acids (aa) that are evenly distributed throughout the whole sequence (Kawai *et al.* 1999; Sanchez *et al.* 2000; Bolduc *et al.* 2003; Chae *et al.* 2003). Upon comparison of the human BI-1 protein with sequences from barley, rice (*Oryza sativa*) and *Arabidopsis thaliana*, 86 invariant amino acids were identified, which, due to their evolutionary conservation, may be relevant for proper conformation and/or function of the protein in defense suppression and/or cell death regulation (Hückelhoven *et al.* 2003). Previous structural analyses of the BI-1 aa

sequence predicted 6-7 transmembrane (TM) spanning regions but no significant functional domains. The so-called BI-1 motif, a short protein segment that stretches from the third to the fourth predicted TM domain, might be relevant since it has considerable structural similarity even to related bacterial proteins (Hückelhoven 2004). In order to get further function-related sequence information, a BLAST database search was carried out to identify short sequence stretches (6 to 18 aa in length) that were nearly identical to BI-1 in other eukaryotic or bacterial proteins with known function. The BI-1 sequence prior to the first TM domain, i.e. between position 25 and 42 (QISPAVQSHLKLVYLTLC), shares a common aa pattern with a MEMBRANE ASSOCIATED ADENYLATE CYCLASE from *Cryptosporidium parvum* (accession number EAK88117). ADENYLATE CYCLASES catalyze the conversion of adenosine triphosphate to the second messenger 3'-5'-cyclic adenosid monophosphate (cAMP). cAMP is known to regulate the opening of cation conducting channels in animals and plants (Zagotta and Siegelbaum 1996; Leng *et al.* 1999; Balagué *et al.* 2003). The GTAIAF stretch at the end of the BI-1 motif (aa 123 to 128) can also be found in so-called RESISTANCE-ASSOCIATED MACROPHAGE PROTEINS of various eukaryotes (e.g. accession number NP_990295) but also in several proteins related to transport processes, e.g. a PROTON-COUPLED DIVALENT METAL ION TRANSPORTER (accession number XP_516089), a bacterial Na^+/H^+ ANTIPORTER (accession number NP_280783), a LIGAND-GATED ION CHANNEL PROTEIN from *Arabidopsis thaliana* (accession number O81078) or a POTASSIUM VOLTAGE-GATED CHANNEL SUBFAMILY B from *Rattus norvegicus* (accession number P15387). In the fifth predicted TM domain, there is an aa pattern (LGGLLSSGLSIL; aa 146 to 157), which also appears to be present at least in parts in a prokaryotic PERMEASE OF THE MAJOR FACILITATOR SUPERFAMILY (accession number ZP_00262974), an ABC-TYPE Mn^{2+}/Zn^{2+} TRANSPORTER (accession number ZP_00164412) or a bacterial type II secretory pathway component (accession number ZP_00063205), but also in a GLYCOSYLPHOSPHATIDYLINOSITOL MATURATION PROTEIN (BST1) from yeast (accession number EAL86917), which is supposed to be a negative regulator of COPII vesicle formation (Elrod-Erickson and Kaiser 1996). The GLLIFLGYMVYDTQ stretch (aa 181 to 194) starts within the sixth predicted TM domain and extends into one of the few short non-TM regions and shares some recognizable sequence similarity with stretches of a putative PLASMA MEMBRANE-TYPE PROTON ATPASE (accession number AAL25803) and an ABC-TYPE DIPEPTIDE TRANSPORT SYSTEM PROTEIN

(accession number ZP_00274241). Finally, the VAVLVRVLIIMLK (aa 219 to 231) sequence is located in the TM stretch close to the C-terminus. A Mn^{2+} AND Fe^{2+} TRANSPORT PROTEIN (accession number AAO75736), a H^{+}/Ca^{2+} EXCHANGER (accession number NP_441468) and a G PROTEIN-COUPLED RECEPTOR (accession number NP_861456) have similar short aa arrangements. Interestingly, it has been noted that also the MLO protein is structurally reminiscent of metazoan G PROTEIN-COUPLED RECEPTORS, which are known to be involved in the transmission of extracellular signals to intracellular signaling systems (Devoto *et al.* 1999).

Based on this information, conserved aa were randomly selected from every sequence stretch to produce 9 BI-1 mutants (Table 2.2). In the transient transformation assay it should be assessed whether they were still able to repress nonhost penetration resistance of barley epidermal cells to *Bgt*. Nine of the variants carried aa exchanges that were introduced into the sequence by means of site-directed mutagenesis of single nucleotides. Two aa within the BI-1 sequence prior to the first TM domain were modified in such a way that proline at position 28 and histidine at position 33 were replaced by alanine and leucine, respectively. The first and the final aa of the short GTAIAF stretch (aa 123 to 128) residing within both, BI-1 motif and the fourth predicted TM domain were changed to arginine or leucine. In addition, the conserved proline at positions 104 within the BI-1 motif was modified to alanine. Two serine residues in the middle of the fifth TM domain were replaced by phenylalanine (position 152) and proline (position 155), respectively. Other aa swaps concerned an asparagine (position 192) between TM domains 6 and 7, which was substituted by alanine, and arginine (position 224) at the end of the seventh TM domain, which was replaced by leucine. Furthermore, the BI-1 C-terminus was truncated by the final 17 aa. This was accomplished by oligo DNA primer derived, PCR mediated introduction of a stop codon right behind the last predicted TM domain at aa position 230 (construct BI-1ΔC). Subsequently, the BI-1 variants were delivered into barley epidermal cells by means of ballistic transformation and their relative functionality in suppressing nonhost penetration resistance to *Bgt* compared to wild-type barley BI-1 was assessed. Except for BI-1G123R and BI-1F128L all BI-1 mutants retained approximate wild-type activity (Figure 3.13). Mean values deviated from relative wild-type susceptibility by up to 13 % (BI-1P28A 108 %, BI-1H33L 98 %, BI-1P104A 112 %; BI-1S152F 100 %, BI-1S155P 87 %, BI-1D192A 87 %, BI-1R224L 103 % relative susceptibility, $P > 0.05$, Student's *t* test). One mutation within the BI-1 motif (BI-1G123R) significantly compromised the ability of BI-1 to increase susceptibility of

barley epidermal cells to *Bgt* (69 % compared to the wild type, $P < 0.05$). The F128L variant exhibited enhanced (127 %) relative defense suppression but this value did not prove to be significant. Truncation of the C-terminus by 17 aa apparently did not influence BI-1 function in resistance suppression (87 % relative susceptibility).

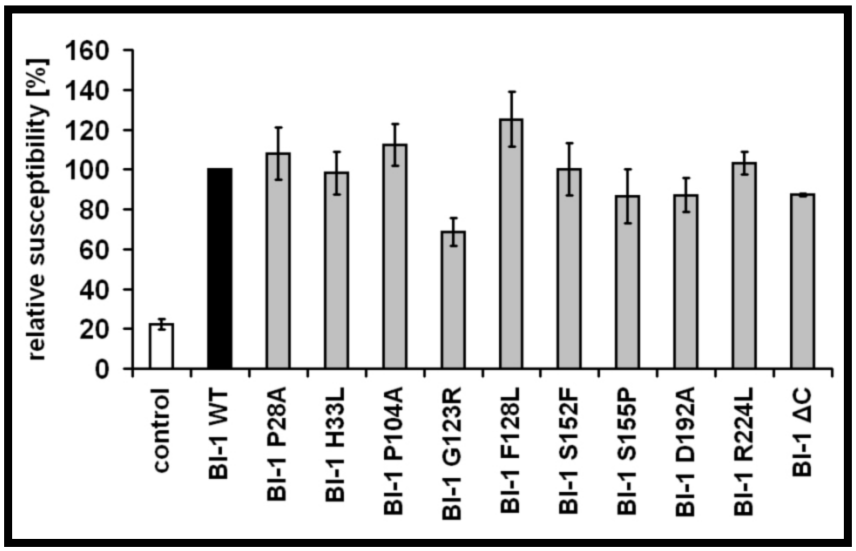


Figure 3.13: Assessment of the functionality of BI-1 mutants in defense suppression. Leaf segments of barley cv. Manchuria were transiently co-transformed with GFP and wild type BI-1 (BI-1 WT, black column) or a mutated BI-1 variant (gray columns). Control cells (white column) were transformed with GFP and empty vector. Nine of the BI-1 mutants carried single substitutions of conserved aa. Mutant BI-1ΔC was C-terminally truncated by 17 aa. Leaf segments were inoculated with *Bgt* spores and GFP cells were microscopically examined in terms of fungal penetration success 48 h later. Columns represent relative values compared to BI-1 WT and bars represent standard errors.

To examine whether truncation of the C-terminus, which carries the KKXX-like motif for ER retention, would affect BI-1 localization, the GFP-BI-1 fusion construct and its modified version GFP-BI-1ΔC were transferred into barley epidermal cells together with *DsRED* as marker. Confocal laser scanning microscopy revealed that, similar to the BI-1 wild-type fusion protein (GFP-BI-1; Figure 3.10), GFP-BI-1ΔC accumulated at the periphery of the nucleus and in a subtle mesh-like pattern throughout the cell, most likely representing the continuous endomembrane system of ER and nuclear envelope (Supplementary figure 3). However, the C-terminus mutant seemed to accumulate additionally in the cell periphery.

Together, these results indicate that rather the BI-1 motif than the cytosolic C-terminus is crucial for BI-1 function in defense suppression, and that loss of the C-terminal 17 aa does not strongly affect subcellular protein localization.

4 Discussion

Nonhost resistance, i.e. the resistance of an entire plant species to all genotypes of a potentially pathogenic microorganism, is the most common form of disease resistance in plants and shows high durability over time. Apart from relying on some constitutive or preformed barriers, nonhost resistance comprises a number of inducible reactions that become activated once the pathogen has overcome the first, preformed line of defense. In some respects, the mechanisms behind nonhost resistance are similar to those expressed in response to the infection with a basically compatible pathogen. However, the molecular basis, that underlies nonhost resistance and determines its constancy, is still largely unknown. In the present study, a macroarray-based approach was pursued in order to comparatively analyze gene expression in host and nonhost interactions of barley with powdery mildew fungi. The experimental design was supposed to be suitable to identify common and divergent elements of compatibility and incompatibility.

The second part of the study focused on the elucidation of the role of the potential cell death regulator BAX INHIBITOR-1 (BI-1) in nonhost resistance. In a candidate gene approach, this conserved protein was identified to be involved in resistance of barley to the appropriate barley powdery mildew fungus. Various molecular and cytological tools were applied to further characterize BI-1 function in the barley-*B. graminis* interaction.

4.1 Macroarray-based identification of differentially regulated genes in the host and nonhost interaction of barley with powdery mildew fungi

Nonhost resistance prevents barley from the invasion of inappropriate *formae speciales* of the grass powdery mildew fungus *Blumeria graminis* (DC) Speer (syn. *Erysiphe graminis* DC) (Tosa *et al.* 1990). The wheat powdery mildew fungus (*B. graminis* f.sp. *tritici*, *Bgt*) is a nonhost pathogen on barley plants. Nonhost resistance here comes along with the formation of cell wall appositions (papillae) at sites of fungal penetration attempts and a hypersensitive cell death reaction (HR) of single attacked cells (Tosa and Shishiyama 1984; Tosa *et al.* 1990; Hückelhoven *et al.*

2001a; Trujillo *et al.* 2004a, b), resistance phenotypes, which can also be observed in *mlo*-mediated broadspectrum (papillae) and *R*-gene dependent race-specific resistance (HR) of barley to avirulent races of the barley powdery mildew fungus *B. graminis* f.sp. *hordei* (*Bgh*) (Kita *et al.* 1981; Peterhänsel *et al.* 1997). Over the past few years, the DNA array technique has become a powerful tool to display gene expression in response to various plant treatments, including pathogen infection. The cDNA macroarray used here was supposed to help to identify pathogen-responsive gene transcripts in barley inoculated with compatible and incompatible *ff. spp.* of the powdery mildew fungus. By comparing the resulting expression profiles one could expect to find genes that were commonly expressed in response to both powdery mildew *ff. spp.* and to trace genes that were specifically expressed in response to only one of them. Thus, this study was aimed at the elucidation of gene regulation during host and nonhost interactions of barley with powdery mildew fungi.

4.1.1 Analysis of gene expression during the interaction of barley with *Bgh* and *Bgt*

In order to trace elements of host or nonhost responses, respectively, probes from barley plants after inoculation with either an appropriate or an inappropriate *forma specialis* of *B. graminis* were used. The macroarray membrane was spotted with 1,536 cDNA fragments that were mainly derived from a cDNA library from epidermal cell layers of chemically-induced barley first leaves. Additional 192 clones were specifically selected for spotting and comprised chemically induced as well as pathogen-responsive genes, some of which served as positive controls for defense related gene expression (Besser *et al.* 2000; Hückelhoven *et al.* 2001b; Eckey *et al.* 2004; Jansen *et al.* 2005). Differential gene expression has already been intensively studied in both compatible and incompatible interactions of barley and wheat with *B. graminis* ff. spp. (Bryngelsson and Green 1989; Dudler *et al.* 1991; Rebmann *et al.* 1991a, b; Bryngelsson *et al.* 1994; Gregersen *et al.* 1997; Molina *et al.* 1999; Hückelhoven *et al.* 2001b; Caldo *et al.* 2004; Eckey *et al.* 2004; Hein *et al.* 2004; Gjetting *et al.* 2004; Jansen *et al.* 2005; Zierold *et al.* 2005). However, a comprehensive overview of cereal transcriptome changes in susceptibility and resistance to powdery mildew fungi is still missing. Here, 102 candidate genes were identified as being induced or repressed at 12 and/or 24 HAI during the interaction

with either the host or the nonhost pathogen or both in relation to mock inoculated barley leaves. For some of the differentially expressed genes transcriptional changes during the attack of powdery mildew fungi had already been demonstrated. An increased transcript accumulation after *Bgh* infection had previously been reported for wheat *WIR2* and *WIR5*, wheat *PR1-1* and a wheat *PEROXIDASE 375* gene as well as for a barley *BASIC PR1* and *PR5* gene, which served as positive controls for pathogen dependent gene induction (Bryngelsson and Green 1989; Bryngelsson *et al.* 1994; Dudler *et al.* 1991; Molina *et al.* 1999; Rebmann *et al.* 1991a, b). Although 80 additional clones were expected to represent positive controls because they had been identified before by differential cDNA-AFLP or subtractive hybridization for *Bgh*-induced genes (Eckey *et al.* 2004; Jansen *et al.* 2005), only 9 of them were identified by differential hybridization of the macroarrays. This might be either explained by the different times used for differential screenings here and in previous studies, or by the fact that the macroarray approach identified only strongly induced or strongly repressed gene expression, whereas cDNA-AFLP might be more sensitive for low-abundance transcripts. Interestingly and in contrast to this study, cDNA-AFLP had identified mainly up-regulated *Bgh*-responsive genes in our laboratory (Eckey *et al.* 2004). However, the relative insensitivity of the macroarray hybridization method was compensated by the advantage of a high reproducibility of the results by independent means. Semi-quantitative methods were used to check pathogen-responsiveness in independent experiments. The comparison of *PR1-b* expression patterns in semi-quantitative RT-PCRs with quantitative Northern blot experiments starting from the same RNA shows that RT-PCR rather underestimates *B. graminis*-induced influences on gene expression. Therefore, positive RT-PCR results could be judged as reliable.

4.1.1.1 Genes up-regulated after inoculation with powdery mildew fungi

The macroarray-based analysis of gene expression after attack of *Bgh* and/or *Bgt* revealed 102 differentially expressed genes. A relatively high number of the genes encoded proteins, which are involved in protein processing, namely chaperones and proteases. Apart from being crucial for plant development and physiology, protein degradation and processing processes are known to be involved in defense and stress responses (Boston *et al.* 1996; van der Hoorn and Jones 2004). Chaperones residing within the endoplasmic reticulum (ER) function in correct folding and

assembly of proteins and protein complexes of the secretory pathway. PROTEIN DISULFIDE ISOMERASE (PDI), LUMENAL BINDING PROTEIN (BIP or HSP70-like) and CALRETICULIN (CRT3) are typical representatives of this protein class and their transcript accumulation has often been observed in connection with ER stress (Kaufman 1999; Shank *et al.* 2001). In tobacco, a rapid but transient accumulation of *PDI*, *CRT3* and *BIP* was observed after treatment with cell wall degrading enzymes, mimicking pathogen stress, which preceded *PR* gene induction and it was suggested that ER chaperones may be required to enable synthesis of PR proteins (Jelitto-Van Dooren *et al*. 1999). Only recently, Wang and co-workers (2005) reported that in Arabidopsis up-regulation of important members of the protein secretory machinery such as *BIP*, *PDI*, *CRT3* and a *CLATHRIN COAT ASSEMBLY PROTEIN* gene (involved in vesicular packaging of secretory proteins) was controlled by *NPR1* (*NONEXPRESSER OF PR GENES 1*), a major regulator of systemic acquired resistance (Cao *et al.* 1998). By using knock-out mutants, the requirement of an intact secretory pathway for resistance induction and its concomitant *PR* gene expression could be demonstrated. It was thus presumed that activation of the secretory machinery was necessary to ensure proper folding and transport of PR proteins. A similar dependency might hold true for the interaction of barley with cereal powdery mildew fungi. This is at least indicated by the elevated expression of molecular chaperones and a *CLATHRIN-COAT ASSEMBLY LIKE PROTEIN* gene on the one hand and a variety of *PR* genes on the other.

PDI is a multifunctional protein present in the ER. It contains thioredoxin domains that aid in the rearrangement of disulfide bonds in proteins to form their native structures (Chen and Hayes 1994). Defense related induction of *PDI* has recently been found in wheat infected with the hemibiotrophic fungal pathogen *Mycosphaerella graminicola* (Ray *et al.* 2003). Some antioxidant properties have been ascribed to PDI, which may be of use in preventing the cell from ER-stress derived from reactive oxygen intermediates (ROI), which are generated during plant-pathogen interactions including barley and *B. graminis* (Hückelhoven and Kogel 2003; Ray *et al.* 2003). Knock-down experiments provide some indication, that PDI is crucially involved in the barley-powdery mildew interaction. Ballistic transfer of double-stranded RNA (dsRNA) into barley epidermal cells initiates the onset of a process called RNA interference (RNAi). During this process, dsRNA is split enzymatically into so-called small interfering RNAs (siRNAs) of 21 to 26 nucleotides.

These siRNAs then direct another enzymatic complex to degrade cognate single-stranded mRNAs, resulting in sequence specific gene silencing (Fire *et al.* 1998; Wang and Metzlaff 2005). The delivery of dsRNA corresponding to a 1 kb *PDI* 5' fragment into epidermal cells of barley cv. Ingrid tend to allow for a higher penetration rate of *Bgh* (Supplementary figure 4 A). However, one needs to consider that *PDI* comprises a gene family (Chen and Hayes 1994). There are around 20 PDI-like proteins in Arabidopsis, rice and maize (Houston *et al.* 2005). Since dsRNA synthesis derived from a relatively large *PDI* fragment (Supplementary table 2), it is likely that RNAi affected more than only one *PDI* sequence. In the converse experiment, in which a single *PDI* gene was overexpressed, penetration efficiency of *Bgh* was not affected (Supplementary figure 4 B). Since powdery mildew fungi already trigger massive *PDI* gene expression, it is questionable, if additional overexpression would have any effect at all.

CALRETICULIN is the major calcium-binding and storage protein in the ER (Kaufman 1999). The protein comprises three major subdomains: a highly conserved N domain, a high-affinity but low-capacity Ca^{2+}-binding P domain, and a low-affinity but high-capacity Ca^{2+}-binding C domain (Michalak *et al*. 1999). Since pathogen-activated signaling implicates the induction of Ca^{2+} fluxes, it is conceivable that the role of calreticulin may also consist of maintaining Ca^{2+} homeostasis by modulating ER Ca^{2+} storage and transport. A CRT3 signal peptide-green fluorescent protein-CRT3 carboxy terminus fusion protein (sGFPHDEL) was detected in the Golgi apparatus of barley after ballistic transformation (Figure 3.5), which affirms the assumed involvement of CRT3 in folding and transport of secreted proteins. Plant CRTs are up-regulated in response to a variety of stress-mediated stimuli, e.g. pathogen-related signaling molecules (Persson *et al*. 2003). Analyses to ascertain CRT3 function in the barley-powdery mildew pathosystem, however, were inconclusive. Both, overexpression and knock-down of *CRT3* did not significantly affect penetration efficiency of *Bgh* into barley epidermal cells (Supplementary table 3 and Supplementary figure 5). Similar to the *PDI* gene, strong *CRT3* transcript accumulation can be observed in connection with *B. graminis* infection, which probably reflects ER stress or activation of the secretory machinery in response to pathogen attack. The already high expression level thus might account for the overexpression results. A BLAST search with the *CRT3* fragment used for the knock-

down experiments revealed that RNAi might have affected more than one gene, since at least one more sequence with considerable homology can be found.

LUMENAL BINDING PROTEIN (BIP) is an ER resident homolog of the HSP70 (heat-shock protein of the 70 kDa-class) protein family. Heat-shock proteins are typically induced upon rapid increase in temperature. HSP70s have chaperone activity: they bind unfolded or misfolded proteins, prevent the aggregation of denatured proteins and assist in protein refolding under stress conditions (Sheffield *et al.* 1990; Goloubinoff *et al.* 1999; Wang *et al.* 2004). Transgenic tobacco plants overexpressing *BIP* were more tolerant to water stress (Alvim *et al.* 2001) and it had already been shown that transcript accumulation of a *BIP* gene was enhanced following pathogen infection (Denecke *et al.* 1995; Jelitto-Van Dooren *et al*. 1999). HSP70 can interact with other co-chaperone proteins that control or support their function in protein folding. The *HSP ASSOCIATED PROTEIN* gene encodes a protein that is predicted to possess a heat shock chaperonin-binding motif at the C-terminal region and a putative zinc-dependent protease, containing a tetratricopeptide repeat (TPR) domain (NCBI Conserved Domain Search, http://www.ncbi.nih.gov). Both, *BIP3* and the *HSP ASSOCIATED PROTEIN* gene displayed increased transcript accumulation in response to inoculation with *Bgh* and *Bgt*. The interaction of HSP70 with TPR-domain proteins has been demonstrated (Scheufler *et al.* 2000). The HSP ASSOCIATED PROTEIN shows sequence similarity to HIP-1 from Arabidopsis (accession number CAB79222), which likely functions to stabilize HSP70 (Webb *et al.* 2001). Therefore, it is conceivable that BIP3 and HSP ASSOCIATED PROTEIN collaborate in a chaperone complex to relieve ER stress induced by powdery mildew infection.

Here, a potentially secreted *CYSTEINE PROTEASE* from barley was identified to be induced by both *formae speciales* of *B. graminis*. Previously, another *CYSTEINE PROTEASE* was found to be induced by both virulent and avirulent *Bgh* (Hückelhoven *et al.* 2001b). CYSTEINE PROTEASES are involved in protein turnover and have important roles in signal transduction, HR and cell death regulation (van der Hoorn and Jones 2004). The barley CYSTEINE PROTEASE identified here seems to be regulated non-specifically but might have a role in extracellular release or amplification of peptide signals.

SUBTILISIN-LIKE SERINE PROTEASES, also termed SUBTILASES, are representatives of the diverse SERINE PROTEASE superfamily (Siezen and

Leunissen 1997). Supposedly, most of the SUBTILASES are targeted to the extracellular matrix (Golldack *et al.* 2003). Only a few plant SUBTILASES have been characterized any further. For some, responsiveness to stress and pathogen attack has been reported (Golldack *et al.* 2003; Jorda *et al.* 1999; Tornero *et al.* 1996). Only recently, a SUBTILISIN-LIKE SERINE PROTEASE was identified during the comparative analysis of the transcriptome of *Bgh*-attacked near-isogenic barley lines, carrying either the functional or the mutated *MLO* gene. The gene was considered to be related to *mlo*-resistance since it was stronger up-regulated in the resistant line BCIngrid-*mlo5* than in the susceptible wild-type cultivar (Zierold *et al.* 2005). It is conceivable that in the apoplast SUBTILASES might either be involved in the proteolysis of proteins damaged by pathogen-generated ROI or in the proteolytic control of H_2O_2-generating enzymes (Golldack *et al.* 2003).

The yeast homolog of barley AUTOPHAGY 8C (ATG8c) is a microtubule-associated protein, which is essential for the delivery of autophagic vesicles to the vacuole (Lang *et al.* 1998). Autophagy is another major, though less selective mechanism for protein degradation in eukaryotic cells. It is responsible for the enclosure of cytoplasmic materials (e.g. proteins and organelles) in double membrane-bound compartments (called autophagosomes) and its targeting to acidic organelles with proteolytic capacity such as vacuoles and lysosomes for degradation. Thus, autophagy supports the cellular recycling and mobilization of amino acids, sugars and lipids during nutrient starvation, other stress situations and cell death (Contento *et al.* 2005; Thompson and Vierstra 2005; van Doorn and Woltering 2005). In yeast, *AUTOPHAGY* genes are either involved in autophagy-related signaling or contribute to the mounting and proper operation of the autophagy machinery (Thompson and Vierstra 2005). A number of AUTOPHAGY homologs have been discovered in plants, among them ATG8c (Thompson and Vierstra 2005). Gene transcript accumulation of Arabidopsis *ATG8c*, which also binds microtubules, was observed in senescing leaves and in response to sucrose starvation (Ketelaar *et al.* 2004; Rose *et al.* 2005). Although autophagic processes seem to coincide with plant developmental PCD, there are only few reports, which associate autophagy with HR or other pathogen-related plant responses (van Doorn and Woltering 2005). Liu and co-workers (2005) found that autophagy was induced in tobacco during *N*-mediated defense against tobacco mosaic virus, and that HR restriction was dependent on the presence of certain autophagy genes. According to macroarray and RT-PCR

analysis employed for this study, the barley *ATG8c* homolog was slightly induced only in the compatible interaction of barley with *Bgh*. Since HR is less frequent in the host interaction compared to the nonhost interaction of barley with powdery mildew fungi (Hückelhoven *et al.* 2001a; Trujillo *et al.* 2004a) induced *ATG8c* expression here could reflect the protein's involvement in HR suppression. Alternatively, since the time of gene induction coincides with the establishment of the fungal feeding organ, it is conceivable that ATG8c influences the mobilization of nutrients in an altered source-sink relationship in the epidermal cell layer.

A *PEROXIREDOXIN* gene was strongly induced after inoculation with both powdery mildew fungi (Figure 3.1). Plant PEROXIREDOXINS, also termed THIOREDOXIN PEROXIDASES, constitute a ubiquitously distributed multigene family of antioxidant enzymes, whose members can accomplish the reduction of hydroperoxides, e.g. H_2O_2 (Rouhier and Jacquot 2005). There are only few reports that connect elevated *PEROXIREDOXIN* expression to pathogen challenge. In pepper (*Capsicum annuum* L.) *PEROXIREDOXIN* expression correlated with H_2O_2 production during HR induced by *Xanthomonas campestris* (Do *et al.* 2003). Jones and co-workers (2004) observed an elevated *PEROXIREDOXIN* transcript accumulation in Arabidopsis in response to challenge with *Pseudomonas syringae*. Among the major H_2O_2 scavenging enzymes in plants there are catalase and ascorbate peroxidase, which are up-regulated in barley leaves in response to powdery mildew infection, probably in order to spatially restrict oxidative damage (Burhenne and Gregersen 2000; Hückelhoven *et al.* 2001b). Due to similar enzymatic traits, an analogous function may also be attributed to barley PEROXIREDOXIN.

According to the macroarray analysis, a couple of genes encoding proteins with putative kinase function were differentially expressed in response to pathogen infection (Supplementary table 1), among them a putative *RECEPTOR-LIKE KINASES 19 (RLK19*) and a *PTI1 (PTO-INTERACTING PROTEIN 1) -LIKE KINASE*. Protein activation and inactivation are crucial regulatory elements of (defense-related) signal transduction cascades. Kinases and phosphorylases act antagonistically by either activating or inactivating specific target proteins through phosphorylation and dephosphorylation, respectively. There are several types of protein kinases that are supposed to be involved in defense signaling, among them mitogen-activated protein (MAP) kinases, calcium dependent protein kinases and various receptor-like kinases (Nürnberger and Scheel 2001; Chen *et al.* 2004). In the

Arabidopsis genome, more than 400 genes encode putative RLKs. Typically, these proteins possess an extracellular receptor domain, often containing leucine-rich repeats (LRR), a transmembrane domain and a cytoplasmic serine/threonine protein kinase domain (Diévart and Clark 2004). Genes encoding receptor-like kinases are responsive to pathogen infection, ROI or SA treatment (Ohtake *et al.* 2000; Chen *et al.* 2004; Jansen *et al.* 2005). Plants employ membrane associated LRR-containing receptors to detect pathogen-derived external signals. The protein's innate kinase domain then relays these signals by initiating downstream signal transduction cascades. Due to the high number of different RLKs in plant genomes, there is only scarce information on specific signaling pathways. Small GTPases of the Rac/Rop family have been identified as potential interactors of RLKs (Trotochaud *et al.* 1999). Upon sensing extracellular signals, small GTPases become activated and then bind and activate other downstream effectors. In doing so, they are related to a series of cellular processes, including those involved in host-pathogen interactions (Ono *et al.* 2001; Yang 2002; Schultheiss *et al.* 2002, 2003b). Serine/threonine kinases and MAP kinases belong to the possible downstream targets for small GTPase-activated signaling (Trotochaud *et al.* 1999), providing a link to activation of transcription factors and induction of *PR* genes or other defense related genes (Zhang and Klessig 2001). It is difficult to speculate on a signal transduction pathway in the barley-powdery mildew interaction that involves RLK19, as long as there is no information available on specific interacting proteins.

Another protein-kinase encoding gene with high homology to a *PTI1* (PTO-INTERACTING PROTEIN 1) gene from soybean was also induced after infection with the powdery mildew fungus. The soybean PTI1 protein, like its counterpart in tomato, is a cytoplasmic protein that possesses a serine/threonine/tyrosine kinase domain. Enhanced gene expression was observed following SA treatment and wounding (Pedley and Martin 2003; Tian *et al.* 2003). PTO is an intracellular R-protein with serine/threonine protein kinase activity that confers resistance to those pathovars of *Pseudomonas syringae* that express and secrete the corresponding AVIRULENCE factor (AVRPTO; Ronald *et al.* 1992; Martin *et al.* 1993). In the course of infection, *P. syringae* transfers so-called effector proteins into the host cell. Effector proteins are usually required for pathogenesis, but, as in the case of AVRPTO, may sometimes represent appropriate targets for recognition by specific R-proteins (Guttman *et al.* 2002). In plants, the interaction of PTO with AVRPTO initiates the phosphorylation of

PTI1, which in turn activates another phosphorylation cascade that eventually results in defense responses such as HR and formation of callose containing papilla-like cell wall protrusions (Staswick 2000; Bogdanove 2002; Xiao *et al.* 2003). Interestingly, overexpression of bacterial AVRPTO in Arabidopsis caused suppression of a set of defense-related host genes and compromised callose deposition in infected leaves (Hauck *et al.* 2003). In the experiments presented here, *PTI1* expression was induced from 8 to 24 HAI with both *Bgh* and *Bgt,* covering the timeframe, in which formation of cell wall appositions in response to fungal attack occurs (Ellingboe 1972; Aist and Bushnell 1991; Hückelhoven *et al.* 1999). It is thus tempting to speculate that also in barley, PTI1 may be involved in signal transduction leading to papilla-associated cell wall defense against powdery mildew fungi.

4.1.1.2 Genes down-regulated after inoculation with powdery mildew fungi

Analysis of the macroarray revealed a considerable number of genes, which were down-regulated during the interaction of barley with at least either of the two powdery mildew fungi. Conceivably, gene suppression can either be initiated by the attacking fungus itself in order to bear down plant defense responses. On the other hand, down-regulation of housekeeping or development-related genes may occur as consequence of a metabolism altered in favor of the activation of defense.

Two of the down-regulated genes identified in the present study, a *BARLEY LIPID TRANSFER PROTEIN 4* (*BLT4*) gene and *GLYCINE-RICH CELL WALL STRUCTURAL PROTEIN* (GRP) gene, encode proteins, which are thought to be involved in the assembly of cuticle or cell wall (Cassab 1998). Both genes were down-regulated 24 and 48 HAI with both the appropriate and inappropriate powdery mildew fungus (Figure 3.2). LIPID TRANSFER PROTEINS (LTPs) are ubiquitously distributed small proteins, which are able to transfer lipids between membranes *in vitro*. Their actual cellular function, however, is still unclear. The fact that LTPs were found to be secreted and located in the cell wall contradicts the assumed involvement in membrane to membrane transport (Kader 1996). A more convincing hypothesis implicates LTPs and cutin biosynthesis. According to this, LTPs participate in the secretion and deposition of lipophilic material required for cutin formation. This is supported by the observation that LTPs transcripts and gene products accumulate preferentially in peripheral cell layers, including epidermis (Molina and García-Olmedo 1993; Gausing *et al.* 1994; Kader 1996). Some of the

LTPs identified in plants possess antifungal activity (Molina *et al.* 1993; Segura *et al.* 1993). It is thus rather astonishing and in contrast to Molina and García-Olmedo (1993) that there was a decrease in *BLT4* transcript accumulation in barley leaves inoculated with *Bgh* and *Bgt*.

A set of plant GRPs have been identified, whose subcellular localization, expression pattern and function can be quite diverse (Sachetto-Martins *et al.* 2000). The protein sequence of the differentially expressed barley *GRP* possesses an N-terminal signal peptide and consists of about 50 % glycine, arranged in short amino acid repeat units, and a considerable number of tyrosine residues. Immunolocalization studies confirmed localization of similar proteins to the interface between plasma membrane and cell wall (Lei and Wu 1991; Condit 1993; Sachetto-Martins *et al.* 2000). According to McDougall and co-workers (1996) the tyrosine residues supposedly participate in the cross-linking of GRPs to aromatic residues of lignin. GRPs have been discussed as structural proteins with important functions in plant vascular system and wound healing (Showalter 1993). Similarly, they could also be involved in pathogen-dependent papilla formation. On the other hand, GRPs can interact with wall-associated kinases, which are induced during plant response to pathogen infection (Anderson *et al.* 2001) and may thus partake in defense-related signaling as well. To my knowledge, down-regulation of GRPs in response to pathogen attack has never been reported. Possibly, the barley GRP functions as a negative regulatory element in defense signal transduction during the barley-powdery mildew interaction and therefore becomes suppressed by the plant. Delivery of *in vitro* synthesized dsRNA corresponding to the full-length sequence of barley GRP, tended not to strongly influence the barley-*Bgh* interaction (Supplementary table 4 and Supplementary figure 6). However, RNAi again might have affected more than one gene, since there is at least one more sequence with considerable homology to the targeted barley GRP.

REMORINS are encoded by large gene families in diverse plant species (Bariola *et al.* 2004). They are plant-specific small, hydrophilic proteins located at the plasma membrane. The N-terminus is rich in prolines, while the C-terminal end forms a coiled coil structure that is expected to interact with other macromolecules (Reymond *et al.* 1996) or to form oligomeric or filamentous protein structures (Bariola *et al.* 2004). REMORINS have been identified during a screening for proteins that bind oligogalacturonides (OGs; Reymond *et al.* 1996). OGs are released from the plant

cell wall during pathogenesis. They represent well-known elicitors of plant defense responses and can trigger the transient formation of ROI such as O_2^-, H_2O_2, and ·OH (Svalheim and Robertsen 1993; Bellincampi *et al.* 2000). OGs are supposed to stimulate REMORIN phosphorylation presumably in the presence of plasma membrane associated serine/threonine protein kinase(s) (Reymond *et al.* 1996). Reymond and co-workers noticed a striking similarity of REMORIN with viral cell-to-cell movement proteins and speculated on the involvement of REMORIN in intercellular communication. Another working group found that in tobacco REMORIN (and interestingly also NADPH OXIDASE, which is likely involved in the production of ROI for defense signaling) accumulated in so-called lipid rafts, functional micro-domains within the plasma membrane, in which sphingolipids and sterols cluster together (Mongrand *et al.* 2004). In animal cells, lipid rafts are thought to be involved in several physiological processes such as vesicular and membrane trafficking, receptor signaling, endocytosis or apoptosis (Garcia *et al.* 2003; van Meer and Sprong 2004), but it has also been proposed that lipid rafts constitute appropriate targets for pathogen entry into the cell (Rosenberger *et al*. 2000; Duncan *et al.* 2004). It has to be noted, that the existence of lipid rafts *in planta* and its importance during the interaction with pathogens still requires unequivocal verification (Martin *et al.* 2005). However, the assumed involvement of REMORIN in elicitor-dependent signal transduction and its accumulation at potential sites of pathogen entry indicate a role of the protein in sensing pathogen attack (Mongrand *et al.* 2004).

4.1.2 General considerations on the macroarray results

A surprisingly large number of the differentially expressed genes identified in this study were down-regulated during the interaction with powdery mildew fungi. This might be due to the fact that the majority of the cDNA fragments on the array membrane derive from a cDNA library of BTH-induced barley plants, which might be enriched in salicylic acid (SA) induced genes (Pieterse and van Loon 2004). It has been shown, that an Arabidopsis factor of papilla formation, which occurs in compatible and incompatible interactions, suppresses the salicylic acid pathway (Nishimura *et al.* 2003). This is supported by other reports where genes, which were induced upon powdery mildew infection, were either not differentially expressed or down-regulated upon treatment with SA or BTH in barley (Besser *et al.* 2000; Hein *et al.* 2004; Bettina Kah and Gregor Langen, Justus-Liebig Universität, Giessen,

Germany, personal communication). Alternatively, down-regulation of house-keeping or development-related genes might represent physiological requirements for allocation of resources for defense. In coherence with this, a large number of house-keeping genes were down-regulated in response to powdery mildew infection. Expression of one representative of this category, a *PHOSPHOGLYCOLATE PHOSPHATASE* (*PGP*) gene, was considerably suppressed 24 and 48 HAI with appropriate *Bgh* and slightly 24 HAI with *Bgt* (Figure 3.1). PGP is a hydrolytic enzyme, which prevents the accumulation of phosphoglycolate (a by-product of the dicarboxylate metabolism) by cleaving it into glycolate and phosphate. Since there are no reports that directly connect PGP to defense, PGP suppression could mirror a decrease in phosphoglycolate production due to pathogen dependent reduction in photosynthetic activity (Matsumura *et al.* 2003; Mysore *et al.* 2003). Consistent with my findings, Zimmerli and co-workers (2004) recently found a decreased biomass production and down-regulation of gene expression in Arabidopsis following inoculation with either virulent *Erysiphe cichoracearum* or with incompatible *Bgh*, respectively. Consistently, they also found little differences in gene expression patterns when Arabidopsis was challenged by the host or nonhost pathogen, respectively. One may speculate that host und nonhost pathogens trigger the same basic defense responses, which might then be specifically suppressed only by the host pathogen *Bgh*, perhaps by targeting the host susceptibility factor MLO that is indispensable for compatibility (Hückelhoven and Kogel 2003; Schulze-Lefert and Panstruga 2003). Gjetting *et al.* (2004) recently compared expression of selected defense genes in single barley cells, which were either penetrated by *Bgh* or not. The study demonstrated that *Bgh* suppresses defense gene expression on the single-cell level. Beyond this, it is possible that *Bgh* suppresses host defense mechanisms that are independent of transcriptional activation. This idea is supported by the observation that penetration resistance, which has a major role in both background and nonhost resistance, was not impaired by the mRNA synthesis inhibitor cordycepin in either susceptible or resistant barley (Schiffer *et al.* 1997).

Lately, several groups have identified an increasing number of barley genes that potentially partake in the barley-*Bgh* interaction (Caldo *et al.* 2004; Eckey *et al.* 2004; Hein *et al.* 2004; Jansen *et al.* 2005). Most recently, Zierold and co-workers (2005) analyzed the epidermal transcriptome of barley in *mlo*-mediated resistance to *Bgh.* They identified more than 300 differentially expressed genes with little qualitative but

quantitative differences in susceptibility-related and resistance-related gene expression. For some of the *Bgh*-responsive genes, expression had also been examined during the barley-*Bgt* nonhost interaction, but apparently there were little differences in transcript accumulation compared to the host situation (Jansen *et al.* 2005; Christina Eckey, Justus-Liebig Universität, Giessen, Germany, personal communication). This observation was confirmed on a larger scale in this study in that there were rather few genes with host or nonhost specific expression patterns. Most genes were similarly induced or repressed and also displayed similar temporal expression patterns, which indicates that they may be expressed in response to common signaling pathways during host and nonhost resistance. However, there was a tendency for higher up-regulation of activated genes in the nonhost interaction at 12 HAI and in the compatible interaction at 24 HAI as well as for stronger down-regulation of repressed genes in the compatible interaction at 24 HAI in this study (see http://www.uni-giessen.de/fbr09/ipaz/ipaz/EichmannetalJPP.htm). It cannot be excluded, that more comprehensive studies would identify significant host or nonhost specific gene expression. Recently, a large-scale microarray study revealed that virulent *Bgh* specifically suppresses non-specifically induced defense-related genes in susceptible hosts (Caldo *et al.* 2004). Defense suppression might occur highly localized because the expression pattern of penetrated cells greatly differs from that of non-penetrated cells in susceptible backgrounds (Gjetting *et al.* 2004). Previous studies already suggested a partially common molecular basis for barley host and nonhost resistance (Elliott *et al.* 2002; Trujillo *et al.* 2004b). Basal background resistance and nonhost resistance of barley, for example, share genetic elements such as *ROR1* and *ROR2* (Peterhänsel *et al.* 1997; Collins *et al.* 2003; Trujillo *et al.* 2004b). Together, the present data support the view that the majority of *B. graminis* induced gene expression is triggered by non-specific pathogen-associated molecules, and that differences in gene expression are mostly of quantitative nature.

4.2 Molecular characterization of BAX INHIBITOR-1 and its role in nonhost resistance of barley to the wheat powdery mildew fungus

The hypersensitive reaction (HR) is a common and effective means to restrict pathogen growth on plants. It is assumed that HR represents a distinct form of programmed cell death (PCD). Besides the strong relation to pathogen defense, HR is not easily distinguishable from programmed cell death that occurs during developmental processes especially senescence. The observation that expression of marker genes for senescence also occurs in connection with HR during plant-pathogen interactions and vice versa provokes the assumption that some overlapping elements in both signaling pathways may exist (Heath 2000b). Especially with regard to the control of necrotrophic pathogens, strong interest on the regulatory mechanisms of plant cell death has emerged. It seems that pathogens themselves may take advantage of signal transduction pathways of their host-plant. *Sclerotinia sclerotiorum*, for example, induces disease in tobacco by activating plant PCD, and tobacco plants could be protected from fungal infection by expression of anti-apoptotic proteins that inhibited this cell death pathway (Dickman *et al.* 2001). Conversely, it is conceivable that biotrophic microbes utilize plant cell-survival mechanisms to support their lifestyle. In this context it was discussed that the biotrophic pathogen *Bgh* might target the barley MLO protein, a negative regulator of cell death, in order to suppress plant defense (Panstruga and Schulze-Lefert 2003). This assumption is even more intelligible if one considers that in barley cell survival and *Bgh*-resistance are regulated antagonistically. It was observed that barley plants lacking the functional MLO protein are almost completely resistant to penetration attempts of *Bgh*, while this trait is often accompanied by a more frequent occurrence of spontaneous leaf cell death, especially in the mesophyll (Wolter *et al.* 1993; Peterhänsel *et al.* 1997). Besides other aspects, this remarkable connection between cell death and defense regulation prompted researchers to investigate the involvement of putative cell death regulators in different kinds of plant-pathogen interactions. In doing so, they were also inspired by cell death regulatory mechanisms in animal systems (Dickman *et al.* 2001; Hückelhoven *et al.* 2001b).

4.2.1 Barley BI-1 delays BAX-induced death of barley epidermal cells

BI-1 is one of a few regulators of PCD that are highly conserved in humans, animals and plants. One can find BI-1 homologs even in lower eukaryotic cells, and proteins with similar domain architecture have been identified in bacteria and viruses (Hückelhoven 2004). Besides in yeast, where BI-1 was originally identified during a screening for suppressors of BAX-induced apoptosis (Xu and Reed 1998), the protein cross-functionally inhibits cell death in plant and animal systems (Kawai-Yamada *et al.* 2001; Bolduc *et al.* 2003; Chae *et al.* 2004; Kawai-Yamada *et al.* 2004; Baek *et al.* 2004). In animals, mitochondria appear to play a critical role in the induction of apoptosis. When BAX becomes activated, the protein usually translocates from the cytosol to the mitochondrial outer membrane, where it clusters and exerts its function by causing membrane permeability for apoptogenic factors such as cytochrome *c* (Breckenridge and Xue 2004; Sharpe *et al.* 2004). In the cytosol these factors activate a cascade of proteolytic enzymes called caspases that eventually leads to protein degradation and loss of cell integrity (Green and Reed 1998; Loeffler and Kroemer 2000). The resulting lethal phenotype includes chromatin condensation, DNA fragmentation, cell shrinkage, membrane blebbing and eventually development of apoptotic bodies (Collins *et al.* 1997; Ligr *et al.* 1998). To verify cell death inhibiting properties of the barley BI-1 homolog, the protein's capability to suppress BAX-induced lethality was examined in yeast and barley, respectively. Yeast is widely accepted as model organism to study cell death involving processes like aging or cell differentiation (Madeo *et al.* 2004). Though devoid of most common regulators of apoptosis themselves, yeast cells execute an apoptosis-like cell death program upon heterologous expression of mammalian pro-apoptotic proteins. The resulting death phenotype displays characteristic features of apoptosis, namely DNA cleavage, chromatin condensation, cellular disorganization, vacuolization and shrinkage of the cytoplasm (Jürgensmeier *et al.* 1997; Ink *et al.* 1997; Madeo *et al.* 2004; Baek *et al.* 2004). Here, co-expression of mammalian BAX, was employed to evaluate the alleged potential of barley BI-1 to suppress BAX-related cell death phenotypes in yeast. Due to the strong lethal effect of BAX, it was necessary to use a galactose inducible expression system, which was adapted from Ligr and co-workers (1998). As expected, galactose-induced expression of mammalian *BAX* completely abolished colony formation on galactose plates, whereas co-expression of the mammalian BAX antagonist *BCL-X_L*, which served as

positive control (Ligr *et al.* 1998), rescued yeast cells from dying. In contrast, co-expression of barley *BI-1* did not support growth of yeast cells harboring *BAX*, although BAX-antagonistic function had been demonstrated previously for several other plant BI-1 homologs, i.e. from Arabidopsis (Sanchez *et al.* 2000; Kawai-Yamada *et al.* 2001), rice (Kawai *et al.* 1999; Chae *et al.* 2003) and tomato (Chae *et al.* 2003). This might be due to several reasons: barley *BI-1* expression in yeast might have been restrained, possibly because of problems with barley codon usage. In fact, production of barley BI-1 protein in transformed yeast cells could not be confirmed by immunoblot analysis. Another important reason for failure of the barley protein to inhibit BAX-induced cell death in yeast may lie within the choice of expression plasmids. The usage of a strong constitutive promoter instead of an inducible one for *BI-1* expression would have provided a pivotal advantage in the race for cell survival.

Concomitant to the yeast experiments, a fast and easy method for testing candidate cell death inhibitors *in planta* should be established. Although BAX and other pro-apoptotic members of the BCL-2 family are apparently not present in plants, they can promote cell death features in plants as well (Lacomme and Santa Cruz 1999; Kawai-Yamada *et al.* 2001; Abramovitch *et al.* 2003; Baek *et al.* 2004; Yoshinaga *et al.* 2005). At first Lacomme and Santa Cruz (1999) noticed striking similarities when they compared *N*-gene mediated HR in response to tobacco mosaic virus (TMV) infection with cell death triggered by *BAX* expression from a viral vector. *BAX*-induced localized tissue and cell collapse appeared 2 to 3 days after inoculation and thus resembled virus induced HR in both timing and phenotype. Upon induction, transgenic Arabidopsis plants or Arabidopsis mesophyll protoplasts expressing mammalian BAX from a dexamethasone (DEX) inducible promoter exhibit strong chlorotization and severe growth reduction at the whole-plant level and hallmarks of cell death such as cytoplasmic shrinkage, increased vacuolation and DNA laddering at the cellular level (Kawai-Yamada *et al.* 2001; Baek *et al.* 2004). In the single cell transient transformation assay in this study, overexpression of *BAX* provoked similar morphological changes in barley epidermal cells already at relatively low concentrations of *BAX* expression plasmid. Although fluorescence of the transformation marker GFP remained unexpectedly stable, one could easily distinguish dead or dying cells from unaffected ones on the basis of cell phenotype and GFP distribution. Upon expression of the mammalian *BAX* gene from a

constitutive promoter, condensation and aggregation of the cytoplasm and the disappearance of cytoplasmic strands and concomitant cytoplasmic streaming became apparent (Figure 3.3). Simultaneous expression of *BAX* and different kinds of potential BAX antagonists influenced cellular integrity differently. While the mammalian opponent of BAX activity, BCL-X_L, almost completely prevented the cell death phenotype, barley BI-1 and its truncated version BI-1ΔC could not sustain cytoplasmic streaming. However, expression of both variants had significant effect on the maintenance of cytoplasmic strands (Figure 3.4). BAX inhibiting properties have also been attributed to plant ASCORBATE PEROXIDASE (APX; Moon *et al.* 2002; see below). In four independent experiments, a barley APX supported cytoplasmic movement and the maintenance of cytoplasmic strands. However, this effect was not statistically significant (Figure 3.4). The devastating effect of *BAX* expression was apparent as soon as GFP accumulation became visible. In order to give *BI-1* expression a head start, different transgenic barley plants, which were stably expressing a GFP-BI-1 fusion construct (albeit at different levels) were used for re-examination of BAX-induced lethality. In contrast to the control plants, some of the transgenic barley lines were quite effective in the preservation of cytoplasmic movement (Figure 3.6). These results indicate that barley BI-1 indeed functions as a BAX antagonist and has the potential to inhibit or at least delay BAX-induced cell death. One can thus expect to obtain stronger BI-1 effects in the transient transformation assay with the combination of constitutive *BI-1* overexpression and *BAX* expression from an inducible promoter.

There are different kinds of development- or pathogen-related cell death in plants, whose exact mechanisms and regulatory elements are largely unknown. It is even more enigmatic, why and how mammalian BAX can act *in planta* despite the obvious lack of other key elements of animal apoptosis. It has always been assumed that the basic molecular mechanisms underlying PCD were evolutionally conserved in plants and animals. For example, cytochrome *c* release from mitochondria in response to cell death activation has been observed in plants. But it still remains to be elucidated whether this is a prerequisite for cell death execution or merely derives from cellular and mitochondrial demise (Balk *et al.* 1999; Jones 2000; Lam *et al.* 2001; Yu *et al.* 2002). Likewise, the importance of caspase-like proteins in plant PCD has not unequivocally been proven yet. The application of caspases-specific peptide inhibitors influences cell death in various plants species upon different lethal

treatments, although no close homologs of apoptosis relevant caspases are present in plant genomes (Lam and del Pozo 2000; Elbaz *et al.* 2002; Watanabe and Lam 2004). It has been speculated that other proteolytic enzymes similar to caspases regulate both initiation and execution of plant PCD. Due to their potential for limited and site-specific proteolysis, SUBTILISIN-LIKE and CYSTEINE PROTEASES, which constitute large gene families in plant genomes, turned out to be suitable candidates for that (Chichkova *et al.* 2004; Coffeen and Wolpert 2004; Schaller 2004; Watanabe and Lam 2004; Hara-Nishimura *et al.* 2005). However, the fact that the resulting death phenotypes in plants and animals are strikingly similar provides some evidence that there is at least partial overlap between the underlying cellular mechanisms. Recent reports suggest two possible mechanisms, through which BAX-induced cell death can be mediated in animal systems. One of them seems to be dependent on the protein's translocation to mitochondria and the production of ROI, while the other one likely proceeds via the ER. Accordingly, at least either of these processes should be influenced by the BAX antagonist BI-1. It has been shown that BAX-induced cell death in yeast, animals and plants was accompanied by the accumulation of ROI, which occurs as a consequence of dysfunction or interruption of mitochondrial transmembrane potential and can amplify the cell death signal (Ligr *et al.* 1998; Madeo *et al.* 1999; Lam *et al.* 2001; Kawai-Yamada *et al.* 2004; Yoshinaga *et al.* 2005). As in animals, BAX operation in plants seems to require the protein's targeting to mitochondria (Lacomme and Santa Cruz 1999; Kawai-Yamada *et al.* 2001; Baek *et al.* 2004; Yoshinaga *et al.* 2005). In Arabidopsis protoplasts, ROI production preceded loss of cell viability and was therefore considered to be cause rather than consequence of cell death (Baek *et al.* 2004). Apparently, BI-1 did not directly scavenge ROI (Baek *et al.* 2004; Kawai-Yamada *et al.* 2004), and the protein was able to inhibit cell death that was independent from ectopic BAX expression but resulted from oxidative stress induced by application of a fungal elicitor, H_2O_2 or salicylic acid (SA). Thus, a function of BI-1 downstream of ROI production was assumed (Chae *et al.* 2003; Matsumura *et al.* 2003; Kawai-Yamada *et al.* 2004). Besides BI-1, at least five other plant proteins possess the ability to suppress lethal activity of BAX in yeast and/or plant systems. Strikingly, all plant BAX-antagonist proteins were able to prevent yeast and plant cell death induced by biotic and abiotic stimuli that caused oxidative stress or by exogenous application of H_2O_2, which can lower the mitochondrial membrane potential leading to the release of cytochrome *c*

and the activation of the caspase pathway in mammalian cells (Florea *et al.* 2005). A soybean ASCORBATE PEROXIDASE prevented cellular demise by inhibiting the accumulation of ROI generated by BAX (Moon *et al.* 2002). A VESICLE-ASSOCIATED MEMBRANE PROTEIN from Arabidopsis blocked BAX-induced death of yeast cells downstream of oxidative burst, most likely by mending oxidatively damaged membranes (Levine *et al.* 2001). Similarly, when co-expressed with *BAX* in yeast cells, a tomato GLUTATHIONE *S*-TRANSFERASE homolog with weak glutathione peroxidase activity maintained cellular integrity, supposedly through the protection of cellular components from oxidative stress (Kampranis *et al.* 2000). Likewise it was shown that an Arabidopsis ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN could prevent cell death induced by BAX and oxidative stress most likely through transcriptional activation of genes involved in ROI detoxification (Pan *et al.* 2001; Ogawa *et al.* 2005). A PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE from tomato exerted cytoprotective function by stopping lipid peroxidation (Chen *et al.* 2004). Cell protection through BI-1 could also be explained by the protein's direct or indirect participation in the maintenance of membrane integrity. In both mammalian and plant cells, BI-1 fusions with the green fluorescing protein (GFP) can be detected predominantly in endomembranes, namely ER and the perinuclear region, which constitute a continuum, but supposedly do not physically interact with mitochondria (Xu and Reed 1998; Kawai-Yamada *et al.* 2001; Bolduc *et al.* 2003). Due to its presence at the interface of ER and Golgi apparatus, BI-1 could well be involved in the regulation of vesicular trafficking and the recycling of membranes (Baek *et al.* 2004). However, BI-1 can interact with other human cell death regulators, e.g. BCL-2 and BCL-X_L, and might affect other signaling molecules downstream of mitochondria as well, thereby monitoring mitochondrial function without physical interaction with these organelles. Interestingly, in Arabidopsis mesophyll protoplasts strong radical scavengers could not completely prevent BAX-mediated cell death and the resulting lethal phenotype was not accompanied by ROI accumulation, indicating an alternative cell death process that is independent of ROI production (Baek *et al.* 2004). In this connection it has been suggested that BAX could operate via the ER in the regulation of apoptosis by triggering massive Ca^{2+} release from this organelle. When taken up by mitochondria close to the ER, high levels of Ca^{2+}, too, modulate mitochondrial permeability transition and promote cytochrome *c* leakage with the resulting caspases activation (Nutt *et al.* 2002a, b;

Oakes *et al.* 2003; Scorrano *et al.* 2003; Zong *et al.* 2003). Recently, Westphalen and colleagues (2005) observed that overexpression of *BI-1* but not of a ΔC-mutant dramatically reduced releasable ER calcium content so that Ca^{2+} amounts in cytosol and mitochondria did not reach apoptosis relevant concentrations in human cells. This proposed mechanism is supported by the assumption that the 6-7 transmembrane domain protein BI-1 might be able to form or modify ion-conducting channels in the ER that can also be influenced by BAX (Nutt *et al.* 2002a, b; Scorrano *et al.* 2003). It remains to be elucidated whether this holds true for the plant system, too. The charged C-terminus of BI-1 is predicted to form a coiled-coil structure that likely extends into the cytosol (Bolduc *et al.* 2003; Chae *et al.* 2003). It might be involved in protein-protein interactions and was shown to be important for cell death regulation (Chae *et al.* 2003; Kawai-Yamada *et al.* 2004) but not for the localization of BI-1 to the ER (Bolduc *et al.* 2003). In the experiments presented here, the C-terminally truncated BI-1 protein was still able to confer some cytoprotection. Therefore, it might be possible that the accumulation of the truncated protein is sufficient to compensate for the assumed loss of antiapoptotic function.

Compared to barley BI-1 the mammalian BCL-X_L protein shows a higher potential to prohibit BAX-dependent lethality in plant cells. This might be due to a more direct cellular effect of this antiapoptotic protein on BAX function. Although BCL-X_L potentially interferes with the apoptosis pathway at different sites (also downstream of mitochondria), it has convincingly been demonstrated that BCL-X_L can directly interact with BAX and that overexpression of the protein prevents mitochondrial membrane permeabilization by inhibition of BAX translocation and/or dimerization (Sato *et al.* 1994; Breckenridge and Xue 2004; Kim 2005). It is conceivable that the prevention of cell death activation is more effective than the containment of death signaling once it has been initiated. Anyway, this and the fact that BI-1 suppresses different innate cell death responses support the view that BI-1 originally evolved as a cell death suppressor that was not supposed to specifically regulate BAX activity (Hückelhoven 2004).

4.2.2 Expression of barley *BI-1* in response to inoculation with *Bgt*

Hückelhoven *et al.* (2001b) examined the expression of putative PCD related genes of barley in the barley-*Bgh* interaction. A barley *BI-1* encoding gene was up-regulated in response to *Bgh*A6 inoculation, especially in barley genotypes that typically

execute HR in response to *Bgh* attack. It was therefore assumed that BI-1 might be involved in restricting the spread of PCD in cells adjacent to cells that underwent HR. *BI-1* gene transcript was constitutively present in barley leaves and transcript accumulation slightly increased with leaf age (Hückelhoven *et al.* 2001b). In the barley-*Bgt* nonhost interaction *BI-1* expression was enhanced after inoculation with spores of the powdery mildew fungus, albeit gene activation appears not very strong (Figure 3.7). This induction correlated with early defense response as was reported for the host situation (Hückelhoven *et al.* 2003). Elevated accumulation of *BI-1* transcript upon pathogen challenge and other stress related stimuli already pointed to the implication of BI-1 in defense and general stress responses (Sanchez *et al.* 2000; Hückelhoven *et al.* 2001b, 2003; Matsumura *et al.* 2003; Xu *et al.* 2003; Kawai-Yamada *et al.* 2004). In animals and plants ER stress occurs together with the accumulation of unfolded or misfolded proteins. Microarray analysis of Arabidopsis plants upon induction of ER stress characterized BI-1 as being a so-called unfolded protein response gene. Gene expression of *BI-1* but also of ER resident chaperones was significantly elevated in this particular situation (Kamauchi *et al.* 2005). Induction of *BI-1* expression was also observed in interactions of Arabidopsis with virulent and avirulent strains of *Pseudomonas syringae* pv. tomato DC3000 and even with non-phytopathogenic bacteria, suggesting a general response of the gene to microbial invasion of the apoplast. In peanut plants, elevated transcript accumulation of BI-1 was associated with resistance to *Cercosporidium personatum*, the causal agent of the late leaf spot disease (Luo *et al.* 2005). On the other hand, *BI-1* was down-regulated during the onset of Chemically Induced Resistance (CIR), which in barley leads to enhanced epidermal cell death and papilla formation in response to *Bgh* infection, and *BI-1* expression recovered after *Bgh*-inoculation of the chemically induced leaves, indicating that BI-1 acts as a negative regulator of penetration resistance (Hückelhoven *et al.* 2003).

4.2.3 *BI-1* overexpression compromises penetration resistance of barley to *Bgt*

Only recently, Hückelhoven *et al.* (2003) could demonstrate a direct participation of *BI-1* in the interplay of plant and pathogen, which was quite reminiscent to the one observed for MLO (Kim *et al.* 2002a). The MLO protein is a modulator of defense

responses to different pathogens and depends on calmodulin binding in *Bgh*-susceptibility (Kim *et al.* 2002b). Functional comparison of BI-1 and MLO revealed strong similarities: The *Mlo* and *BI-1* gene are similarly expressed in response to wounding, pathogen challenge and ROI as well as during leaf aging (Hückelhoven *et al.* 2001b; 2003; Piffanelli *et al.* 2002; Sanchez *et al.* 2000). In barley, overexpression of both the *BI-1* and the *Mlo* gene confers super-susceptibility to *Bgh*, and both genes, when overexpressed in *mlo*-genotypes, restore accessibility of the fungus to epidermal cells (Shirasu *et al.* 1999b; Hückelhoven *et al.* 2003). Besides, the *mlo*-mediated defense mechanism seems to be active in the nonhost interaction with *Bgt*, because some isolates of *Bgt* are restricted earlier on *mlo*-barley as compared to *Mlo*-barley, where these isolates paradoxically trigger a cell death response (Hückelhoven *et al.* 2001a; Peterhänsel *et al.* 1997). This is comprehensible since the functional MLO acts as a suppressor of penetration resistance and developmental cell death but not as a suppressor of *R*-gene mediated HR that might be involved in post penetration defense (Matsumura and Tosa 1995; Peterhänsel *et al.* 1997). Vice versa, overexpression of functional *MLO* suppressed penetration resistance in the nonhost interaction of barley and wheat with inappropriate *formae speciales* of *B. graminis* (Elliott *et al.* 2002). As demonstrated here, the same holds true for *BI-1* when overexpressed in epidermal cells of various barley cultivars inoculated with *Bgt* (Figure 3.8). When expressed from its endogenous promoter, however, *Bgt*-induced expression of *BI-1* is either misplaced or insufficient to induce accessibility to the fungus. The observation that BI-1 affects penetration resistance stronger in barley cultivar Manchuria than in Ingrid might be explained by a lower background penetration resistance of Manchuria to *Bgt* and *Bgh* that can be detected microscopically after inoculation of seven days old plants (Trujillo *et al.* 2004a, b). Interestingly, and in analogy with the regulator of developmental cell death in barley, *MLO*, this defense suppression only affected papilla-related penetration resistance, and thus early defense mechanisms. Until now, it remains elusive how BI-1 suppresses papilla-based defense to powdery mildew fungi and whether the protein participates in the same signal transduction pathway that is also regulated by MLO. To address this question, experiments were conducted, in which both putative cell death regulator genes were simultaneously overexpressed. It turned out, that by 48 HAI the penetration efficiency of *Bgt* in cells simultaneously expressing *Mlo* and *BI-1* was only slightly enhanced when compared to the discrete effects of the genes on

penetration success (Figure 3.9 A). Therefore, it is unlikely, that MLO and BI-1 act synergistically. Both proteins rather appear to target a similar mechanism. One may speculate that BI-1 interferes with the MLO-pathway downstream of MLO (Hückelhoven *et al.* 2003). This is supported by the fact that *BI-1* overexpression weakens penetration resistance to *Bgt* even in the *mlo5*-genotype that does not express MLO. *BI-1* expression in BCIngrid-*mlo5* induced more than 20 % susceptibility to *Bgh* (Hückelhoven *et al.* 2003) but only 7 % susceptibility to *Bgt* (Figure 3.8 B) demonstrating that BI-1 is more efficient when an appropriate fungus attacks. However, the fact that simultaneous overexpression of *BI-1* and *MLO* slightly enhanced fungal development at late infection times (corresponding roughly to 6 days after penetration) indicates a possible teamwork of both proteins in post penetration defense suppression and maintenance of single cell compatibility (Figure 3.9 B). However, there was only little sporulation of *Bgt* on *MLO* or *BI-1* overexpressing cells. This shows that both proteins neither alone nor together were sufficient to abolish post penetration defense completely.

It has been demonstrated that in barley leaves in contrast to MLO, which is located at the plasma membrane of epidermal cells (Devoto *et al.* 1999), *BI-1* is predominantly expressed in mesophyll tissue and only weakly in the epidermis, which renders any physical interaction of the proteins rather unlikely. But considering that the *mlo*-mutation also affects mesophyll cell death as indicated by spontaneous leaf lesions in *mlo*-plants (Wolter *et al.* 1993; Peterhänsel *et al.* 1997; Piffanelli *et al.* 2002) it is conceivable that the *Mlo*-dependent cell survival pathway may also involve some signal exchange between epidermis and mesophyll and the participation of *BI-1*. Possibly, BI-1, when overexpressed in epidermal cells, interferes with this cell-survival pathway that is negatively linked to penetration resistance. The fact that overexpression of *BI-1* but not of the mammalian BAX antagonist *BCL-X_L* compromised papilla formation (data not shown) indicates that BI-1 function in defense suppression is specific. It should be examined, however, whether other barley proteins with potentially BAX antagonistic properties such as VESICLE-ASSOCIATED MEMBRANE PROTEIN (Levine *et al.* 2001) affect defense responses to powdery mildew fungus.

4.2.4 Subcellular localization of GFP-BI-1 fusion proteins

Computer programs predict eukaryotic BI-1 proteins to be integral membrane proteins with 6-7 membrane spanning domains and a cytoplasmic C-terminus (Kawai *et al.* 1999; Bolduc *et al.* 2003; Chae *et al.* 2003; Hückelhoven *et al.* 2003; Coupe *et al.* 2004). In accordance with other reports, a GFP-BI-1 fusion protein expressed in barley epidermal cells was predominantly localized to the nuclear envelope and a net-shaped structure within the cytoplasm, most probably representing the ER (Figure 3.10 A). Similar patterns of BI-1 distribution in plant and yeast cells were found by other researchers (Bolduc *et al.* 2003; Kawai-Yamada *et al.* 2001). The charged C-terminus of BI-1 likely extends into the cytosol (Bolduc *et al.* 2003; Chae *et al.* 2003). This is supported by computational analyses (Bolduc *et al.* 2003; Hückelhoven *et al.* 2003), but also by the finding that digitonin digestion of tobacco cells, which permeabilizes the plasma membrane but not endomembranes allowed anti GFP-immunodetection of BI-1-GFP (C-terminal GFP fusion) in the ER (Bolduc *et al.* 2003). In addition, barley and other BI-1 proteins possess RXR and/or KKXX-related motifs close to the C-terminus representing structures that typically mediate ER retention of membrane proteins with the C-terminus extending into the cytoplasm (Shikano and Li 2003; Hückelhoven 2004).

GFP-BI-1 localization was not only examined in unstressed cells, but also in epidermal cells attacked by either the appropriate or the inappropriate powdery mildew fungus. The GFP-BI-1 fusion protein frequently accumulated beneath the appressorial germ tube and in the immediate vicinity of papillae. One could also observe that immature haustoria were enclosed by a green fluorescing envelope (Figure 3.11). This BI-1 accumulation around haustoria could be specific and thus required for the establishment of susceptibility. However, Leckie *et al.* (1995) already reported on the reorganization of the host ER, which was forming a dense network around the developing extra haustorial complex. Since it could possibly function in membrane assembly and cell signaling, as well as in the regulation of cytosolic Ca^{2+}, the ER was supposed to either contribute to the formation of the extra haustorial membrane or in signaling between plant and fungus (Green *et al.* 2002). Similarly, Takemoto *et al.* (2003) found an aggregation of ER membrane and the accumulation of Golgi bodies in Arabidopsis at sites of infection with *Peronospora parasitica*, suggesting that production and secretion of plant materials were activated around the penetration site. The cortical ER network is also linked to the actin scaffold, which

provides a path for Golgi and vesicular trafficking (Boevink *et al*. 1998). The cytoskeleton plays a crucial role in plant-microbe interactions. In response to attack, actin cables polarize to the sites of attempted fungal penetration, most likely in order to focus cellular defense and vesicular transport on the endangered spots (Collins *et al.* 2003; Yun *et al.* 2003; Opalski *et al.* 2005). In any case, there was more subtle actin reorganization around fungal haustoria when susceptible host cells were penetrated (Opalski *et al.* 2005). Parasite entry into the epidermal cell is the critical step in pathogenesis. Deposition of cell wall material and accumulation of phenolic compounds or reactive oxygen species in papillae are usually effective means to withstand attempted fungal penetration. In epidermal cells attacked by germinating spores of the powdery mildew fungus, GFP tagged Arabidopsis PEN1 or its ortholog in barley, ROR2, focally accumulate beneath sites of attempted penetration (Assaad *et al.* 2004; Bhat *et al.* 2005). Both proteins are required for penetration resistance in plant nonhost interactions with powdery mildew fungi (Collins *et al.* 2003; Trujillo *et al.* 2004b; Nürnberger and Lipka 2005). Consistently, both proteins encode syntaxin Syp121, a protein of the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) family, which is likely involved in polarized vesicular secretion events during papilla formation. In the converse situation, pathogens sometimes require the presence of certain host proteins, so-called susceptibility factors, which support their invasion. Only recently, Bhat *et al.* (2005) observed a similar focal accumulation of the negative regulator of papilla formation, MLO, underneath the appressorium. This accumulation turned out to be specific and independent of actin polarization. The authors suggested that the powdery mildew fungus, upon release of unknown effectors from the appressorium, triggers the recruitment of certain plasma membrane proteins and by this means creates a plasma membrane microdomain (reminiscent of lipid rafts, see chapter 4.1.1.2), which facilitates fungal entry into the host cell (Bhat *et al.* 2005; Panstruga 2005). At the moment it is not definitively clear, whether GFP-BI-1 accumulation around haustoria and/or in the vicinity of attacking appressoria is also a specific requirement for defense suppression or rather the consequence of more general cellular reorganization processes (e.g. actin polarization) or whether BI-1 is even located in lipid raft-like membrane domains. One can state, however, that BI-1 is present at the critical sites of pathogen entry where it potentially fulfills its specific function in susceptibility, probably in cooperation with MLO.

4.2.5 Overexpression of *BI-1* modulates local H_2O_2 accumulation

Previous findings indicate that the effectiveness of cell wall appositions largely depends on the presence of hydrogen peroxide (H_2O_2), which is thought to be responsible for local cell wall re-enforcement by oxidative cross-linking of proteins and phenolic compounds (Thordal-Christensen *et al.* 1997; Hückelhoven *et al.* 1999; Zeyen *et al.* 2002). Therefore, it was investigated, whether *BI-1* expression evoked any changes in pathogen related H_2O_2 accumulation patterns by combining the established single cell transformation assay (Schweizer *et al.* 1999) with H_2O_2-specific DAB staining (Thordal-Christensen *et al.* 1997). BI-1-dependent suppression of penetration resistance is quite distinct in *mlo*-mediated resistance, which usually rests upon formation of effective papilla underneath the appressorial germ tube (APG; Hückelhoven *et al.* 2003). Therefore, barley *mlo* genotype BCIngrid-*mlo5* was employed for these experiments. Overexpression of *BI-1* caused significant elevation of penetration frequency from 5 % in control cells to around 20 % (Figure 3.12 A). These values were quite consistent with those published previously (Hückelhoven *et al.* 2003). Strikingly, the increased penetration efficiency coincided with a significantly reduced number of interaction sites that showed DAB staining beneath the appressorial germ tube. Similar results were obtained with overexpression of *MLO*, whereby the effects were even more pronounced (Figure 3.12 B). When examining powdery mildew associated H_2O_2 accumulation in different barley genotypes Hückelhoven *et al.* (1999; 2000b) found that penetration efficiency was significantly higher in cells expressing the functional *MLO* gene, while frequency of DAB staining beneath appressoria was markedly reduced, which is consistent with my results. Although by this time it cannot unequivocally be excluded that decrease in papilla staining is consequence rather than cause of enhanced susceptibility of transformed cells, the results suggest that both MLO and BI-1 are able to modulate local accumulation of H_2O_2 during the interaction with barley powdery mildew. This is supported by the fact that the comparably big difference in defense suppression initiated by BI-1 and MLO, respectively, is not reflected in the reduction of H_2O_2 accumulation (Figure 3.12 A and B). Expression of both genes, *MLO* and *BI-1*, is considerably enhanced in response to fungal attack (Piffanelli *et al.* 2002; Kim *et al.* 2002a; Hückelhoven *et al.* 2003), and MLO has been discussed as target protein for *Bgh* to trigger accessibility (Kim *et al.* 2002b; Hückelhoven 2005). It is thus conceivable that the fungus uses MLO and probably (as a consequence) BI-1 in

order to down-regulate H_2O_2 and consequently penetration resistance. From animal and plant systems it is known that BI-1 supposedly does not directly scavenge ROI production during cell death execution (Baek *et al.* 2004; Kawai-Yamada *et al.* 2004). It is thus likely that BI-1 rather influences cellular processes, which are involved in H_2O_2 production and/or papilla formation. The cytosolic free Ca^{2+} levels represent an appropriate control point for this purpose. Increased cytosolic free Ca^{2+} levels are known to be involved in the control of defense responses via induction of $O_2^{\cdot-}$ and H_2O_2 production (Blume *et al.* 2000). Ca^{2+} also regulates other events that are crucial for the establishment of penetration resistance. Organization and dynamics of the actin cytoskeleton, for example are governed by Ca^{2+} dependent protein kinases or get promoted by constructive and deconstructive proteins, which are dependent on Ca^{2+} gradients (Grabski *et al.* 1998; Staiger 2000). Consequently, Ca^{2+} has considerable impact on secretion and vesicular transport of proteins and other defense compounds to sites of attempted fungal ingress. It is conceivable that, due to its potential to limit Ca^{2+} release from the ER, BI-1 might exert some influence on these processes, thereby impairing the formation of cell wall appositions.

4.2.6 The BI-1 motif is important for protein function in powdery mildew susceptibility

Structure-function analysis by means of site-directed mutagenesis or truncation of protein residues represents a means to provide insights into the contribution of specific amino acids (aa) and/or domains to protein function (Elliott *et al.* 2005). High evolutionary conservation of single aa especially across kingdoms indicates their probable relevance for protein activity. Plant BI-1 homologs are up to 42 % identical to the human representative (Bolduc *et al.* 2003). Comparison of the deduced aa sequence of barley BI-1 with other plant homologs and the human protein reveals 86 invariant aa (out of 247), which, due to their high conservation, may be crucial for execution of protein function (Hückelhoven *et al.* 2003). Only limited information is available concerning structural and functional domains of the cell death antagonist protein. Computational analyses of deduced BI-1 sequences predicted 6-7 transmembrane (TM) domains. Apart from the C-terminus, which resides within the cytoplasm and is required for cell death inhibition and probably protein-protein interactions (Yu *et al.* 2002; Bolduc *et al.* 2003; Chae *et al.* 2003; Kawai-Yamada *et*

al. 2004), no specific property has been ascribed to any other protein domain. A short aa stretch between the third and fourth predicted TM span shows particular structural conservation since it is already present in bacterial proteins and is therefore referred to as BI-1 motif (Hückelhoven 2004). The search for short nearly exact matches in protein databases (http://www.ncbi.nih.gov/BLAST) can provide further hints on protein properties by detecting conserved sequence segments in proteins with assigned function. Within the BI-1 sequence, five short aa stretches considerably resembled motifs in proteins of various prokaryotic or eukaryotic organisms. All of these proteins are predicted to be membrane integral. The fact that one can attribute channel function or transport processes to most of them supports the assumption that the BI-1 protein takes the shape of an ion conducting channel inside the ER membrane to regulate cytosolic Ca^{2+} levels (Xu and Reed 1998; Bolduc *et al.* 2003; Hückelhoven 2004). Nine conserved aa were selected for site directed mutagenesis. The aa were rather randomly distributed within the protein but all sequence stretches discussed above were modified at least in one aa. The set of mutants was then individually tested in the transient transformation assay to assess whether the corresponding proteins were still able to affect penetration resistance of barley to the inappropriate *Bgt*. A single aa exchange within the BI-1 motif at position 123 significantly reduced the ability of BI-1 to suppress nonhost penetration resistance, mutant F128L was slightly but not significantly hyperactive, while all the other mutants approximately retained wild type function (Figure 3.13). This indicates that the BI-1 motif might be crucial for defense suppression. To explain partial loss of function, one may speculate that the mutation either prevents correct folding of the protein (by modifying the structural basis for the building of intramolecular bridges and bonds) and (consequently) its localization, or hampers the binding of putative interacting (agonist) polypeptides. It has recently been shown that invariant aa in the MLO protein were necessary for the proteins functionality, supposedly since they were either required for the formation of putative ligand binding sites or intramolecular conformational changes (Elliott *et al.* 2005). The subcellular localization of GFP-BI-1G123R und GFP-BI-1F128L fusion constructs was not examined. The clear residual effect of the one mutant and the slight hyperactivity of the other, however, argue against complete improper folding and relocation of the proteins. At the moment, the exact impact of local TM domain modifications on protein conformation remains elusive. C-terminal truncation of the BI-1 protein by 17

aa (including a KKXX-like ER-retention motif) apparently did not affect defense suppression, and, as indicated by the localization of a GFP-BI-1ΔC fusion protein, it apparently did not alter the protein's adhesion to the endomembrane system, which is in concordance with Bolduc *et al.* (2003). However, besides typical ER-localization, a tendency for stronger accumulation of GFP-BI-1ΔC in the cell periphery was observed.

4.2.7 General considerations on the BI-1 results

It has been assumed that the MLO protein negatively regulates both penetration resistance to *B. graminis* and PCD in barley. This was supported by the observation that loss of MLO function in *mlo* mutant barley plants confers race unspecific penetration resistance to the barley powdery mildew fungus on the one hand, and promotes premature leaf senescence on the other. The present model of MLO function proposes that the powdery mildew fungus exploits the assumed control element of an endogenous (developmental) cell death pathway for pathogenicity. The exact mechanism behind this assumption remains elusive. It has been shown, however, that MLO function in defense suppression involves both focal protein accumulation at sites of fungal attack and interaction with ROR2, a mediator of membrane fusion events (Bhat *et al.* 2005). While located to the plant plasma membrane underneath the attacking appressorium, MLO may either support development of the haustorium or suppress the formation of cell wall appositions (Panstruga 2005). In striking congruence with that, the barley BI-1 protein displays cytoprotective properties and negatively interferes with a defense pathway that supports cell wall-associated defense. According to the models proposed in figures 4.1 and 4.2, the dual function of BI-1 can best be explained by its potential to modulate intracellular Ca^{2+} dynamics, which influence both, cell death regulation and processes that are involved in penetration resistance. Although reports that provide direct evidence for BI-1-mediated Ca^{2+} regulation are still rare (Westphalen *et al.* 2005), BI-1 localization to the cells major Ca^{2+} reservoir and the alleged potential for pore or channel formation support this assumption. Whether the powdery mildew fungus also targets BI-1 for defense suppression, as it is supposed for MLO, is not clear. It is also conceivable that BI-1 functions in dependency of the MLO protein. Given that BI-1 functions as a down-stream element of MLO, it might be part of a negative feed back regulation of pathogen induced Ca^{2+} release from the ER, which helps plants to contain excess defense responses (Figure 4.2).

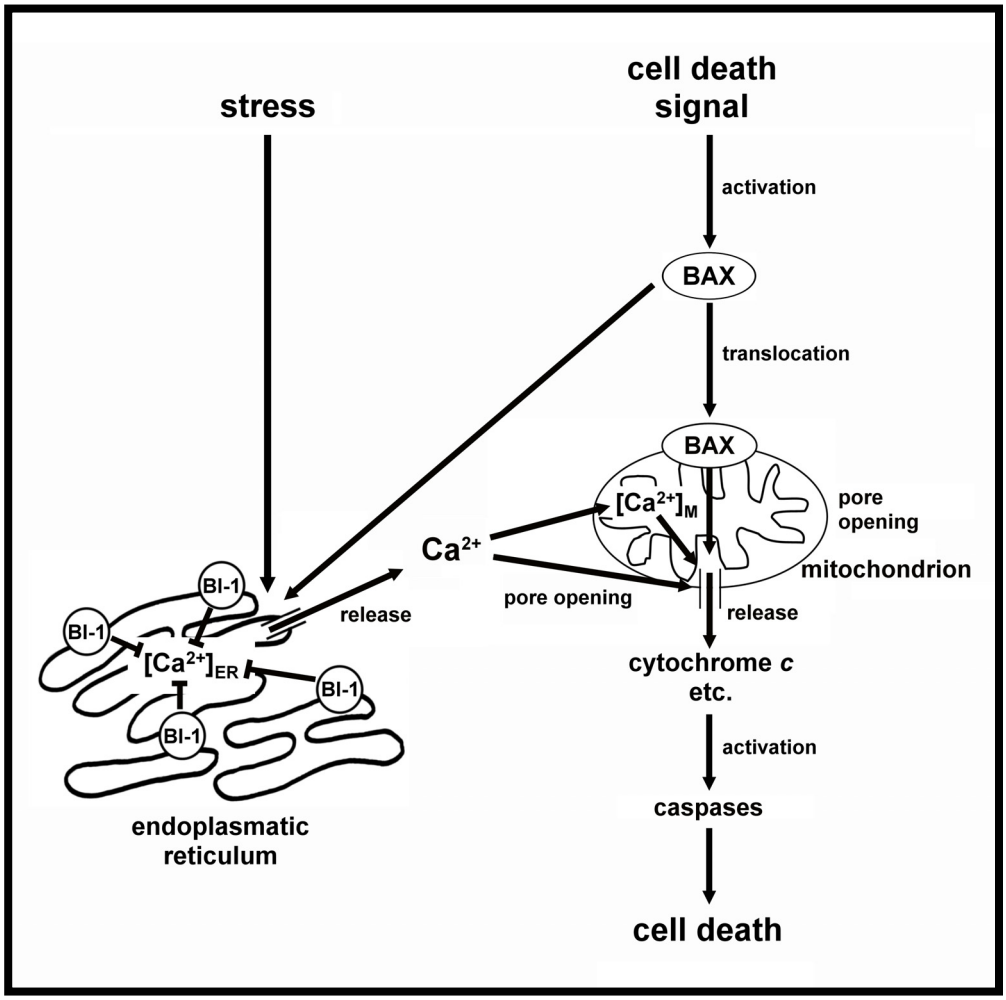


Figure 4.1: Schematic representation of the suggested function of BI-1 as suppressor of cell death processes in animal systems. Upon perception of specific cell death signals, the BAX protein becomes activated and translocates to the mitochondrial outer membrane. Here, it initiates the opening of so-called permeability transition pores, through which cytochrome *c* and other pro-apoptotic factors get released. In the cytosol, these pro-apoptotic factors activate a cascade of proteolytic caspases that eventually results in protein degradation and loss of cell integrity. Stress factors like heat, cold, pathogen attack, reactive oxygen intermediates etc. cause ER (endoplasmic reticulum) stress that is accompanied by calcium (Ca^{2+}) release from the organelle. Likewise, the BAX protein itself can trigger Ca^{2+} release from the ER. High levels of Ca^{2+} can also mediate pore opening and cytochrome *c* release from mitochondria. This occurs either as a direct effect of an elevated cytosolic Ca^{2+} level or as a result of Ca^{2+} uptake, which increases the concentration of Ca^{2+} within the mitochondrion ($[Ca^{2+}]_M$). It has been suggested that BI-1 can limit the amount of Ca^{2+}, which can be released from the ER, thus preventing Ca^{2+}-dependent execution of mitochondrial cell death.

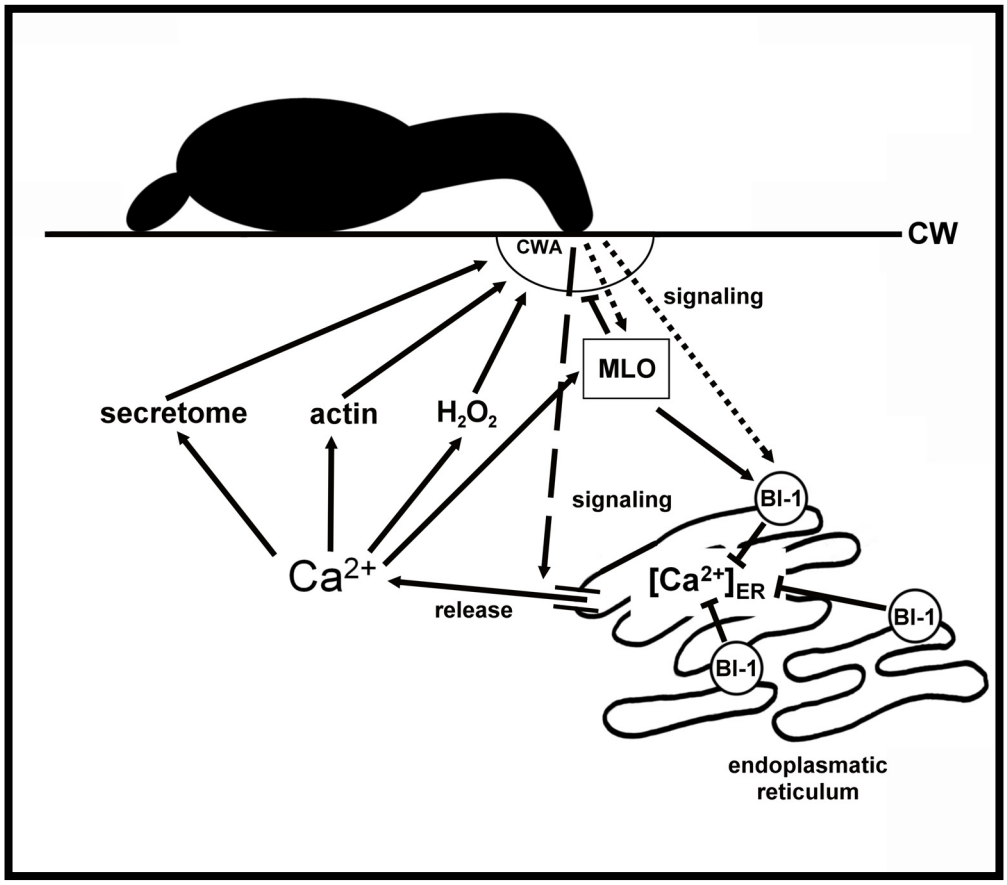


Figure 4.2: Schematic representation of the suggested function of BI-1 in suppression of penetration resistance during the interaction of barley with the powdery mildew fungus. During its attempt to penetrate the cell wall (CW), the fungus unspecifically activates a signaling cascade that, inter alia through the initiation of Ca^{2+} release from the endoplasmic reticulum (ER), elevates the cytoplasmic calcium (Ca^{2+}) level. Cytoplasmic calcium itself is involved in the activation of H_2O_2 production, actin reorganization and the secretion and vesicular transport of defense compounds, which all represent factors that contribute to the formation of cell wall apposition (CWA), also referred to as papilla. Overexpression of *BI-1* in barley epidermal cells is suggested to limit the amount of releasable Ca^{2+} within the ER ($[Ca^{2+}]_{ER}$) and to thus interfere with the processes leading to papilla formation. The fungal targeting or Ca^{2+}-dependent indirect activation of the MLO protein is known to negatively regulate penetration resistance. Similarly, the model proposes that in plants, BI-1 expression and function is regulated either directly, through transcriptional activation in response to fungal attack, or indirectly as a consequence of MLO activation, or both.

5 Summary / Zusammenfassung

Different *formae speciales* of the powdery mildew fungus *Blumeria graminis* undergo basic-compatible or basic-incompatible (nonhost) interactions with barley. In the present work, a macroarray based approach was followed to comparatively analyze the expression of 1,536 barley gene transcripts in the early host interaction with *Blumeria graminis* f.sp. *hordei* (*Bgh*) and the nonhost pathogen *Blumeria graminis f.sp. tritici* (*Bgt*), respectively. The cDNA fragments on the macroarray mainly derived from epidermal peels of plants pre-treated with the chemical resistance activating compound acibenzolar-*S*-methyl, and were therefore expected to be enriched with defense-related transcripts. 102 spots corresponding to 94 genes repeatedly gave *B. graminis*-responsive signals on the macroarray at 12 and/or 24 hours after inoculation. In independent expression analyses, the differential expression of 18 arbitrarily selected genes could be confirmed. The temporal expression profile of the majority of the genes was similar in the compatible and the incompatible interaction. The data support the view that background resistance in compatible interactions and nonhost resistance require common genetic and mechanistic elements of plant defense.

BAX INHIBITOR-1 (BI-1) proteins are negative regulators of programmed cell death in mammals and plants. When overexpressed in epidermal cells of barley, BI-1 suppresses non-specific background resistance and *mlo*-mediated penetration resistance to the biotrophic fungal pathogen *Bgh*. It could be demonstrated that overexpression of *BI-1* partially protects barley cells from cell death and breaks nonhost resistance of barley epidermal cells to the nonhost pathogen *Bgt*. The degree of transgene-induced accessibility was thereby similar to the effect achieved by overexpression of the defense suppressor gene *MLO* and could not be further enhanced by simultaneous expression of both *BI-1* and *MLO*. Furthermore, results indicate that during defense suppression, BI-1 modulates defense-associated hydrogen peroxide accumulation underneath the site of attempted fungal penetration. In barley epidermal cells, a functional green fluorescing GFP-BI-1 fusion protein accumulated in endomembranes and the nuclear envelope and was found in the vicinity of the site of fungal attack and/or around intracellular fungal structures. Together, enhanced expression of barley *BI-1* suppresses nonhost resistance to Bgt, linking barley nonhost penetration resistance with cell death regulation.

In Abhängigkeit von der *forma specialis* des angreifenden Mehltaupilzes ist die Interaktion von Gerste mit dem potenziellen Pathogen entweder basiskompatibel oder basis-inkompatibel. Für eine vergleichende Expressionsanalyse von 1.536 Gerstengenen in der frühen Wirtinteraktion mit *Blumeria graminis* f.sp. *hordei* (*Bgh*) und der Nichtwirtinteraktion mit *Blumeria graminis f.sp. tritici* (*Bgt*) wurde in dieser Arbeit ein auf der Macroarraytechnik basierender Versuchsansatz verfolgt. Die auf die Arraymembranen aufgebrachten cDNA Fragmente stammten hauptsächlich aus Epidermisgewebe von Pflanzen, die mit dem Resistenzinduktor Acibenzolar-*S*-methyl vorbehandelt worden waren und daher mit abwehrrelevanten Gentranskripten angereichert sein sollten. 102 cDNA Fragmente von 94 Genen zeigten zu den Zeitpunkten 12 und/oder 24 Stunden nach Inokulation Responsivität auf Infektion mit *B. graminis*. In unhabhängigen Expressionsstudien konnte die differentielle Expression von 18 zufällig ausgewählten Genen bestätigt werden. Die Mehrheit der Gene zeigte sowohl in der kompatiblen als auch in der inkompatiblen Interaktion ein ähnliches Expressionsmuster. Die Ergebnisse unterstützen die Annahme, dass sowohl die Hintergrundresistenz in der kompatiblen Interaktion als auch die Nichtwirtresistenz von ähnlichen genetischen Elementen bestimmt werden.

BAX INHIBITOR-1 (BI-1) Proteine sind als negative Regulatoren des Programmierten Zelltods in Tieren und Pflanzen bekannt. Die Überexpression des *BI-1* Gens beeinträchtigt sowohl die unspezifische Hintergrundresistenz als auch die *mlo*-vermittelte Penetrationsresistenz von Gerste gegenüber *Bgh*. Neben der zellschützenden Wirkung von Gersten BI-1 konnte in dieser Arbeit gezeigt werden, dass durch die Überexpression des *BI-1* Gens sogar die Nichtwirtresistenz von Gerstenepidermiszellen gegenüber *Bgt* gebrochen werden kann. Durch Einschleusen des Transgens wurde dabei ein ähnliches Suszeptibilitätsniveau erreicht, wie durch die Überexpression des Abwehrsuppressorgens *MLO*, wobei die gleichzeitige Überexpression der beiden Gene keinen weiteren Resistenz-supprimierenden Einfluss hatte. Weitere Ergebnisse zeigten, dass BI-1 im Zuge der Abwehrsuppression die lokale Akkumulation von Wasserstoffperoxid am Ort der pilzlichen Attacke zu beeinflussen scheint. In Epidermiszellen von Gerste akkumuliert ein funktionelles grün fluoreszierendes GFP-BI-1 Fusionsprotein in Endomembranen und in der Hülle des Zellkerns. Man findet es ebenfalls am Ort der pilzlichen Attacke und/oder an intrazellulären pilzlichen Strukturen. Die erhöhte Expression des Zelltodsuppressorgens *BI-1* beeinträchtigt also die Nichtwirtresistenz von Gerste gegenüber *Bgt* und verknüpft damit die Regulation von Nichtwirtinteraktion und Programmiertem Zelltod.

6 References

ABAD, L.R., D'URZO, M.P., LIU, D., NARASIMHAN, M.L., REUVENI, M., ZHU, J.K., NIU, X.M., SINGH, N.K., HASEGAWA, P.M. AND BRESSAN, R.A. (1996) Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Sci. 118: 11-23.

ABRAMOVITCH, R.B., KIM, Y.J., CHEN, S., DICKMAN, M.B. AND MARTIN, G.B. (2003) *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. EMBO J. 22: 60-69.

AIST, J.R. (1976) Papillae and related wound plugs of plant cells. Annu. Rev. Phytopathol. 14: 145-163.

AIST, J.R. AND BUSHNELL, W.R. (1991) Invasion of plant hosts by powdery mildew fungi and cellular mechanism of resistance. In: The Fungal Spore and Disease Initiation in Plants and Animals (Cole, G.T. and Hoch, H.C. Eds.), Plenum Press, New York, pp. 321-345.

ALTPETER, F., VARSHNEY, A., ABDERHALDEN, O., DOUCHKOV, D., SAUTTER, C., KUMLEHN, J., DUDLER, R. AND SCHWEIZER, P. (2005) Stable expression of a defense-related gene in wheat epidermis under transcriptional control of a novel promoter confers pathogen resistance. Plant Mol. Biol. 57: 271-283.

ALVAREZ, M.E., PENNELL, R.I., MEIJER, P.J., ISHIKAWA, A., DIXON, R.A. AND LAMB, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92: 773-784.

ALVIM, F.C., CAROLINO, S.M., CASCARDO, J.C., NUNES, C.C., MARTINEZ, C.A., OTONI, W.C. AND FONTES, E.P. (2001) Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. Plant Physiol. 126: 1042-1054.

ANDERSON, C.M., WAGNER, T.A., PERRET, M., HE, Z.H., HE, D. AND KOHORN, B.D. (2001) WAKs: cell wall-associated kinases linking the cytoplasm to the extracellular matrix. Plant Mol. Biol. 47: 197-206.

ASSAAD, F.F., QIU, J.L., YOUNGS, H., EHRHARDT, D., ZIMMERLI, L., KALDE, M., WANNER, G., PECK, S.C., EDWARDS, H., RAMONELL, K., SOMERVILLE, C.R. AND THORDAL-CHRISTENSEN, H. (2004) The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Mol. Biol. Cell 15: 5118-5129.

AZEVEDO, C., SADANANDOM, A., KITAGAWA, K., FREIALDENHOVEN, A., SHIRASU, K. AND SCHULZE-LEFERT, P. (2002) The RAR1 interactor SGT1, an essential component of *R* gene-triggered disease resistance. Science 295: 2073-2076.

BAEK, D., NAM, J., KOO, Y.D., KIM, D.H., LEE, J., JEONG, J.C., KWAK, S.S., CHUNG, W.S., LIM, C.O., BAHK, J.D., HONG, J.C., LEE, S.Y., KAWAI-YAMADA, M., UCHIMIYA, H. AND YUN, D.J. (2004) Bax-induced cell death of *Arabidopsis* is meditated through reactive oxygen-dependent and -independent processes. Plant Mol. Biol. 56: 15-27.

BALAGUÉ, C., LIN, B., ALCON, C., FLOTTES, G., MALMSTROM, S., KOHLER, C., NEUHAUS, G., PELLETIER, G., GAYMARD, F. AND ROBY, D. (2003) HLM1, an essential signaling component in the hypersensitive

response, is a member of the cyclic nucleotide-gated channel ion channel family. Plant Cell 15: 365-379.

BALK, J., LEAVER, C.J. AND MCCABE, P.F. (1999) Translocation of cytochrome *c* from the mitochondria to the cytosol occurs during heat-induced programmed cell death in cucumber plants. FEBS Lett. 463: 151-154.

BARIOLA, P.A., RETELSKA, D., STASIAK, A., KAMMERER, R.A., FLEMING, A., HIJRI, M., FRANK, S. AND FARMER, E.E. (2004) Remorins form a novel family of coiled coil-forming oligomeric and filamentous proteins associated with apical, vascular and embryonic tissues in plants. Plant Mol. Biol. 55: 579-594.

BEDINI, E., DE CASTRO, C., ERBS, G., MANGONI, L., DOW, J.M., NEWMAN, M.A., PARRILLI, M. AND UNVERZAGT, C. (2005) Structure-dependent modulation of a pathogen response in plants by synthetic O-antigen polysaccharides. J. Am. Chem. Soc. 127: 2414-2416.

BELLINCAMPI, D., DIPIERRO, N., SALVI, G., CERVONE, F. AND DE LORENZO, G. (2000) Extracellular H_2O_2 induced by oligogalacturonides is not involved in the inhibition of the auxin-regulated *rolB* gene expression in tobacco leaf explants. Plant Physiol. 122: 1379-1385.

BESSER, K., JAROSCH, B., LANGEN, G. AND KOGEL, K-H. (2000) Expression analysis of genes induced in barley after chemical activation reveals distinct disease resistance pathways. Mol. Plant Pathol. 1: 277-286.

BHAT, R.A., MIKLIS, M., SCHMELZER, E., SCHULZE-LEFERT, P. AND PANSTRUGA, R. (2005) Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. Proc. Natl. Acad. Sci. U.S.A. 102: 3135-3140.

BIERI, S., MAUCH, S., SHEN, Q.H., PEART, J., DEVOTO, A., CASAIS, C., CERON, F., SCHULZE, S., STEINBIß, H.H., SHIRASU, K. AND SCHULZE-LEFERT, P. (2004) RAR1 positively controls steady state levels of barley MLA resistance proteins and enables sufficient MLA6 accumulation for effective resistance. Plant Cell 16: 3480-3495.

BLUME, B., NÜRNBERGER, T., NASS, N. AND SCHEEL, D. (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell 12: 425-440.

BOEVINK, P., OPARKA, K., SANTA CRUZ, S., MARTIN, B., BETTERIDGE, A. AND HAWES, C. (1998) Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. Plant J. 15: 441-447.

BOGDANOVE, A.J. (2002) Protein-protein interactions in pathogen recognition by plants. Plant Mol. Biol. 50: 981-989.

BOHLMANN, H. AND APEL, K. (1987) Isolation and characterization of cDNAs coding for leaf specific thionins closely related to the endosperm-specific hordothionin of barley (*Hordeum vulgare* L.). Mol. Gen. Genet. 207: 446-454.

BOLDUC, N., OUELLET, M., PITRE, F. AND BRISSON, L.F. (2003) Molecular characterization of two plant BI-1 homologues which suppress Bax-induced apoptosis in human 293 cells. Planta 216: 377-386.

BOSTON, R.S., VIITANEN, P.V. AND VIERLING, E. (1996) Molecular chaperones and protein folding in plants. Plant Mol. Biol. 32: 191-222.

BRANDIZZI, F., IRONS, S.L., JOHANSEN, J., KOTZER, A. AND NEUMANN, U. (2004) GFP is the way to glow: bioimaging of the plant endomembrane system. J. Microsc. 214: 138-158.

BRAUN, U., COOK, R.T.A., IMMAN, A.J. AND SHIN, H.D. (2002) The taxonomy of the powdery mildew fungi. In: The Powdery Mildews – A Comprehensive Treatise (Bélanger, R.R., Bushnell, W.R., Dik, A.J. and Carver, T.L.W. Eds.), St. Paul, Minnesota, pp. 13-55.

BRECKENRIDGE, D.G. AND XUE, D (2004) Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases. Curr. Opin.Cell Biol. 16: 647-652.

BRECKENRIDGE, D.G., GERMAIN, M., MATHAI, J.P., NGUYEN, M. AND SHORE, G.C. (2003) Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 22: 8608-8618.

BRIGGS, S.P. AND JOHAL, G.S. (1994) Genetic patterns of plant host-parasite interactions. Trends Genet. 10: 12-16.

BROEKAERT, W.F., TERRAS, F.R.G., CAMMUNE, B.P.A. AND OSBORN, R.W. (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol. 108: 1353-1358.

BRYNGELSSON, T. AND GREEN, B. (1989) Characterization of a pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. Physiol. Mol. Plant Pathol. 35: 45-52

BRYNGELSSON, T., SOMMER-KNUDSEN, J., GREGERSEN, P.L., COLLINGE, D.B., EK, B. AND THORDAL-CHRISTENSEN, H. (1994) Purification, characterization, and molecular cloning of basic PR-1-type pathogenesis-related proteins from barley. Mol. Plant-Microbe Interact. 7: 267-275.

BURHENNE, K. AND GREGERSEN, P.L. (2000) Up-regulation of the ascorbate-dependent antioxidative system in barley leaves during powdery mildew infection. Mol. Plant Pathol. 1: 303-314.

BÜSCHGES, R., HOLLRICHER, K., PANSTRUGA, R., SIMONS, G., WOLTER, M., FRIJTERS, A., VAN DAELEN, R., VAN DER LEE, T., DIERGAARDE, P., GROENENDIJK, J., TÖPSCH, S., VOS, P., SALAMINI, F. AND SCHULZE-LEFERT, P. (1997) The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell 88: 695-705.

BUSH, D.S. (1995) Calcium regulation in plant cells and its role in signaling. Ann. Rev. Plant Physiol. Plant Mol. Biol. 46: 95-122.

BUSHNELL, W.A. AND BERQUIST, S.E. (1975) Aggregation of host cytoplasm and the formation of papillae and haustoria in powdery mildew of barley. Phytopathology 65: 310-318.

CALDO, R.A., NETTLETON, D. AND WISE, R.P. (2004) Interaction-dependent gene expression in *Mla*-specified response to barley powdery mildew. Plant Cell 16: 2514-2528.

CAO, H., LI, X. AND DONG, X. (1998) Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl. Acad. Sci. U.S.A. 95: 6531-6536.

CASSAB, G.I. (1998) Plant cell wall proteins. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 281-309.

CHAE, H.J., KE, N., KIM, H.R., CHEN, S., GODZIK, A., DICKMAN, M. AND REED, J.C. (2003) Evolutionarily conserved cytoprotection provided by *Bax Inhibitor-1* homologs from animals, plants, and yeast. Gene 323: 101-13.

CHAE, H.J., KIM, H.R., XU, C., BAILLY-MAITRE, B., KRAJEWSKA, M., KRAJEWSKI, S., BANARES, S., CUI, J., DIGICAYLIOGLU, M., KE, N., KITADA, S., MONOSOV, E., THOMAS, M., KRESS, C.L., BABENDURE, J.R.,

TSIEN, R.Y., LIPTON, S.A. AND REED, J.C. (2004) BI-1 regulates an apoptosis pathway linked to endoplasmic reticulum stress. Mol. Cell 15: 355-366.

CHEN, F. AND HAYES, P.M. (1994) Nucleotide sequence and developmental expression of duplicated genes encoding protein disulfide isomerase in barley (*Hordeum vulgare* L.). Plant Physiol. 106: 1705-1706.

CHEN, F., HAYES, P.M., MULROONEY, D.M. AND PAN, A. (1994) Identification and characterization of cDNA clones encoding plant calreticulin in barley. Plant Cell 6: 835-843.

CHEN, S., VAGHCHHIPAWALA, Z., LI, W., ASARD, H. AND DICKMAN, M.B. (2004) Tomato phospholipid hydroperoxide glutathione peroxidase inhibits cell death induced by Bax and oxidative stresses in yeast and plants. Plant Physiol. 135: 1630-1641.

CHICHKOVA, N.V., KIM, S.H., TITOVA, E.S., KALKUM, M., MOROZOV, V.S., RUBTSOV, Y.P., KALININA, N.O., TALIANSKY, M.E. AND VARTAPETIAN, A.B. (2004) A plant caspase-like protease activated during the hypersensitive response. Plant Cell 16: 157-171.

CLOSE, T.J., WANAMAKER, S.I., CALDO, R.A., TURNER, S.M., ASHLOCK, D.A., DICKERSON, J.A., WING, R.A., MUEHLBAUER, G.J., KLEINHOFS, A. AND WISE, R.P. (2004) A new resource for cereal genomics: 22K barley GeneChip comes of age. Plant Physiol. 134: 960-968.

COFFEEN, W.C. AND WOLPERT, T.J. (2004) Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in *Avena sativa*. Plant Cell 16: 857-873.

COLLINS, J.A., SCHANDI, C.A., YOUNG, K.K., VESELY, J. AND WILLINGHAM, M.C. (1997) Major DNA fragmentation is a late event in apoptosis. J. Histochem. Cytochem. 45: 923-934.

COLLINS, N.C., THORDAL-CHRISTENSEN, H., LIPKA, V., BAU, S., KOMBRINK, E., QIU, J.-L., HÜCKELHOVEN, R., STEIN, M., FREIALDENHOVEN, A., SOMERVILLE, S.C. AND SCHULZE-LEFERT, P. (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425: 973-977.

CONDIT, C.M. (1993) Developmental expression and localization of petunia glycine-rich protein 1. Plant Cell 5: 277-288.

CONTENTO, A.L., XIONG, Y. AND BASSHAM, D.C. (2005) Visualization of autophagy in *Arabidopsis* using the fluorescent dye monodansylcadaverine and a GFP-AtATG8e fusion protein. Plant J. 42: 598-608.

CORPAS, F.J., BARROSO, J.B. AND DEL RIO, L.A. (2001) Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. Trends Plant Sci. 6: 145-150.

COUPE, S.A., WATSON, L.M., RYAN, D.J., PINKNEY, T.T. AND EASON, J.R. (2004) Molecular analysis of programmed cell death during senescence in *Arabidopsis thaliana* and *Brassica oleracea*: cloning broccoli *LSD1*, *Bax inhibitor* and *serine palmitoyltransferase* homologues. J. Exp. Bot. 55: 59-68.

CZYMMEK, K.J., BOURETT, T.M., SWEIGARD, J.A., CARROLL, A. AND HOWARD, R. J. (2002) Utility of cytoplasmic fluorescent proteins for live cell imaging of *Magnaporthe grisea* in planta. Mycologia 94: 280-289.

DANGL, J.L. AND JONES, J.D.G. (2001) Plant pathogens and integrated defense responses to infection. Nature 411: 826-833.

DANGL, J.L., DIETRICH, R.A. AND RICHBERG, M.H. (1996) Death don't have no mercy: Cell death programs in plant-microbe interactions. Plant Cell 8: 1793-1807.

DAVIDSON, A.D., MANNERS, J.M., SIMPSON, R.S. AND SCOTT, K.J. (1987) cDNA cloning of mRNAs induced in resistant barley during infection by *Erysiphe graminis* f.sp. *hordei*. Plant Mol. Biol. 8: 77-85.

DENECKE, J., CARLSSON, L.E., VIDAL, S., HOGLUND, A.S., EK, B., VAN ZEIJL, M.J., SINJORGO, K.M. AND PALVA, E.T. (1995) The tobacco homolog of mammalian calreticulin is present in protein complexes *in vivo*. Plant Cell 7: 391-406.

DEVOTO, A., PIFFANELLI, P., NILSSON, I., WALLIN, E., PANSTRUGA, R., VON HEIJNE, G. AND SCHULZE-LEFERT, P. (1999) Topology, subcellular localization, and sequence diversity of the *Mlo* family in plants. J. Biol. Chem. 274: 34993-35004.

DEVOTO, A., HARTMANN, H. A., PIFFANELLI, P., ELLIOTT, C., SIMMONS, C., TARAMINO, G., GOH, C. S., COHEN, F. E., EMERSON, B. C., SCHULZE-LEFERT, P. AND PANSTRUGA, R. (2003) Molecular phylogeny and evolution of the plant-specific seven-transmembrane MLO family. J. Mol. Evol. 56: 77-88.

DICKMAN, M.B., PARK, Y.K., OLTERSDORF, T., LI, W., CLEMENTE, T. AND FRENCH, R. (2001) Abrogation of disease development in plants expressing animal antiapoptotic genes. Proc. Natl. Acad. Sci. U.S.A. 98: 6957-6962.

DIETRICH, C. AND MAISS, E. (2002) Red fluorescent protein DsRed from *Discosoma* sp. as a reporter protein in higher plants. Biotechniques 32: 286, 288-290, 292-293.

DIÉVART, A. AND CLARK, S.E. (2004) LRR-containing receptors regulating plant development and defense. Development 131: 251-261.

DO, H.M., HONG, J.K., JUNG, H.W., KIM, S.H., HAM, J.H. AND HWANG, B.K. (2003) Expression of peroxidase-like genes, H_2O_2 production, and peroxidase activity during the hypersensitive response to *Xanthomonas campestris* pv. *vesicatoria* in *Capsicum annuum*. Mol. Plant-Microbe Interact. 16: 196-205.

DUDLER, R., HERTIG, C., REBMANN, G., BULL, J. AND MAUCH, F. (1991) A pathogen-induced wheat gene encodes a protein homologous to glutathione-S-transferases. Mol. Plant-Microbe Interact. 4: 14-18.

DUNCAN, M.J., LI, G., SHIN, J.S., CARSON, J.L. AND ABRAHAM, S.N. (2004) Bacterial penetration of bladder epithelium through lipid rafts. J. Biol. Chem. 279: 18944-18951.

ECKEY, C., KORELL, M., LEIB, K., BIEDENKOPF, D., JANSEN, C., LANGEN, G. AND KOGEL, K.-H. (2004) Identification of powdery mildew-induced barley genes by cDNA-AFLP: functional assessment of an early expressed MAP kinase. Plant Mol. Biol. 55: 1-15.

ELBAZ, M., AVNI, A. AND WEIL, M. (2002) Constitutive caspase-like machinery executes programmed cell death in plant cells. Cell Death Differ. 9: 726-733.

ELLINGBOE, A.H. (1972) Genetics and physiology of primary infection by *Erysiphe graminis*. Phytopathology 62: 401-406.

ELLIOTT, C., ZHOU, F., SPIELMEYER, W., PANSTRUGA, R. AND SCHULZE-LEFERT, P. (2002) Functional conservation of wheat and rice *Mlo* orthologs in defense modulation to the powdery mildew fungus. Mol. Plant-Microbe Interact. 15: 1069-1077.

ELLIOTT, C., MÜLLER, J., MIKLIS, M., BHAT, R.A., SCHULZE-LEFERT, P. AND PANSTRUGA R. (2005) Conserved extracellular cysteine residues and cytoplasmic loop-loop interplay are required for functionality of the heptahelical MLO protein. Biochem. J. 385: 243-254.

ELLIS, J. AND JONES, D. (1998) Structure and function of proteins controlling strain-specific pathogen resistance in plants. Curr. Opin. Plant Biol. 1: 288-293.

ELROD-ERICKSON, M.J. AND KAISER, C.A. (1996) Genes that control the fidelity of endoplasmic reticulum to Golgi transport identified as suppressors of vesicle budding mutations. Mol. Biol. Cell 7: 1043-1058.

FELIX, G., DURAN, J.D., VOLKO, S. AND BOLLER, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. 18: 265-276.

FIRE, A., XU, S., MONTGOMERY, M.K., KOSTAS, S.A., DRIVER, S.E. AND MELLO, C.C. (1998) Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. Nature 391: 806-811.

FLOR, H.H. (1955) Host-parasite interaction in flax rust - ist genetic and other implications. Phytopathology 45: 680-685.

FLOR, H.H. (1971) Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9: 275-296.

FLOREA, A.M., YAMOAH, E.N. AND DOPP, E. (2005) Intracellular calcium disturbances induced by arsenic and its methylated derivatives in relation to genomic damage and apoptosis induction. Environ. Health Perspect. 113: 659-664.

FREIALDENHOVEN, A., SCHERAG, B., HOLLRICHER, K., COLLINGE, D.B., THORDAL-CHRISTENSEN, H. AND SCHULZE-LEFERT, P. (1994) *Nar-1* and *Nar-2*, two loci required for *Mla12*-specified race-specific resistance to powdery mildew in barley. Plant Cell 6: 983-994.

FREIALDENHOVEN, A., PETERHÄNSEL, C., KURTH, J., KREUZALER, F. AND SCHULZE-LEFERT, P. (1996) Identification of genes required for the function of non-race-specific *mlo* resistance to powdery mildew in barley. Plant Cell 8: 5-14.

FRYE, C.A., TANG, D. AND INNES, R.W. (2001) Negative regulation of defense responses in plants by a conserved MAPKK kinase. Proc. Natl. Acad. Sci. U.S.A. 98: 373-378.

GALLOIS, P., MAKISHIMA, T., HECHT, V., DESPRES, B., LAUDIÉ, M., NISHIMOTO, T. AND COOKE, R. (1997) An *Arabidopsis thaliana* cDNA complementing a hamster apoptosis suppressor mutant. Plant J. 11: 1325-1331.

GARCIA, A., CAYLA, X., FLEISCHER, A., GUERGNON, J., CANAS, F.A.F., REBOLLO, M.P., RONCAL, F. AND REBOLLO, A. (2003) Rafts: a simple way to control apoptosis by subcellular redistribution. Biochimie 85: 727-731

GAUSING, K. (1994) Lipid transfer protein genes specifically expressed in barley leaves and coleoptiles. Planta 192: 574-580.

GJETTING, T., CARVER, T.L,. SKØT, L. AND LYNGKJÆR, M.F. (2004) Differential gene expression in individual papilla-resistant and powdery mildew-infected barley epidermal cells. Mol. Plant-Microbe Interact. 17: 729-738.

GOLLDACK, D., VERA, P. AND DIETZ, K.J. (2003) Expression of *subtilisin-like serine proteases* in *Arabidopsis thaliana* is cell-specific and responds to jasmonic acid and heavy metals with developmental differences. Physiol. Plant. 118: 64-73.

GOLOUBINOFF, P., MOGK, A., BEN-ZVI, A.P., TOMOYASU, T. AND BUKAU, B. (1999) Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc. Natl. Acad. Sci. U.S.A. 96: 13732-13737.

GOULD, A. (2004) Plant pathogenic fungi. In: Plant Pathology – Concepts and laboratory exercise (Trigiano, R.N., Windham, M.T. and Windham, A.S. Eds.), CRC Press, Boca Raton, USA, pp. 75-89.

GOVRIN, E.M. AND LEVINE, A. (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr. Biol. 10: 751-757.

GRABSKI, S., ARNOYS, E., BUSCH, B. AND SCHINDLER, M. (1998) Regulation of actin tension in plant cells by kinases and phosphatases. Plant Physiol. 116: 279-290.

GRANT, M., BROWN, I., ADAMS, S., KNIGHT, M., AINSLIE, A. AND MANSFIELD, J. (2000) The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J. 23: 441-450.

GRANT, M.R., GODIARD, L., STRABUE, E., ASHFIELD, T., LEWALD, J., SATTLER, A., INNES, R.W. AND DANGL, J.L. (1995) Structure of the Arabidopsis *RPM1* gene enabling dual specificity disease resistance. Science 269: 843-846.

GREEN, D.R. AND KROEMER, G. (2004) The pathophysiology of mitochondrial cell death. Science 305: 626-629.

GREEN, D.R. AND REED, J.C. (1998) Mitochondria and apoptosis. Science 281: 1309-1312.

GREEN, J.R., CARVER, T.L.W. AND GURR, S.J. (2002) The formation and function of infection structures. In: The Powdery Mildews – A Comprehensive Treatise (Bélanger, R.R., Bushnell, W.R., Dik, A.J. and Carver, T.L.W. Eds.), St. Paul, Minnesota, pp. 66-82.

GREENBERG, J.T. AND YAO, N. (2004) The role and regulation of programmed cell death in plant-pathogen interactions. Cell. Microbiol. 6: 201-211.

GREGERSEN, P.L., THORDAL-CHRISTENSEN, H., FORSTER, H. AND COLLINGE, D.B. (1997) Differential gene transcript accumulation in barley leaf epidermis and mesophyll in response to attack by *Blumeria* (syn. *Erysiphe graminis* f.sp. *hordei*). Physiol. Mol. Plant Pathol. 51: 85-97.

GREY, W.M. (2002) Plant defence: a new weapon in the arsenal. Curr. Biol. 14: 352-354.

GROOM, Q.J., TORRES, M.A., FORDHAM-SKELTON, A.P., HAMMOND-KOSAK, K.E., ROBINSON, N.J. AND JONES, J.D.G. (1996) *RbohA*, a rice homologue of mammalian *gp91phox* respiratory burst oxidase gene. Plant J. 10: 515-522.

GUTTMAN, D.S., VINATZER, B.A., SARKAR, S.F., RANALL, M.V., KETTLER, G. AND GREENBERG, J.T. (2002) A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. Science 295: 1722-1726.

HAMMOND-KOSACK, K.E. AND JONES, J.D.G. (1996) Resistance gene-dependent plant defense responses. Plant Cell 8: 1773-1791.

HAMMOND-KOSACK, K.E. AND JONES, J.D.G. (1997) Plant disease resistance genes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 575-607.

HARA-NISHIMURA, I., HATSUGAI, N., NAKAUNE, S., KUROYANAGI, M. AND NISHIMURA, M. (2005) Vacuolar processing enzyme: an executor of plant cell death. Curr. Opin. Plant Biol. 8: 404-408.

HARLAN, J.R. AND ZOHARY, D. (1966) Distribution of wild wheats and barley. Science 153: 1074-1080.

HASELOFF, J., SIEMERING, K., PRASHER, D. AND HODGE, S. (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Sci. U.S.A. 94: 2122-2127.

HAUCK, P., THILMONY, R. AND HE, S.Y. (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. Proc. Natl. Acad. Sci. U.S.A. 100: 8577-8582.

HEATH MC (1981) A generalized concept of host-parasite specificity. Phytopathology 71: 1121-1123.

HEATH, M.C. (1991) The role of gene-for-gene interactions in the determination of host species specificity. Phytopathology 81: 127-130.

HEATH, M.C. (2000a) Hypersensitive response-related cell death. Plant Mol. Biol. 44: 321-334.

HEATH, M.C. (2000b) Nonhost resistance and nonspecific plant defenses. Curr. Opin. Plant Biol. 3: 315-319.

HEIN, I., CAMPBELL, E.I., WOODHEAD, M., HEDLEY, P.E., YOUNG, V., MORRIS, W.L., RAMSAY, L., STOCKHAUS, J., LYON, G.D., NEWTON, A.C. AND BIRCH, P.R. (2004) Characterisation of early transcriptional changes involving multiple signalling pathways in the *Mla13* barley interaction with powdery mildew (*Blumeria graminis* f.sp. *hordei*). Planta 218: 803-813.

HEO, W.D., LEE, S.H., KIM, M.C., KIM, J.C., CHUNG, W.S., CHUN, H.J., LEE, K.J., PARK, C.Y., PARK, H.C., CHOI, J.Y. AND CHO, M.J. (1999) Involvement of specific calmodulin isoforms in salicylic acid-independent activation of plant disease resistance responses. Proc. Natl. Acad. Sci. U.S.A. 96: 766-771.

HESS, W.R. AND BÖRNER, T. (1998) Cloning and characterization of expression of cytosolic *ascorbate peroxidase* cDNA from barley. Plant Physiol. 118: 329.

HOEBERICHTS, F.A. AND WOLTERING, E.J. (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. Bioessays 25: 47-57.

HOUSTON, N.L., FAN, C., XIANG, J.Q., SCHULZE, J.M., JUNG, R. AND BOSTON, R.S. (2005) Phylogenetic analyses identify 10 classes of the protein disulfide isomerase family in plants, including single-domain protein disulfide isomerase-related proteins. Plant Physiol. 137: 762-778.

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

HÜCKELHOVEN, R. (2005) Powdery mildew susceptibility and biotrophic infection strategies. FEMS Microbiol. Lett. 245: 9-17.

HÜCKELHOVEN, R. AND KOGEL, K.-H. (1998) Tissue-specific superoxide generation at interaction sites in resistant and susceptible nearisogenic barley lines attacked by the powdery mildew fungus (*Erysiphe graminis* f.sp. *hordei*). Mol. Plant-Microbe Interact. 11: 292-300.

HÜCKELHOVEN, R. AND KOGEL, K.-H. (2003) Reactive oxygen intermediates in plant-microbe interactions: Who is who in powdery mildew resistance? Planta 216: 891-902.

HÜCKELHOVEN, R., FODOR, J., PREIS, C. AND KOGEL, K.-H. (1999) Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. Plant Physiol. 119: 1251-1260.

HÜCKELHOVEN, R., FODOR, J., TRUJILLO, M. AND KOGEL, K.-H. (2000a) 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.

HÜCKELHOVEN, R., TRUJILLO, M. AND KOGEL, K.-H. (2000b) 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.

HÜCKELHOVEN R., DECHERT, C. AND KOGEL, K.-H. (2001a) 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.

HÜCKELHOVEN,R., DECHERT, C., TRUJILLO, M. AND KOGEL, K.-H. (2001b) Differential expression of putative cell death regulator genes in near-isogenic, resistant and susceptible barley lines during interaction with the powdery mildew fungus. Plant Mol. Biol. 47: 739-748.

HÜCKELHOVEN, R., DECHERT, C. AND KOGEL, K.-H. (2003) Overexpression of barley *BAX inhibitor 1* induces breakdown of *mlo*-mediated penetration resistance to *Blumeria graminis*. Proc. Natl. Acad. Sci. U.S.A. 100: 5555-5560.

INK, B., ZORNIG, M., BAUM, B., HAJIBAGHERI, N., JAMES, C., CHITTENDEN, T. AND EVAN, G. (1997) Human *Bak* induces cell death in *Schizosaccharomyces pombe* with morphological changes similar to those with apoptosis in mammalian cells. Mol. Cell. Biol. 17: 2468-2474.

JABS, T., DIETRICH, R.A. AND DANGL, J.L. (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. Science 273: 1853-1856.

JACOBS, A.K., LIPKA, V., BURTON, R.A., PANSTRUGA, R., STRIZHOV, N., SCHULZE-LEFERT, P. AND FINCHER, G.B. (2003) An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. Plant Cell 15: 2503-2513.

JANSEN, C., KORELL, M., ECKEY, C., BIEDENKOPF, D. AND KOGEL, K.-H. (2005): Identification and transcriptional analysis of powdery mildew-induced barley genes. Plant Sci. 168: 373-380.

JAROSCH, B., KOGEL, K.-H. AND SCHAFFRATH, U. (1999) The ambivalence of the barley *Mlo* locus: Mutations conferring resistance against powdery mildew (*Blumeria graminis* f.sp. *hordei*) enhance susceptibility to the rice blast fungus *Magnaporthe grisea*. Mol. Plant-Microbe Interact. 12: 508-514.

JELITTO-VAN DOOREN, E.P., VIDAL, S. AND DENECKE, J. (1999) Anticipating endoplasmic reticulum stress. A novel early response before pathogenesis-related gene induction. Plant Cell. 11: 1935-1944.

JONES, A. (2000) Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? Trends Plant Sci. 5: 225-230.

JONES, D.A. AND TAKEMOTO, D. (2004) Plant innate immunity - Direct and indirect recognition of general and specific pathogen-associated molecules. Curr. Opin. Immunol. 16: 48-62.

JONES, A.M., THOMAS, V., TRUMAN, B., LILLEY, K., MANSFIELD, J. AND GRANT, M. (2004) Specific changes in the *Arabidopsis* proteome in response to bacterial challenge: differentiating basal and *R*-gene mediated resistance. Phytochemistry 65: 1805-1816.

JORDA, L., COEGO, A., CONEJERO, V. AND VERA, P. (1999) A genomic cluster containing four differentially regulated subtilisin-like processing protease genes is in tomato plants. J. Biol. Chem. 274: 2360-2365.

JØRGENSEN, J.H. (1992) Discovery, characterization and exploitation of *Mlo* powdery mildew resistance in barley. Euphytica 63: 141-152.

JØRGENSEN, J.H. (1994) Genetics of powdery mildew resistance in barley. Crit. Rev. Plant Sci. 13: 97-119.

JÜRGENSMEIER, J.M., S. KRAJEWSKI, R. ARMSTRONG, G.M. WILSON, T. OLTERSDORF, L.C. FRITZ, J.C. REED AND OTTILIE, S. (1997) Bax- and Bak-induced cell death in the fission yeast *Schizosaccharomyces pombe*. Mol. Biol. Cell 8: 325-329.

KADER, J.C. (1996) Lipid-transfer proteins in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 627-654.

KAMAUCHI, S., NAKATANI, H., NAKANO, C. AND URADE, R. (2005) Gene expression in response to endoplasmic reticulum stress in *Arabidopsis thaliana*. FEBS J. 272: 3461-3476.

KAMPRANIS, S.C., DAMIANOVA, R., ATALLAH, M., TOBY, G., KONDI, G., TSICHLIS, P.N. AND MAKRIS, A.M. (2000) A novel plant glutathione S-transferase/peroxidase suppresses Bax lethality in yeast. J. Biol. Chem. 275: 29207-29216.

KAUFMAN, R.J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. 13: 1211-1233.

KAWAI, M., PAN, L., REED, J.C. AND UCHIMIYA, H. (1999) Evolutionally conserved plant homologue of the *Bax inhibitor-1* (*BI-1*) gene capable of suppressing Bax-induced cell death in yeast. FEBS Lett. 464: 143-147.

KAWAI-YAMADA, M., JIN, L., YOSHINAGA, K., HIRATA, A. AND UCHIMIYA, H. (2001) Mammalian Bax-induced plant cell death can be down-regulated by overexpression of *Arabidopsis Bax Inhibitor-1* (*AtBI-1*). Proc. Natl. Acad. Sci. U.S.A. 98: 12295-12300.

KAWAI-YAMADA, M., OHORI, Y. AND UCHIMIYA, H. (2004) Dissection of *Arabidopsis Bax inhibitor-1* suppressing Bax-, hydrogen peroxide-, and salicylic acid-induced cell death. Plant Cell 16: 21-32.

KETELAAR, T., VOSS, C., DIMMOCK, S.A., THUMM, M. AND HUSSEY, P.J. (2004) *Arabidopsis* homologues of the autophagy protein Atg8 are a novel family of microtubule binding proteins. FEBS Lett. 567: 302-306.

KIM, R. (2005) Unknotting the roles of Bcl-2 and Bcl-xL in cell death. Biochem. Biophys. Res. Commun. 333: 336-343.

KIM, M.C., LEE, S.H., KIM, J.K., CHUN, H.J., OK, H.M., MOON, B.C., KANG, C.H., CHUNG, W.S., PARK, C.Y., CHOI, M.S., KANG, Y.H., KOO, S.C., KOO, Y.C., JUNG, J.C., SCHULZE-LEFERT, P. AND CHO, M.J. (2002a) *Mlo*, a modulator of plant defense and cell death, is a novel calmodulin-binding protein: Isolation and characterization of a rice *Mlo* homologue. J. Biol. Chem. 277: 9304-9314.

KIM, M.C., PANSTRUGA, R., ELLIOTT, C., MÜLLER, J., DEVOTO, A., YOON, H.W., PARK, H., CHO, M.J. AND SCHULZE-LEFERT, P. (2002b) Calmodulin interacts with MLO to regulate defense against mildew in barley. Nature 416: 447-450.

KITA, N., TOYODA, H. AND SHISHIYAMA, J. (1981) Chronological analysis of cytological responses in powdery-mildewed barley leaves. Can. J. Bot. 59: 1761-1768.

KJEMTRUP, S., NIMCHUK, Z. AND DANGL, J.L. (2000) Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. Curr. Opin. Microbiol. 3: 73-78.

KOBAYASHI, Y., KOBAYASHI, I. AND KUNOH, H. (1993) Recognition of a pathogen and nonpathogen by barley coleoptile cells. II. Alteration of cytoplasmic strands in coleoptile cells caused by the pathogen, *Erysiphe graminis* and the nonpathogen, *E. pisi* prior to penetration. Physiol. Mol. Plant Pathol. 43: 243-254.

KOBAYASHI, Y., KOBAYASHI, I., FUNAKI, Y., FUJIMOTO, S., TAKEMOTO, T. AND KUNOH, H. (1997) Dynamic reorganization of microfilaments and microtubules is necessary for the expression of non-host resistance in barley coleoptile cells. Plant J. 11: 525-537.

KOGA, H., ZEYEN, R.J., BUSHNELL, W.R. AND AHLSTRAND, G.G. (1988) Hypersensitive cell death, autofluorescence, and insoluble silicon accumulation in barley leaf epidermal cells under attack by *Erysiphe graminis* f.sp. *hordei*. Physiol. Mol. Plant Pathol. 32: 395-409.

KOGA, H., BUSNELL, W.R. AND ZEYEN, R.J. (1990) Specificity of cell type and timing of events associated with papilla formation and the hypersensitive reaction in leaves of *Hordeum vulgare* attacked by *Erysiphe graminis* f.sp. *hordei*. Can. J. Bot. 68: 2344-2352.

KRAGH, K.M., JACOBSEN, M.S. AND MIKKELSEN, J.D. (1990) Induction, purification and characterization of barley leaf chitinase. Plant Sci. 71: 55-68.

KUĆ, J. (1995) Phytoalexins, stress metabolism and disease resistance in plants. Annu. Rev. Phytopathol. 33: 275-297.

KUMAR, J., HÜCKELHOVEN, R., BECKHOVE, U., NAGARAJAN, S. AND 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. Phytopathology 91: 127-133.

KUNOH, H., HAYASHIMOTO, A., HARUI, M. AND ISHIZAKI, H. (1985) Induced susceptibility and enhanced inaccessibility at the cellular level in barley coleoptiles. I. The significance of timing fungal invasion. Physiol. Plant Pathol. 27: 43-54.

KUNOH, H., YAMAMORI, K. AND ISHIZAKI, H. (1982) Cytological studies of the early stages of powdery mildew in barley and wheat. VIII. Autofluorescence at penetration sites of *Erysiphe graminis* f.sp. *hordei* on living barley coleoptiles. Physiol. Plant Pathol. 21: 373-379.

LACOMME, C. AND SANTA CRUZ, S. (1999) Bax-induced cell death in tobacco is similar to the hypersensitive response. Proc. Natl. Acad. Sci. U.S.A. 96: 7956-7961.

LAHAYE, T. AND BONAS, U. (2001) Molecular secrets of bacterial type III effector proteins. Trends Plant Sci. 6: 479-485.

LAM, E. AND DEL POZO, O. (2000) Caspase-like protease involvement in the control of plant cell death. Plant Mol. Biol. 44: 417-428.

LAM, E., KATO, N. AND LAWTON, M. (2001) Programmed cell death, mitochondria and the plant hypersensitive response. Nature 411: 848-853.

LAMB, C. AND DIXON, R.A. (1997) The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 251-275.

LANG, T., SCHAEFFELER, E., BERNREUTHER, D., BREDSCHNEIDER, M., WOLF, D.H. AND THUMM M. (1998) Aut2p and Aut7p, two novel microtubule-associated proteins are essential for delivery of autophagic vesicles to the vacuole. EMBO J. 17: 3597-3607.

LECKIE, C.P., CALLOW, J.A. AND GREEN, J.R. (1995) Re-organization of the endoplasmic reticulum in pea leaf epidermal cells infected by the powdery mildew fungus *Erysiphe pisi*. New Phytol. 131: 211-221.

LEI, M. AND WU, R. (1991) A novel glycine-rich cell wall protein gene in rice. Plant Mol. Biol. 16: 187-198.

LENG, Q., MERCIER, R.W., YAO, W. AND BERKOWITZ, G.A. (1999) Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. Plant Physiol. 121: 753-761.

LEVINE, A., BELENGHI, B., DAMARI-WEISLER, H. AND GRANOT, D. (2001) Vesicle associated membrane protein of *Arabidopsis* suppresses Bax-induced apoptosis in yeast downstream of oxidative burst. J. Biol. Chem. 276: 46284-46289.

LEVINE, A., PENNELL, R.I., ALVAREZ, M.E., PALMER, R. AND LAMB, C. (1996) Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr. Biol. 6: 427-437.

LIGR, M., MADEO, F., FRÖHLICH, E., HILT, W., FRÖHLICH, K.U. AND WOLF, D.H. (1998) Mammalian Bax triggers apoptotic changes in yeast. FEBS Lett. 438: 61-65.

LIU, Y., SCHIFF, M., CZYMMEK, K., TALLOCZY, Z., LEVINE, B. AND DINESH-KUMAR, S.P. (2005) Autophagy regulates programmed cell death during the plant innate immune response. Cell 121: 567-577.

LOEFFLER, M. AND KROEMER, G. (2000) The mitocondrion in cell death control: certainties and incognita. Exp. Cell Res. 256: 19-26.

LUO, M., DANG, P., BAUSHER, M. G., HOLBROOK, C. C., LEE, R. D., LYNCH, R. E. AND GUO, B. Z. (2005) Identification of transcripts involved in resistance responses to leaf spot disease caused by *Cercosporidium personatum* in peanut (*Arachis hypogaea*). Phytopathology 95: 381-387.

LYNGKJÆR, H.F. AND CARVER, T.L.W. (1999) Modification of *mlo5* resistance to *Blumeria graminis* attack in barley as a consequence of induced accessibility and inaccessibility. Physiol. Mol. Plant Pathol. 55: 163-174.

MADEO, F., FRÖHLICH ,E., LIGR, M., GREY, M., SIGRIST, S.J., WOLF, D.H. AND FRÖHLICH, K.U. (1999) Oxygen stress: a regulator of apoptosis in yeast. J. Cell. Biol. 145: 757-767.

MADEO, F., HERKER, E., WISSING, S., JUNGWIRTH, H., EISENBERG, T. AND FRÖHLICH, K.U. (2004) Apoptosis in yeast. Curr. Opin. Microbiol. 7: 655-660.

MARTIN, G.B., BROMMONSCHENKEL, S.H., CHUNWONGSE, J., FRARY, A., GANAL, M.W., SPIVEY, R., WU, T., EARLE, E.D. AND TANKSLEY, S.D. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262: 1432-1436.

MARTIN, S.W., GLOVER, B.J. AND DAVIES, J.M. (2005) Lipid microdomains - plant membranes get organized. Trends Plant Sci. 10: 263-265.

MATSUMURA, K. AND TOSA, Y. (1995) The rye mildew fungus carries avirulence genes corresponding to wheat genes for resistance to races of wheat mildew fungus. Phytopathology 85: 753-756.

MATSUMURA, H., NIRASAWA, S., KIBA, A., URASAKI, N., SAITOH, H., ITO, M., KAWAI-YAMADA, M., UCHIMIYA, H. AND TERAUCHI, R. (2003) Overexpression of *Bax inhibitor* suppresses the fungal elicitor-induced cell death in rice (*Oryza sativa* L) cells. Plant J. 33: 425-434.

MCDOUGALL, G.J., STEWART, D. AND MORRISON, I.M. (1996) Tyrosine residues enhance cross-linking of synthetic proteins into lignin-like dehydrogenation products. Phytochemistry 41: 43 47.

MCKEEN, W.E. AND RIMMER, S.R. (1973) Initial penetration process in powdery mildew infection of susceptible barley leaves. Phytopathology 63: 1049-1053.

MICHALAK, M., CORBETT, E.F., MESAELI, N., NAKAMURA, K. AND OPAS, M. (1999) Calreticulin: one protein, one gene, many functions. Biochem. J. 344: 281-292.

MITTLER, R., VANDERAUWERA, S., GOLLERY, M. AND VAN BREUSEGEM, F. (2004) Reactive oxygen gene network of plants. Trends Plant Sci. 9: 490-498.

MOLINA, A. AND GARCÍA-OLMEDO, F. (1993) Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. Plant J. 4: 983-991.

MOLINA, A., SEGURA, A., GARCÍA-OLMEDO, F. (1993) Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. FEBS Lett. 316: 119-122.

MOLINA, A., GÖRLACH, J., VOLRATH, S. AND RYALS, J. (1999) Wheat genes encoding two types of PR-1 proteins are pathogen inducible, but do not respond to activators of systemic acquired resistance. Mol. Plant-Microbe Interact. 12: 53-58.

MØLLER, I.M. (2001) Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52: 561-591.

MONGRAND, S., MOREL, J., LAROCHE, J., CLAVEROL, S., CARDE, J.P., HARTMANN, M.A., BONNEU, M., SIMON-PLAS, F., LESSIRE, R. AND BESSOULE, J.J. (2004) Lipid rafts in higher plant cells: purification and characterization of Triton X-100-insoluble microdomains from tobacco plasma membrane. J. Biol. Chem. 279: 36277-36286.

MOON, H., BAEK, D., LEE, B., PRASAD, D.T., LEE, S.Y., CHO, M.J., LIM, C.O., CHOI, M.S., BAHK, J., KIM, M.O., HONG, J.C. AND YUN, D.J. (2002) Soybean ascorbate peroxidase suppresses Bax-induced apoptosis in yeast by inhibiting oxygen radical generation. Biochem. Biophys. Res. Commun. 290: 457-462.

MYSORE, K.S., D'ASCENZO, M.D., HE, X. AND MARTIN, G.B. (2003) Overexpression of the disease resistance gene *Pto* in tomato induces gene expression changes similar to immune responses in human and fruitfly. Plant Physiol. 132: 1901-1912.

NEBENFÜHR, A., GALLAGHER, L.A., DUNAHAY, T.G., FROHLICK, J.A., MAZURKIEWICZ, A.M., MEEHL, J.B. AND STAEHELIN, L.A. (1999) Stop-and-go movements of plant Golgi stacks are mediated by the acto-myosin system. Plant Physiol. 121: 1127-1142.

NIDERMAN, T., GENETET, I., BRUYERE, T., GEES, R., STINTZI, A., LEGRAND, M., FRITIG, B. AND MOSINGER, E. (1995) Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. Plant Physiol. 108: 17-27.

NIELSEN, K., OLSEN, O. AND OLIVER, R. (1999) A transient expression system to assay putative antifungal genes on powdery mildew infected barley leaves. Physiol. Mol. Plant Pathol. 54: 1-12.

NIKS, R.E. (1988) Nonhost plant species as donor for resistance to pathogens with narrow host range. II. Concepts and evidence on the genetic basis of nonhost resistance. Euphytica 37: 89-99.

NIMCHUK, Z., ROHMER, L., CHANG, J.H. AND DANGL, J.L. (2001) Knowing the dancer from the dance: R-gene products and their interactions with other proteins from host and pathogen. Curr. Opin. Plant Biol. 4: 288-294.

NISHIMURA, M.T., STEIN, M., HOU, B.H., VOGEL, J.P., EDWARDS, H. AND SOMERVILLE, S.C. (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science 301: 969-972.

NOCTOR, G. AND FOYER, C.H. (1998) Ascorbate and glutathione: Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 249-279.

NÜRNBERGER, T. AND BRUNNER, F. (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Curr. Opin. Plant Biol. 5: 318-324.

NÜRNBERGER, T. AND LIPKA, V. (2005) Non-host resistance in plants: new insights into an old phenomenon. Mol. Plant Pathol. 6: 335-345.

NÜRNBERGER, T. AND SCHEEL, D. (2001) Signal transmission in the plant immune response. Trends Plant Sci. 6: 372-379.

NUTT, L.K., CHANDRA, J., PATAER, A., FANG, B., ROTH, J.A., SWISHER, S.G., O'NEIL, R.G. AND MCCONKEY, D.J. (2002a) Bax-mediated Ca^{2+} mobilization promotes cytochrome *c* release during apoptosis. J. Biol. Chem. 277: 20301-20308.

NUTT, L.K., PATAER, A., PAHLER, J., FANG, B., ROTH, J., MCCONKEY, D.J. AND SWISHER, S.G. (2002b) Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca^{2+} stores. J. Biol. Chem. 277: 9219-9225.

OAKES, S.A., OPFERMAN, J.T., POZZAN, T., KORSMEYER, S.J. AND SCORRANO, L. (2003) Regulation of endoplasmic reticulum Ca^{2+} dynamics by proapoptotic BCL-2 family members. Biochem. Pharmacol. 66: 1335-1340.

OERKE, E.C. AND DEHNE, H.W. (1997) Global crop production and the efficacy of crop protection: Current situation of future trends. Eur. J. Plant Pathol. 103: 203-215.

OGAWA, T., PAN, L., KAWAI-YAMADA, M., YU, L.H., YAMAMURA, S., KOYAMA, T., KITAJIMA, S., OHME-TAKAGI, M., SATO, F. AND UCHIMIYA, H. (2005) Functional analysis of *Arabidopsis* ethylene-responsive element binding protein conferring resistance to Bax and abiotic stress-induced plant cell death. Plant Physiol. 138: 1436-1445.

OHTAKE, Y., TAKAHASHI, T. AND KOMEDA, Y. (2000) Salicylic acid induces the expression of a number of receptor-like kinase genes in Arabidopsis thaliana. Plant Cell Physiol. 41: 1038-1044.

OLESEN, K.L., CARVER, T.L.W., LYNGKJÆR, M.F. (2003) Fungal suppression of resistance against inappropriate *Blumeria graminis* formae speciales in barley, oat and wheat. Physiol. Mol. Plant Pathol. 62: 37-50.

OLIVER, R.P. AND IPCHO, S.V.S. (2004) *Arabidopsis* pathology breathes new life into the necrotrophs vs. biotrophs classification of fungal pathogens. Mol. Plant Pathol. 5: 347-352.

ONO, E., WONG, H.L., KAWASAKI, T., HASEGAWA, M., KODAMA, O. AND SHIMAMOTO, K. (2001) Essential role of the small GTPase Rac in disease resistance of rice. Proc. Natl. Acad. Sci. U.S.A. 98: 759-764.

OPALSKI, K.S., SCHULTHEISS, H., KOGEL, K.-H. AND HÜCKELHOVEN, R. (2005): The receptorlike MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley

attacked by the biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei*. Plant J. 41: 291-303.

ORZÁEZ, D. AND GRANELL, A. (1997) The plant homologue of the defender against apoptotic death gene is down-regulated during senescence of flower petals. FEBS Lett. 404: 275-278.

PAN, L., KAWAI, M., YU, L.H., KIM, K.M., HIRATA, A., UMEDA, M. AND UCHIMIYA, H. (2001) The *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP) can function as a dominant suppressor of Bax-induced cell death of yeast. FEBS Lett. 508: 375-378.

PANSTRUGA, R. (2004) A golden shot: how ballistic single cell transformation boosts the molecular analysis of cereal-mildew interactions. Mol. Plant Pathol. 5: 141-148.

PANSTRUGA, R. (2005) Serpentine plant MLO proteins as entry portals for powdery mildew fungi. Biochem. Soc. Trans. 33: 389-392.

PANSTRUGA, R. AND SCHULZE-LEFERT, P. (2003) Corruption of host seven-transmembrane proteins by pathogenic microbes: a common theme in animals and plants? Microbes Infect. 5: 429-437.

PEART, J.R., LU, R., SADANANDOM, A., MALCUIT, I., MOFFETT, P., BRICE, D.C., SCHAUSER, L., JAGGARD, D.A.W., XIAO, S., COLEMAN, M.J., DOW, M., JONES, J.D.G., SHIRASU, K. AND BAULCOMBE, D.C. (2002) Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc. Natl. Acad. Sci. U.S.A. 99: 10865-10869.

PEDLEY, K.F. AND MARTIN, G.B (2003) Molecular basis of *Pto*-mediated resistance to bacterial speck disease in tomato. Annu. Rev. Phytopathol. 41: 215-243.

PEDRAS, M.S. AND AHIAHONU, P.W. (2005) Metabolism and detoxification of phytoalexins and analogs by phytopathogenic fungi. Phytochemistry 66 : 391-411.

PERSSON, S., WYATT, S.E., LOVE, J., THOMPSON, W.F., ROBERTSON, D. AND BOSS, W.F. (2001) The Ca^{2+} status of the Endoplasmic Reticulum is altered by induction of calreticulin expression in transgenic plants. Plant Physiol. 126: 1092-1104.

PERSSON, S., ROSENQUIST, M., SVENSSON, K., GALVAO, R., BOSS, W.F. AND SOMMARIN, M. (2003) Phylogenetic analyses and expression studies reveal two distinct groups of calreticulin isoforms in higher plants. Plant Physiol. 133: 1385-1396.

PETERHÄNSEL, C., FREIALDENHOVEN, A., KURTH, J., KOLSCH, R. AND SCHULZE-LEFERT, P. (1997) Interaction analyses of genes required for resistance responses to powdery mildew in barley reveal distinct pathways leading to leaf cell death. Plant Cell 9: 1397-1409.

PIEDRAS, P., RIVAS, S., DRÖGE, S., HILLMER, S. AND JONES, J.D.G. (2000) Functional, c–myc epitope tagged *Cf-9* resistance gene products are plasmamembrane localized and glycosylated. Plant J. 21: 529-536.

PIETERSE, C.M. AND VAN LOON, L.C. (2004) *NPR1*: the spider in the web of induced resistance signaling pathways. Curr. Opin. Plant Biol. 7: 456-464.

PIFFANELLI, P., ZHOU, F., CASAIS, C., ORME, J., JAROSCH, B., SCHAFFRATH, U., COLLINS, N.C., PANSTRUGA, R. AND SCHULZE-LEFERT, P. (2002) The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. Plant Physiol. 129: 1076-1085.

POTOKINA, E., SREENIVASULU, N., ALTSCHMIED, L., MICHALEK, W. AND GRANER, A. (2002) Differential gene expression during seed germination in barley (*Hordeum vulgare* L.). Funct. Integr. Genomics 2: 28-39.

PRICE, A.H., TAYLOR, S., RIPLEY, S.J., GRIFFITHS, A., TREWAVAS, A.J. AND KNIGHT, M.R. (1994) Oxidative signals in tobacco increase cytosolic calcium. Plant Cell 6: 1301-1310.

RAUSCHER, M., ADAM, A.L., WIRTZ, S., GUGGENHEIM, R., MENDGEN, K. AND DEISING, H.B. (1999) PR-1 protein inhibits the differentiation of rust infection hyphae in leaves of acquired resistant broad bean. Plant J. 19: 625-33.

RAY, S., ANDERSON, J.M., URMEEV, F.I. AND GOODWIN, S.B. (2003) Rapid induction of a protein disulfide isomerase and defense-related genes in wheat in response to the hemibiotrophic fungal pathogen *Mycosphaerella graminicola*. Plant Mol. Biol. 53: 701-714.

REBMANN, G., HERTIG, C., BULL, J., MAUCH, F. AND DUDLER, R. (1991a) Cloning and sequencing of cDNAs encoding a pathogen-induced putative peroxidase of wheat (*Triticum aestivum* L.). Plant Mol. Biol. 16: 329-331.

REBMANN, G., MAUCH, F. AND DUDLER, R. (1991b) Sequence of a wheat cDNA encoding a pathogen-induced thaumatin-like protein. Plant Mol. Biol. 17: 283-285.

REYMOND, P., KUNZ, B., PAUL-PLETZER, K., GRIMM, R., ECKERSKORN, C. AND FARMER, E.E. (1996) Cloning of a cDNA encoding a plasma membrane-associated, uronide binding phosphoprotein with physical properties similar to viral movement proteins. Plant Cell 8: 2265-2276.

RONALD, P.C., SALMERON, J.M., CARLAND, F.M. AND STASKAWICZ, B.J. (1992) The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J. Bacteriol. 174: 1604-1611.

ROSE, T.L., BONNEAU, L, DER, C. MARTY-MAZARS, D. AND MARTY, F. (2005) Starvation-induced expression of autophagy-related genes in *Arabidopsis*. Biology of the Cell DOI: 10.1042/BC20040516.

ROSENBERGER, C.M., BRUMELL, J.H. AND FINLAY, B.B. (2000) Microbial pathogenesis: Lipid rafts as pathogen portals. Curr. Biol 10: R823-R825.

ROUHIER, N. AND JACQUOT, J.P. (2005) The plant multigenic family of thiol peroxidases. Free Radic. Biol. Med. 38: 1413-1421.

SACHETTO-MARTINS, G., FRANCO, L.O. AND DE OLIVEIRA, D.E. (2000) Plant glycine-rich proteins: a family or just proteins with a common motif? Biochim. Biophys. Acta 1492: 1-14.

SAGI, M. AND FLUHR, R. (2001) Superoxide production by plant homologues of the gp91(phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiol. 126: 1281-1290.

SAGI, M., DAVYDOV, O., ORAZOVA, S., YESBERGENOVA, Z., OPHIR, R., STRATMANN, J.W. AND FLUHR, R. (2004) Plant respiratory burst oxidase homologs impinge on wound responsiveness and development in *Lycopersicon esculentum*. Plant Cell 16: 616-628.

SAINT-JORE, C.M., EVINS, J., BATOKO, H., BRANDIZZI, F., MOORE, I. AND HAWES, C. (2002) Redistribution of membrane proteins between the Golgi apparatus and endoplasmic reticulum in plants is reversible and not dependent on cytoskeletal networks. Plant J. 29: 661-678.

SANCHEZ, P., DE TORRES ZABALA, M. AND GRANT, M. (2000) AtBI-1, a plant homologue of Bax inhibitor-1, suppresses Bax-induced cell death in yeast and is rapidly upregulated during wounding and pathogen challenge. Plant J. 21: 393-399.

SATIAT-JEUNEMAITRE, B., BOEVINK, P. AND HAWES, C. (1999) Membrane trafficking in higher plant cells: GFP and antibodies, partners for probing the secretory pathway. Biochimie 81: 597-605.

SATO, T., HANADA, M., BODRUG, S., IRIE, S., IWAMA, N., BOISE, L.H., THOMPSON, C.B., GOLEMIS, E., FONG, L., WANG, H.G. AND REED, J.C. (1994) Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. Proc. Natl. Acad. Sci. U.S.A. 91: 9238-9242.

SCHÄFER, P., HÜCKELHOVEN, R. AND KOGEL, K.-H. (2004) The white barley mutant *Albostrians* shows a supersusceptible but symptomless interaction phenotype with the hemibiotrophic fungus *Bipolaris sorokiniana*. Mol. Plant-Microbe Interact. 17: 366-373.

SCHALLER, A. (2004) A cut above the rest: the regulatory function of plant proteases. Planta 220: 183-197.

SCHEUFLER, C., BRINKER, A., BOURENKOV, G., PEGORARO, S., MORODER, L., BARTUNIK, H., HARTL, F.U. AND MOAREFI, I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell 101: 199-210.

SCHIFFER, R., GÖRG, R., JAROSCH, B., BECKHOVE, U., BAHRENBERG, G., KOGEL, K.-H. AND SCHULZE-LEFERT, P. (1997) Tissue dependence and differential cordycepin sensitivity of race-specific resistance responses in the barley-powdery mildew interaction. Mol. Plant-Microbe Interact. 10: 830-839.

SCHLÖSSER, E. (1997) Allgemeine Phytopathologie. Georg Thieme Verlag, Stuttgart.

SCHOPFER, P., LISZKAY, A., BECHTOLD, M., FRAHRY, G. AND WAGNER, A. (2002) Evidence that hydroxyl radicals mediate auxin-induced extension growth. Planta 214: 821-828.

SCHULTHEISS, H., DECHERT, C., KOGEL, K.-H. AND 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.

SCHULTHEISS, H., DECHERT, C., KIRÁLY, L., FODOR, J., MICHEL, K., KOGEL, K.-H. AND HÜCKELHOVEN, R. (2003a) Functional assessment of the pathogenesis-related protein PR-1b in barley. Plant Sci. 165: 1275-1280.

SCHULTHEISS, H., DECHERT, C., KOGEL, K.-H. AND HÜCKELHOVEN, R. (2003b) Functional analysis of barley RAC/ROP G-protein family members in susceptibility to the powdery mildew fungus. Plant J. 36: 589-601.

SCHULTHEISS, H., HENSEL, G., IMANI, J., BROEDERS, S., SONNEWALD, U., KOGEL, K.-H., KUMLEHN, J. AND HÜCKELHOVEN, R. (in press) Ectopic expression of constitutively activated RACB in barley enhances susceptibility to powdery mildew and abiotic stress. Plant Physiol.

SCHULZE-LEFERT, P. AND PANSTRUGA, R. (2003) Establishment of biotrophy by parasitic fungi and reprogramming of host cells for disease resistance. Annu. Rev. Phytopathol. 41: 641-667.

SCHWEIZER, P., POKORNY, J., ABDERHALDEN, O. AND DUDLER, R. (1999) A transient assay system for the functional assessment of defense-related genes in wheat. Mol. Plant-Microbe Interact. 12: 647-654.

SCHWEIZER, P., KMECL, A., CARPITA, N. AND DUDLER, R. (2000a) A soluble carbohydrate elicitor from *Blumeria graminis* f.sp. *tritici* is recognized by a broad range of cereals. Physiol. Mol Plant Pathol. 56: 157-167.

SCHWEIZER, P., POKORNY, J., SCHULZE-LEFERT, P. AND DUDLER, R. (2000b) Double-stranded RNA interferes with gene function at the single-cell level in cereals. Plant J. 24: 895-903.

SCORRANO, L., OAKES, S.A., OPFERMAN, J.T., CHENG, E.H., SORCINELLI, M.D., POZZAN, T. AND KORSMEYER, S.J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 300: 135-139.

SEGURA, A., MORENO, M. AND GARCIA-OLMEDO, F. (1993) Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. FEBS Lett. 332: 243-246.

SHANK, K.J., SU, P. BRGLEZ I., BOSS, W.F., DEWEY, R.E. AND BOSTON, R.S. (2001) Induction of lipid metabolic enzymes during the endoplasmic reticulum stress response in plants. Plant Physiol. 126: 267-277.

SHARPE, J.C., ARNOULT, D. AND YOULE, R.J. (2004) Control of mitochondrial permeability by Bcl-2 family members. Biochim. Biophys. Acta 1644: 107-113.

SHEFFIELD, W.P., SHORE, G.C. AND RANDALL, S.K. (1990) Mitochondrial precursor protein. Effects of 70-kilodalton heat shock protein on polypeptide folding, aggregation, and import competence. J. Biol. Chem. 265: 11069-11076.

SHIKANO, S. AND LI, M. (2003) Membrane receptor trafficking: Evidence of proximal and distal zones conferred by two independent endoplasmic reticulum localization signals. Proc. Natl. Acad. Sci. U.S.A. 100: 5783-5788.

SHIRASU, K. AND SCHULZE-LEFERT, P. (2003) Complex formation, promiscuity and multi-functionality: Protein interactions in disease-resistance pathways. Trends Plant Sci. 8: 252-258.

SHIRASU, K., LAHAYE, T., TAN, M. W., ZHOU, F., AZEVADO, C. AND SCHULZE-LEFERT, P. (1999a) A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell 99: 355-366.

SHIRASU, K., NIELSEN, K., PIFFANELLI, P., OLIVER, R. AND SCHULZE-LEFERT, P. (1999b) Cell-autonomous complementation of *mlo* resistance using a biolistic transient expression system. Plant J. 17: 293-299.

SHOWALTER, A.M. (1993) Structure and function of plant cell wall proteins. Plant Cell 5: 9-23.

SIEZEN, R.J. AND LEUNISSEN, J.A. (1997) Subtilases: the superfamily of subtilisin-like serine proteases. Protein Sci. 6: 501-523.

SIMON-PLAS, F., ELMAYAN, T. AND BLEIN, J.P. (2002) The plasma membrane oxidase NtrbohD is responsible for AOS production in elicited tobacco cells. Plant J. 31: 137-147.

SONG, W.Y., WANG, G.L., CHEN, L.L., KIM, H.S., HOLSTEN, T., WANG, T., ZHAI, W.X., ZHU, L. H., FANQUET, C. AND RONALD, P. (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270: 1804-1806.

SREENIVASULU, N., ALTSCHMIED, L., PANITZ, R., HÄHNEL, U., MICHALEK, W., WESCHKE, W. AND WOBUS, U. (2002) Identification of genes specifically expressed in maternal and filial tissues of barley caryopses: a cDNA array analysis. Mol. Genet. Genomics 266: 758-767.

STAIGER, C.J. (2000) Signaling to the actin cytoskeleton in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51: 257-288.

STASWICK, P. (2000) Two expressed soybean genes with high sequence identity to tomato *Pti1* kinase lack autophosphorylation activity. Arch. Biochem. Biophys. 383: 233-237.

SVALHEIM, O. AND ROBERTSEN, B. (1993) Elicitation of H_2O_2 production in cucumber hypocotyl segments by oligo-1,4-α-D-galacturonides and an oligo-β-glucan preparation from cell walls of *Phytophthora megasperma* f.sp. *glycinea*. Physiol. Plant. 88: 675-681.

TAKEMOTO, D. AND HARDHAM, A.R. (2004) The cytoskeleton as a regulator and target of biotic interactions in plants. Plant Physiol. 136: 3864-3876.

TAKEMOTO, D., JONES, D.A. AND HARDHAM, A.R. (2003) GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of *Arabidopsis* by oomycete pathogens. Plant J. 33: 775-792.

THOMPSON, A.R. AND VIERSTRA, R.D. (2005) Autophagic recycling: lessons from yeast help define the process in plants. Curr. Opin. Plant Biol. 8: 165-173.

THORDAL-CHRISTENSEN, H. (2003) Fresh insights into processes of nonhost resistance. Curr. Opin. Plant Biol. 6: 351-357.

THORDAL-CHRISTENSEN, H., BRANDT, J., CHO, B. H., RASMUSSEN, S. K., GREGERSEN, P. L., SMEDEGAARD-PETERSEN, V. AND COLLINGE, D. B. (1992) cDNA cloning and characterization of two barley peroxidase transcripts induced differentially by the powdery mildew fungus *Erysiphe graminis*. Physiol. Mol. Plant Pathol. 40: 395-409.

THORDAL-CHRISTENSEN, H., ZHANG, Z., WIE, Y. AND COLLINGE, D.B. (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J. 11: 1187-1194.

TIAN, A.G., LUO, G.Z., WANG, Y.J., ZHANG, J.S., GAI, J.Y. AND CHEN, S.Y. (2003) Isolation and characterization of a *Pti1* homologue from soybean. J. Exp. Bot. 55: 535-537.

TÖR, M., YEMM, A. AND HOLUB, E. (2003) The role of proteolysis in *R* gene mediated defense in plants. Mol. Plant Pathol. 4: 287-296.

TORNERO, P., CONEJERO, V. AND VERA, P. (1996) Identification of a new pathogen-induced member of the subtilisin-like processing protease family from plants. J. Biol. Chem. 272: 14412-14419.

TORRES, M.A., DANGL, J.L. AND JONES, J.D. (2002) *Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. U.S.A. 99: 517-522.

TOSA, Y. (1992) A model for the evolution of *formae speciales* and races. Phytopathology 82: 728-730.

TOSA, Y. AND SHISHIYAMA, J. (1984) Defense reactions of barley cultivars to an inappropriate *forma specialis* of the powdery mildew fungus of gramineous plants. Can. J. Bot. 62: 2114-2117.

TOSA, Y., AKIYAMA, T. AND OGURA, H. (1990) Cytological aspects of interactions between *formae speciales* of *Erysiphe graminis* and genera of gramineous plants, and their evolutionary implications. Can. J. Bot. 68: 1249-1253.

TOYODA, K., COLLINS, N.C., TAKAHASHI, A. AND SHIRASU, K. (2002) Resistance and susceptibility of plants to fungal pathogens. Transgenic Res. 11: 567-582.

TROTOCHAUD, A.E., HAO, T., WU, G., YANG, Z. AND CLARK, S.E. (1999) The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into signaling complex that includes KAPP and Rho-related protein. Plant Cell 11: 393-405

TRUJILLO, M., KOGEL, K.-H. AND HÜCKELHOVEN, R. (2004a) 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.

TRUJILLO, M., TROEGER, M., NIKS, R., KOGEL, K-H. AND HÜCKELHOVEN, R. (2004b) Mechanistic and genetic overlap of barley host and non-host resistance to *Blumeria graminis.* Mol. Plant Pathol. 5: 389-396.

TSUJIMOTO, Y. AND SHIMIZU, S. (2000) Bcl-2 family: life-or-death switch. FEBS Let. 466: 6-10.

VAN DER HOORN, R.A. AND JONES, J.D. (2004) The plant proteolytic machinery and its role in defence. Curr. Opin. Plant Biol. 7: 400-407.

VAN DOORN, W.G. AND WOLTERING, E.J. (2005) Many ways to exit? Cell death categories in plants. Trends Plant Sci. 10: 117-122.

VAN LOON, L.C AND VAN STRIEN, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55: 85-97.

VAN LOON, L.C., PIERPOINT, W.S., BOLLER, T. AND CONEJERO, V. (1994) Recommendations for naming plant pathogenesis related proteins. Plant Mol. Biol. Rep. 12: 245-264.

VAN MEER, G. AND SPRONG, H. (2004) Membrane lipids and vesicular traffic. Curr. Opin. Cell Biol. 16: 373-378.

VANETTEN, H.D., MANSFIELD, J.W., BAILEY, J.A. AND FARMER, E.E. (1994) Two Classes of Plant Antibiotics: Phytoalexins versus "Phytoanticipins". Plant Cell 6: 1191-1192.

VOGEL, J.P., RAAB, T.K., SCHIFF, C. AND SOMERVILLE, S.C. (2002) *PMR6*, a pectate lyase-like gene required for powdery mildew susceptibility in Arabidopsis. Plant Cell 14: 2095-2106.

VON RÖPENACK, E., PARR, A. AND SCHULZE-LEFERT P. (1998) Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. J. Biol. Chem. 273: 9013-9022.

WALTON, J.D. (1996) Host-selective toxins: Agents of compatibility. Plant Cell 8: 1723-1733.

WANG, M.B. AND METZLAFF, M. (2005) RNA silencing and antiviral defense in plants. Curr. Opin. Plant Biol. 8: 216-222.

WANG, W., VINOCUR, B., SHOSEYOV, O. AND ALTMAN, A. (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9: 244-252.

WANG, D., WEAVER, N.D., KESARWANI, M. AND DONG, X. (2005) Induction of protein secretory pathway is required for systemic acquired resistance. Science 308: 1036-1040.

WATANABE, N. AND LAM, E. (2004) Recent advance in the study of caspases-like proteases and Bax inhibitor-1 in plants: their possible roles as regulator of programmed cell death. Mol. Plant Pathol. 5: 65-70.

WEBB, M.A., CAVALETTO, J.M., KLANRIT, P. AND THOMPSON, G.A. (2001) Orthologs in *Arabidopsis thaliana* of the Hsp70 interacting protein Hip. Cell Stress Chaperones 6: 247-255.

WEI, F., GOBELMAN-WERNER, K., MORROLL, S.M., KURTH, J., MAO, L., WING, R., LEISTER, D., SCHULZE-LEFERT, P. AND WISE, R.P. (1999) The *Mla* (powdery mildew) resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb DNA interval on chromosome 5S (1HS) of barley. Genetics 153: 1929-1948.

WESTPHALEN, B.C., WESSIG, J., LEYPOLDT, F., ARNOLD, S. AND METHNER, A. (2005) BI-1 protects cells from oxygen glucose deprivation by reducing the calcium content of the endoplasmic reticulum. Cell Death Differ. 12: 304-306.

WHITHAM, S., DINESH-KUMAR, S.P., CHOI, D., HEHL, R., CORR, C. AND BAKER, B. (1994) The product of the tobacco mosaic virus resistance gene *N*: Similarity to Toll and the Interleukin-1 receptor. Cell 78: 1011-1015.

WOLTER, M., HOLLRICHER, K., SALAMINI, F. AND SCHULZE-LEFERT, P. (1993) The *mlo* resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defense mimic phenotype. Mol. Gen. Genet. 239: 122-128.

WOLTERING, E.J., VAN DER BENT, A. AND HOEBERICHTS, F.A. (2002) Do plant caspases exist? Plant Physiol. 130: 1764-1769.

WYATT, S.E., TSOU, P.L. AND ROBERTSON, D. (2002) Expression of the high capacity calcium-binding domain of calreticulin increases bioavailable calcium stores in plants. Transgenic Res. 11: 1-10.

XIAO, F., LU, M., LI, J., ZHAO, T., YI, S.Y., THARA, V.K., TANG, X. AND ZHOU, J.M. (2003) *Pto* mutants differentially activate *Prf*-dependent, *avrPto*-independent resistance and gene-for-gene resistance. Plant Physiol. 131: 1239-1249.

XU, Q. AND REED, J.C. (1998) Bax Inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast. Mol. Cell 1: 337-346.

XU, P., BLANCAFLOR, E.B. AND ROOSSINCK, M.J. (2003) In spite of induced multiple defense responses, tomato plants infected with cucumber mosaic virus and D satellite RNA succumb to systemic necrosis. Mol. Plant-Microbe Interact. 16: 467-476.

YANG, Z. (2002) Small GTPases: versatile signaling switches in plants. Plant Cell 14: S375-S388.

YOSHINAGA, K., ARIMURA, S., HIRATA, A., NIWA, Y., YUN, D.J., TSUTSUMI, N., UCHIMIYA, H. AND KAWAI-YAMADA, M. (2005) Mammalian Bax initiates plant cell death through organelle destruction. Plant Cell Reports DOI: 10.1007/s00299-005-0948-6.

YOSHIOKA, H., NUMATA, N., NAKAJIMA, K., KATOU, S., KAWAKITA, K., ROWLAND, O., JONES, J.D. AND DOKE, N. (2003) *Nicotiana benthamiana gp91phox* homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell 15: 706-718.

YOUNG, N.D. (2000) The genetic architecture of resistance. Curr. Opin. Plant Biol. 3: 285-290.

YU, L.H., KAWAI-YAMADA, M., NAITO, M., WATANABE, K., REED, J.C. AND UCHIMIYA, H. (2002) Induction of mammalian cell death by a plant Bax inhibitor. FEBS Lett. 512: 308-312.

YUN, B.W., ATKINSON, H.A., GABORIT, C., GREENLAND, A., READ, N.D., PALLAS, J.A. AND LOAKE, G.J. (2003) Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in *Arabidopsis* against wheat powdery mildew. Plant J. 34: 768-777.

ZAGOTTA, W.N. AND SIEGELBAUM, S.A. (1996) Structure and function of cyclic nucleotide-gated channels. Annu. Rev. Neurosci. 19: 235-263.

ZEYEN, R.J., CARVER, T.L.W. AND LYNGKJÆR, M.F. (2002) Epidermal cell papillae. In: The Powdery Mildews – A Comprehensive Treatise (Bélanger, R.R., Bushnell, W.R., Dik, A.J. and Carver, T.L.W. Eds.), St. Paul, Minnesota, pp. 107-125.

ZHANG, S. AND KLESSIG, D.F. (2001) MAPK cascades in plant defense signaling. Trends Plant Sci. 6: 520-527.

ZHANG, Z., HENDERSON, C. AND GURR, S.J. (2004) *Blumeria graminis* secretes an extracellular catalase during infection of barley: potential role in suppression of host defence. Mol. Plant Pathol. 5: 537-547.

ZHENG, H., KUNST, L., HAWES, C. AND MOORE, I. (2004) A GFP-based assay reveals a role for RHD3 in transport between the endoplasmic reticulum and Golgi apparatus. Plant J. 37: 398-414.

ZHOU, F., ZHANG, Z., GREGERSEN, P.L., MIKKELSEN, J.D., DE NEERGAARD, E., COLLINGE, D.B. AND THORDAL-CHRISTENSEN, H. (1998) Molecular characterization of the oxalate oxidase involved in the response of barley to the powdery mildew fungus. Plant Physiol. 117: 33-41.

ZIEROLD, U., SCHOLZ, U. AND SCHWEIZER, P. (2005) Transcriptome analysis of *mlo*-mediated resistance in the epidermis of barley. Mol. Plant Pathol. 6: 139-151.

ZIMMERLI, L., STEIN, M., LIPKA, V., SCHULZE-LEFERT, P. AND SOMERVILLE, S. (2004) Host and non-host pathogens elicit different jasmonate/ethylene responses in *Arabidopsis*. Plant J. 40: 633-646.

ZONG, W.X., LI, C., HATZIVASSILIOU, G., LINDSTEN, T., YU, Q.C., YUAN, J. AND THOMPSON, C.B. (2003) Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J. Cell Biol. 162: 59-69.

7 Supplement

>CALRETICULIN 3

MCSSRRRGDRQLQLLHRLLALSSLLLLASGEVIFEERFEDGWETRWVKSD 50
WKRSEGKAGTFKHTAGKYSGDP[DDKGIQTTIDARHFAISAKIPEFSNKGR 100
TLVVQYSIKFEQEIECGGGYIKLMSGYVNQKKYSGDTPYSLMFGPDICGT 150
QTKKLHLILSYQGQNYPIKKDLQCETDRLTHVYTFILRPDASYSLLVDNR 200
ERESGSMYTDWDILPPRKIKDVGAKKPKDWDDREYIED]PDAVKPEGYDSI 250
PREIPDPKDKKPDTWDDDDDGIWKPRRIPNPAYKGQWKRKKIKNPNYKGK 300
WKIPWIDNPEFEDDPDLYVLKPLKYIGIEVWQVKAGSVFDNILICDDPEY 350
AKQVADETWGANKEAEKEAFEEAEKERKAREDKEAQQAREEGERRRRERG 400
DRHRGRDHYKDRYKRRNRDHWDDYHDEL 450

>GFP

MSMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFI 50
CTTGKLPVPWPTLVTTLTYGVQCFARYPDHMKQHDFFKSAMPEGYVQERT 100
IFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNS 150
HKVYITADKQKNGIKVNFKTRHNIEDGSVQLADHYQQNTPIGDGPVLLPD 200
NHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKD 250

Supplementary figure 1: Deduced amino acid sequences of barley *CALRETICULIN 3* (*CRT3*, TIGR accession TC131592) and *GFP*, which were used for the construction of the marker protein sGFPHdel. sGFPHdel was constructed by replacing amino acids 73 to 238 (**[ ]**) of CRT3 by GFP, lacking the stop-codon (chapter 2.3.9.1). This procedure maintained the presumptive N-terminal signal peptide and the C-terminal HDEL sequence (shaded in black, respectively). For nucleotide sequence see Supplementary table 3.

Supplementary Table 1: Part 1 **List of total 94 genes (102 ESTs) that were 2.5 fold differentially expressed upon B. graminis inoculation in at least two biological repetitions and with expression levels significantly different from controls.**

Spot (a)	Clone ID	accession number	2.5 fold differential expression (b)	Homology to TIGR-entry (c)	Description TIGR	putative function	Identity (in %) to TIGR-entry	Score to TIGR-entry	E-value to TIGR entry	Blast X hit acc.
D - 04 : 7	Hv000015MK	AJ278561	Bgh 12 (4), 24 (3); Bgt 12 (3), 24 (3)	>barley|TC141581	ice recrystallisation inhibition protein	stress/defence/cell death	99	3019	7.3e-132	
J - 14 : 7	Hv000052MK	BE558443	Bgh 24 (3); Bgt 24 (3)	>barley|TC139154	peroxidase	stress/defence/cell death	94	3628	1.5e-159	AAW52715
D - 09 : 7	Ta0009EX	X58394	Bgh 24 (3); Bgt 24 (2)		wheat WIR2, thaumatin-like	stress/defence/cell death				
J - 13 : 3	GAN002J13F	CX631428	Bgh 12 (1), 24 (3); Bgt 12 (1), 24 (2)	>barley|TC140269	Wheatwin2 precursor (Pathogenesis-related protein 4b)	stress/defence/cell death	94	1379	9.7e-58	A43474
H - 11 : 7	Hv0002EX	X74940	Bgh 24 (3); Bgt 24 (3)		barley basic PR1 protein	stress/defence/cell death				
B - 20 : 7	HvD00177	CX631938	Bgh 24 (3); Bgt 24 (1)	>barley|TC134442	subtilase	protein processing	99	1638	1.5e-69	BAD29425
L - 07 : 7	Ta0005EX	X53675	Bgh 24 (2); Bgt 24 (2)		wheat peroxidase	stress/defence/cell death				
H - 24 : 1	GAN001H24F	DN154963	Bgh 24 (2); Bgt 24 (1)	>barley|TC149289	cytochrome P450	stress/defence/cell death	96	1246	6.3e-52	BAD27939
F - 11 : 7	Hv0001EX	AJ276225	Bgh 24 (2); Bgt 24 (1)	>barley|TC132287	Pathogenesis-related protein 1A/1B precursor	stress/defence/cell death	99	2968	1.7e-129	AAW21722
J - 02 : 7	Hv00004MK	AJ582216	Bgh 12 (2), 24 (3); Bgt 12 (2), 24 (3)	>barley|TC139571	benzothiadiazole-induced protein (clone WCI-5)	stress/defence/cell death	99	4283	4.4e-189	T06278
B - 11 : 7	Ta0016EX	AJ007348	Bgh 24 (3); Bgt 24 (3)	>barley|TC235336	Pathogenisis-related protein 1.1 precursor	stress/defence/cell death	96	3708	1.8e-162	CAA07473
H - 07 : 7	Ta0003SM	AAD28733	Bgh 24 (3)		wheat Chitinase Klasse IV	stress/defence/cell death				
L - 10 : 1	GAN001L10F	DN154953	Bgh 12 (2), 24 (2); Bgt 12 (2), 24 (3)	>barley|TC146841	peroxiredoxin	stress/defence/cell death	99	656	5.5e-25	NP_916886
I - 14 : 3	GAN002I14F	CX631775	Bgh 12 (4), 24 (2); Bgt 12 (3), 24 (2)	>barley|TC146674	Protein disulfide isomerase precursor (PDI)	chaperone	98	1053	2.9e-43	P80284
C - 14 : 1	GAN001C14F	DN155094	Bgh 24 (3); Bgt 24 (1)	>wheat|TC243314	nuclear protein-like	membrane traffic / signaling	81	268	5.2e-06	XP_465274
C - 04 : 1	GAN001C04F	DN155085	Bgh 24 (2); Bgt 24 (1)	>barley|TC139603	early drought induced protein	stress/defence/cell death	92	1025	2.8e-41	AAM46895
J - 14 : 3	GAN002J14F	CX631429	Bgh 12 (2), 24 (3); Bgt 12 (2), 24 (2)	>barley|TC141343	chimeric: weakly similar to DNA damage response protein kinase DUN1	stress/defence/cell death	99	1493	3.4e-63	BAD86970
J - 14 : 3	GAN002J14F	CX631429	Bgh 12 (2), 24 (3); Bgt 12 (2), 24 (2)	>barley|TC147719	ribosomal protein-like	housekeeping	97	1034	3.0e-42	AAM63516
C - 21 : 5	GAN003C21F	DN764056	Bgh 24 (2)	>barley|TC140801	LRK19	stress/defence/cell death	72	954	8.3e-39	AAK20738
F - 09 : 7	Ta0010EX	X56012	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (1)		wheat WIR5, gluth.-S-transferase	stress/defence/cell death				
J - 13 : 1	GAN001J13F	DN154922	Bgh 24 (2); Bgt 24 (1)	>barley|TC148309	cysteine proteinase	protein processing	100	585	2.4e-34	XP_450799
J - 21 : 7	HvD00135	DN764096	Bgh 24 (2); Bgt 24 (1)	>barley|TC147916	plastidic ATP/ADP transporter	housekeeping	100	2085	4.5e-90	BAD68186
B - 03 : 5	GAN003B03F	CX631712	Bgh 24 (2)	>barley|TC150618	DNA-binding protein	membrane traffic / signaling	96	1556	1.3e-65	CAA88326
J - 12 : 7	Hv000044MK	AJ428906	Bgh 24 (3); Bgt 12 (2), 24 (1)	>barley|TC148222	Solute carrier family 2, facilitated glucose transporter, member 8	housekeeping	99	1431	1.6e-60	AAL14615
B - 22 : 5	GAN003B22F	CX631280	Bgh 24 (3)	>barley|TC147949	hypothetical protein	unclassified	99	1417	1.6e-59	BAD29572
A - 17 : 7	GAN004A17F	CX631938	Bgh 24 (3)	>barley|TC134442	subtilase	protein processing	99	1638	1.5e-69	BAD29425
D - 17 : 7	HvD00150	DN764077	Bgh 12 (2), 24 (3); Bgt 12 (2), 24 (2)	>barley|TC146346	Leucin-rich repeat protein	stress/defence/cell death	99	2798	4.9e-122	AAU82111
F - 19 : 1	GAN001F19F	DN155011	Bgh 24 (2); Bgt 24 (2)	>barley|TC135900	chimeric: putative sodium transporter	housekeeping	98	1349	9.5e-57	CAD37183
F - 19 : 1	GAN001F19F	DN155011	Bgh 24 (2); Bgt 24 (2)	>barley|TC140033	no significant similarity found	unclassified	95	755	1.3e-29	
B - 06 : 7	Hv000024MK	AV947463	Bgh 12 (3), 24 (3); Bgt 12 (3), 24 (2)	>barley|TC146402	unknown protein contains GDSL-like lipase/acylhydrolase domain	stress/defence/cell death	80	1585	2.3e-67	BAD69424
A - 14 : 1	GAN001A14F	DN155049	Bgh 12 (1), 24 (2); Bgt 24 (1)	>barley|TC139491	tetrafunctional protein of glyoxysomal fatty acid beta-oxidation	housekeeping	99	1256	1.3e-52	NP_908896
B - 14 : 7	Hv000056MK	AJ428911	Bgh 24 (2)	>barley|TC147208	FK506-binding protein 2-1 precursor (Rotamase); putative immunophilin	chaperone	99	1121	3.9e-46	CAD42633
A - 12 : 7	GAN004A12F	CX631934	Bgh 12 (2), 24 (3); Bgt 12 (1), 24 (1)	>barley|TC14027	serine carboxypeptidase D precursor	protein processing	98	2274	1.8e-98	CAA70815
C - 12 : 1	GAN001C12F	DN155092	Bgh 24 (2); Bgt 24 (1)	>barley|BJ466050	hypothetical protein	unclassified	99	1938	7.9e-83	NP_910168
J - 06 : 7	Hv000020MK	AJ582221	Bgh 12 (2), 24 (2); Bgt 12 (2), 24 (1)	>barley|TC149589	WIR1-like protein	stress/defence/cell death	98	3406	2.9e-149	T06989
A - 21 : 7	GAN004A21F	CX631942	Bgh 24 (2); Bgt 24 (1)	>barley|TC138668	chimeric: aminopropyl transferase	housekeeping	99	1372	9.0e-58	BAD28219
A - 21 : 7	GAN004A21F	CX631942	Bgh 24 (2); Bgt 24 (1)	>barley|TC142058	no significant similarity found.	unclassified	65	262	0.00037	
B - 19 : 1	GAN001B19F	DN155076	Bgh 24 (3); Bgt 24 (1)	>barley|TC146767	chimeric: diphosphonucleotide phosphatase	housekeeping	91	1146	3.1e-47	XP_483478
B - 19 : 1	GAN001B19F	DN155076	Bgh 24 (3); Bgt 24 (1)	>barley|TC147949	hypothetical protein	unclassified	70	486	8.8e-17	BAD29572
L - 08 : 1	GAN001L08F	DN154951	Bgh 24 (1); Bgt 12 (1), 24 (2)	>barley|TC148309	cysteine proteinase	protein processing	100	1055	2.1e-43	XP_450799
A - 06 : 5	GAN003A06F	CX631299	Bgh 24 (2)	>barley|TC134157	unknown protein	unclassified	98	2021	1.1e-86	XP_478523
I - 14 : 1	GAN001I14F	DN154968	Bgh 24 (2); Bgt 24 (2)	>barley|TC134227	conserved oligomeric Golgi complex subunit 5	membrane traffic / signaling	99	1251	2.7e-52	AAG52007
F - 15 : 7	HvD00126	DN764070	Bgh 12 (2), 24 (1)	>barley|TC132885	no significant similarity; weakly similar to nucleotide exchange factor RasGEF C	unclassified	99	1032	8.2e-42	
D - 13 : 7	HvD00108	CX629764	Bgh 24 (2)	>barley|TC147148	autophagy	membrane traffic / signaling	99	2146	2.1e-92	AAP80854
O - 14 : 3	GAN002O14F	CX631311	Bgh 12 (1), 24 (1); Bgt 12 (3), 24 (1)	>barley|TC131592	Calreticulin 3 precursor	chaperone	96	939	3.3e-38	NP_915149
N - 07 : 3	GAN002N07F	CX631351	Bgh 24 (1); Bgt 12 (1), 24 (1)	>barley|TC139411	chimeric: luminal binding protein 3 precursor (BiP3)	chaperone	95	1421	1.0e-59	AAB63469
N - 07 : 3	GAN002N07F	CX631351	Bgh 24 (1); Bgt 12 (1), 24 (1)	>barley|TC136544	At4g32960	unclassified	98	1544	4.6e-65	BAD69045
B - 13 : 5	GAN003B13F	CX631722	Bgh 12 (1), 24 (1)	>barley|TC148309	chimeric: cysteine proteinase	protein processing	99	926	1.4e-37	XP_450799
B - 13 : 5	GAN003B13F	CX631722	Bgh 12 (1), 24 (1)	>barley|TC142886	putaive mitochondrial inner membrane protein	housekeeping	99	1641	1.1e-69	AAP54652
A - 02 : 7	GAN004A02F	DN764055	Bgh 12 (2), 24 (1); Bgt 12 (2), 24 (2)	>wheat|CA745664	no significant similarity found.	unclassified	73	144	5,6	
B - 05 : 5	GAN003B05F	CX631714	Bgh 12 (2), 24 (2); Bgt 12 (1), 24 (2)	>barley|TC139736	photosystem II	housekeeping	98	1235	3.3e-51	P31336
D - 03 : 7	Hv0000370GL	AJ888869	Bgh 12 (1), 24 (1); Bgt 12 (2), 24 (1)	>barley|TC131350	fructose 1,6-bisphosphate aldolase	housekeeping	97	1167	1.8e-48	T03679
G - 03 : 7	GAN004G03F	CX632048	Bgh 12 (1), 24 (2); Bgt 24 (1)	>barley|BF262274	similar to hypothetical protein	unclassified	99	1498	5.5e-63	
C - 16 : 3	GAN002C16F	CX631802	Bgh 24 (1); Bgt 24 (2)	>barley|TC139836	dihydrolipoamide dehydrogenase	housekeeping	98	1447	3.8e-61	NP_908725
O - 02 : 1	GAN001O02F	DN764057	Bgh 12 (2), 24 (2); Bgt 12 (1), 24 (1)	>barley|CA006281	PR-domain zinc finger protein 12	membrane traffic / signaling	91	965	6.4e-39	
B - 01 : 7	Hv000193GL	AJ888867	Bgh 24 (2); Bgt 12 (1), 24 (1)	>barley|TC146760	g5bf protein	membrane traffic / signaling	98	1159	9.5e-48	AAL32648
B - 01 : 3	GAN002B01F	CX631823	Bgh 12 (1), 24 (2); Bgt 24 (2)	>barley|TC131776	methyl binding domain protein MBD109	membrane traffic / signaling	97	1329	6.9e-56	AAM93219
C - 05 : 3	GAN002C05F	CX631548	Bgh 12 (2), 24 (2); Bgt 12 (1), 24 (2)	>barley|TC140441	PQBP-1 protein (Nuclear protein containing a WW domain)	housekeeping	94	1814	1.1e-77	AAP54879

(a): Yellow shadowed boxes indicate potentially chimeric clones

(b): Differential expression at 12 or 24 HAI is indicated for *Bgh*, *Bgt* or both. The number of biological and technical repetitions that gave positive results is given in brackets.

Red numbers indicate up-regulation, green numbers indicate down-regulation. Magenta colored boxes indicate ESTs that were identified only in one biological repetition but were confirmed by further ESTs of the identical unigene or by independent means such as RT-PCR

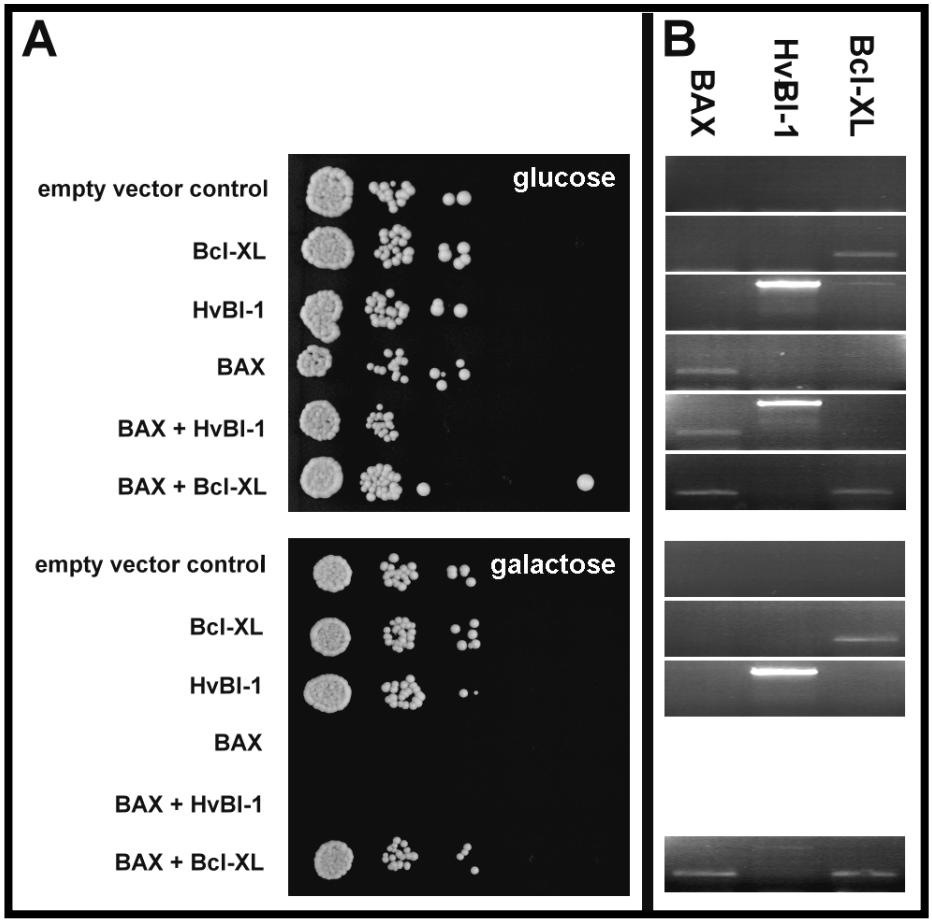
Supplementary Table 1: Part 2 **List of total 94 genes (102 ESTs) that were 2.5 fold differentially expressed upon B. graminis inoculation in at least two biological repetitions and with expression levels significantly different from controls.**

Spot (a)	Clone ID	accession number	2.5 fold differential expression (b)	Homology to TIGR-entry (c)	Description TIGR	putative function	Identity (in %) to TIGR-entry	Score to TIGR-entry	E-value to TIGR entry	Blast X hit acc.
I - 20 : 3	GAN002I20F	CX631398	Bgh 24 (1); Bgt 12 (1)	>barley|TC132933	Glycine-rich cell wall structural protein 1.8 precursor (GRP 1.8)	unclassified/cell wall	97	1331	1.1e-55	BAD62279
B - 02 : 3	GAN002B02F	CX631824	Bgh 12 (1), 24 (2); Bgt 12 (2), 24 (2)	>barley|TC146378	Phosphoglycerate kinase, chloroplast precursor	housekeeping	99	1197	7.7e-50	CAA51931
K - 07 : 5	GAN003K07F	CX631596	Bgh 12 (2) ; Bgt 12 (1)	>barley|TC147770	OSJNBa0083N12.20 protein (OSJNBa0041A02.4 protein)	unclassified	98	852	5.4e-34	XP_473766
J - 03 : 7	Hv000383GL	AJ888868	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (2)	>barley|TC146758	g5bf gene	membrane traffic / signaling	99	3992	4.2e-176	CAA75602
P - 17 : 7	HvD00174	DN764083	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (1)	>barley|TC146796	barley lipid transfer protein 4 (BLT4) precursor	stress/defence/cell death	100	860	2.5e-34	AAA03283
L - 21 : 1	GAN001L21F	DN154896	Bgh 24 (2); Bgt 24 (2)	>barley|TC144635	extra-large G-protein-like	membrane traffic / signaling	98	1276	4.7e-53	BAD45831
N - 16 : 5	GAN003N16F	CX631875	Bgh 12 (1), 24 (2); Bgt 12 (1)	>wheat|TC187167	chimeric: adhesive/proline-rich protein homolog	unclassified	57	167	7,8	
N - 16 : 5	GAN003N16F	CX631875	Bgh 12 (1), 24 (2); Bgt 12 (1)	>barley|TC108992	60S ribosomal protein L7 [imported]	unclassified	98	1239	7.6e-51	AAW50989
J - 23 : 7	HvD00171	DN764104	Bgh 24 (2); Bgt 12 (1), 24 (1)	>barley|BE422360	Sedoheptulose-1 7-bisphosphatase chloroplast precursor	housekeeping	96	1378	8.4e-58	CAA46507
P - 16 : 7	Hv000057MK	BE230944	Bgh 12 (1), 24 (2); Bgt 24 (1)	>barley|TC132362	NADPH thioredoxin reductase	stress/defence/cell death	98	2885	3.5e-126	CAE46765
O - 18 : 1	GAN001O18F	DN154856	Bgh 24 (2); Bgt 12 (1)	>barley|TC139611	HSP associated protein like	chaperone	94	1212	1.4e-50	AAM65016
H - 01 : 7	Hv0000306GL	AJ888870	Bgh 12 (1), 24 (2); Bgt 24 (2)	>barley|BE421610	Chlorophyll A-B binding protein of LHCII type III chloroplast precursor (CAB).	housekeeping	99	1571	1.1e-66	P27523
L - 01 : 5	GAN003L01F	CX631227	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (1)	>wheat|TC240498	hydrolase-like protein	stress/defence/cell death	93	1821	4.2e-77	XP_450624
H - 18 : 1	GAN001H18F	DN764059	Bgh 24 (2); Bgt 24 (2)	>barley|TC148625	unknown	unclassified	90	1378	7.3e-58	XP_473722
F - 10 : 5	GAN003F10F	CX631628	Bgh 24 (2); Bgt 12 (1), 24 (2)	>barley|TC146886	chimeric: expressed protein	unclassified	100	880	1.6e-35	
F - 10 : 5	GAN003F10F	CX631628	Bgh 24 (2); Bgt 12 (1), 24 (2)	>barley|TC139102	light-harvesting complex IIa protein	housekeeping	99	666	1.1e-25	1908421A
D - 01 : 7	Hv0000213GL	AJ250282	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (3)	>barley|TC146853	acid phosphatase	housekeeping	97	5563	6.2e-247	CAB71336
H - 11 : 5	GAN003H11F	CX631673	Bgh 24 (3); Bgt 24 (1)	>barley|BE411670	No significant similarity found.	unclassified	99	1502	4.5e-63	
K - 02 : 5	GAN003K02F	CX631591	Bgh 12 (2), 24 (2); Bgt 12 (2), 24 (1)	>wheat|CK193543	Chimeric: weakly similar to unnamed protein product	unclassified	90	961	3.7e-38	AAM64580
K - 02 : 5	GAN003K02F	CX631591	Bgh 12 (2), 24 (2); Bgt 12 (2), 24 (1)	>barley|TC133311	similar to OSJNBa0089K24.9	unclassified	98	966	1.5e-38	AAU44337
P - 01 : 7	Hv000339GL	AJ888872	Bgh 12 (2), 24 (2); Bgt 12 (2)	>barley|TC147282	cyclophilin-like protein	chaperone	97	2017	1.0e-86	AAP44535
L - 22 : 1	GAN001L22F	DN154897	Bgh 24 (2); Bgt 24 (1)	>barley|TC140500	chimeric: early nodulin	membrane traffic / signaling	99	1736	7.8e-74	XP_464756
L - 22 : 1	GAN001L22F	DN154897	Bgh 24 (2); Bgt 24 (1)	>barley|TC146506	AT5g36790/f5h8_20, phosphoglycolate phosphatase activity	housekeeping	98	1362	5.1e-57	NP_198495
M - 01 : 3	GAN002M01F	CX631367	Bgh 24 (2); Bgt 24 (2)	>barley|TC139685	gamma hydroxybutyrate dehydrogenase-like protein	housekeeping	98	1422	6.4e-60	AAO72678
N - 17 : 5	GAN003N17F	CX631876	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (1)	>barley|TC123790	CDT1 protein	unclassified	100	1380	3.0e-57	AAO72697
L - 11 : 5	GAN003L11F	CX631826	Bgh 24 (2); Bgt 24 (1)	>barley|TC131364	Carbonic anhydrase, chloroplast precursor (Carbonate dehydratase)	housekeeping	98	1704	8.0e-73	P40880
L - 09 : 3	GAN002L09F	CX631389	Bgh 24 (2); Bgt 24 (2)	>barley|TC146512	chlorophyll a/b-binding protein WCAB precursor	housekeeping	94	1280	1.9e-53	AAB18209
N - 20 : 1	GAN001N20F	DN154835	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (1)	>barley|AV834240	chimeric: no significant similarity	unclassified	93	790	1.7e-30	
N - 20 : 1	GAN001N20F	DN154835	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (1)	>barley|TC133311	OSJNBa0089K24.9 protein	unclassified	97	974	1.5e-39	AAU44337
L - 02 : 5	GAN003L02F	CX631228	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (1)	>barley|CB881671	weakly similar to Hypothetical protein	unclassified	99	1737	5.0e-73	NP_911952
M - 16 : 3	GAN002M16F	CX631752	Bgh 24 (2); Bgt 24 (1)	>barley|TC131399	Ferredoxin-NADP(H) oxidoreductase	housekeeping	98	1936	3.7e-83	CAD30025
N - 11 : 5	GAN003N11F	CX631871	Bgh 12 (2), 24 (1); Bgt 12 (2), 24 (1)	>barley|BE231124	homologue to unnamed protein product	unclassified	91	1415	9.5e-59	
L - 21 : 5	GAN003L21F	CX631836	Bgh 24 (2); Bgt 24 (1)	none	chimeric: none	unclassified				
L - 21 : 5	GAN003L21F	CX631836	Bgh 24 (2); Bgt 24 (1)	>barley|BU994621	none	unclassified	75	536	1.4e-19	
M - 08 : 5	GAN003M08F	CX631846	Bgh 12 (1), 24 (1); Bgt 12 (1)	>barley|TC132933	Glycine-rich cell wall structural protein 1.8 precursor (GRP 1.8)	unclassified/cell wall	98	1209	4.1e-50	BAD62279
K - 14 : 3	GAN002K14F	CX631377	Bgh 12 (1), 24 (3); Bgt 12 (1), 24 (3)	>barley|TC120550	P0674H09.7	unclassified	98	1378	1.8e-57	BAD82172
O - 08 : 1	GAN001O08F	DN154846	Bgh 12 (1), 24 (3); Bgt 12 (1)	>barley|TC133627	clathrin coat assembly like protein	membrane traffic / signaling	100	915	7.8e-37	BAC79917
M - 18 : 5	GAN003M18F	CX631855	Bgh 24 (3); Bgt 12 (1), 24 (2)	>barley|TC110399	remorin	membrane traffic / signaling	99	1858	7.0e-79	XP_466996
P - 02 : 5	GAN003P02F	CX631907	Bgh 24 (3); Bgt 24 (2)	>barley|TC131827	GCPE protein	housekeeping	99	1752	3.6e-75	XP_466605
N - 20 : 5	GAN003N20F	CX631879	Bgh 24 (2); Bgt 24 (1)	>barley|TC150468	lipase	stress/defence/cell death	99	1272	3.3e-53	NP_916589
P - 09 : 5	GAN003P09F	CX631914	Bgh 12 (2), 24 (2); Bgt 12 (1), 24 (1)	>barley|TC130762	chimeric: ubiquitin-conjugating enzyme UBC2	protein processing	98	897	3.6e-36	AAU82109
P - 09 : 5	GAN003P09F	CX631914	Bgh 12 (2), 24 (2); Bgt 12 (1), 24 (1)	>barley|TC134774	ATP sulfurylase	housekeeping	98	1202	1.1e-49	AAM63309
N - 05 : 1	GAN001N05F	DN154884	Bgh 12 (2), 24 (3); Bgt 12 (2), 24 (1)	>barley|TC113486	unknown	unclassified	97	1091	1.2e-44	AAT77883
P - 03 : 1	GAN001P03F	DN764058	Bgh 12 (2), 24 (2); Bgt 12 (2), 24 (2)	>wheat|TC238290	Rwp34	unclassified	78	444	6.7e-15	AAU26102
M - 08 : 1	GAN001M08F	DN154904	Bgh 24 (3); Bgt 12 (1), 24 (2)	>barley|TC123168	arginyl-tRNA synthetase	housekeeping	98	1306	5.8e-54	XP_475642
K - 08 : 5	GAN003K08F	CX631597	Bgh 12 (2), 24 (1); Bgt 12 (1), 24 (1)	>barley|TC147045	weakly similar to poly(A) binding protein interacting protein 1, isoform 1	membrane traffic / signaling	99	1632	7.3e-69	AAU11824
N - 06 : 1	GAN001N06F	DN154885	Bgh 12 (1), 24 (2); Bgt 12 (1)	>barley|TC132933	Glycine-rich cell wall structural protein 1.8 precursor (GRP 1.8)	unclassified/cell wall	99	961	5.9e-39	BAD62279
O - 09 : 3	GAN002O09F	CX631306	Bgh 12 (1), 24 (3); Bgt 12 (1), 24 (2)	>barley|TC132360	alpha-glucan phosphorylase, H isozyme (starch phosphorylase H)	housekeeping	99	1616	9.5e-69	AAP03064
O - 06 : 5	GAN003O06F	CX631889	Bgh 12 (2), 24 (1); Bgt 12 (2), 24 (1)	>barley|TC140373	AT4g27700/T29A15_190	unclassified	99	1161	4.6e-48	BAD33770
P - 23 : 5	GAN003P23F	CX631928	Bgh 12 (2), 24 (2); Bgt 12 (1), 24 (2)	>barley|TC150218	unknown protein	unclassified	99	1638	2.0e-69	XP_475462
N - 19 : 5	GAN003N19F	CX631878	Bgh 12 (1), 24 (3); Bgt 24 (2)	>barley|TC131052	glycine rich protein	unclassified/cell wall	97	1195	2.2e-49	CAA8855
O - 07 : 3	GAN002O07F	CX631334	Bgh 12 (1), 24 (1); Bgt 12 (1), 24 (1)	>barley|TC132933	Glycine-rich cell wall structural protein 1.8 precursor (GRP 1.8)	unclassified/cell wall	97	1297	3.9e-54	BAD62279
N - 03 : 7	Hv0000403GL	AJ888871	Bgh 12 (2), 24 (3); Bgt 12 (2), 24 (3)	>barley|TC147768	IAP100; putative chloroplast inner envelope protein	unclassified	96	3034	9.2e-133	AAG13554
P - 08 : 5	GAN003P08F	CX631913	Bgh 12 (2), 24 (1); Bgt 12 (1)	>barley|TC132933	Glycine-rich cell wall structural protein 1.8 precursor (GRP 1.8)	unclassified/cell wall	98	1319	4.0e-55	BAD62279
P - 13 : 7	HvD00115	DN155185	Bgh 12 (1), 24 (2); Bgt 12 (1), 24 (2)	>barley|BE421330	Oxygen-evolving enhancer protein 3-1, chloroplast precursor (OEE3)	housekeeping	99	971	2.0e-39	XP_478627
P - 06 : 7	Hv000017MK	AJ582214	Bgh 12 (2), 24 (3); Bgt 12 (2), 24 (2)	>barley|TC146499	chlorophyll a/b-binding protein II precursor	housekeeping	98	3543	1.2e-155	AAT81763
K - 18 : 3	GAN002K18F	CX631758	Bgh 12 (1), 24 (3); Bgt 12 (2, 24 (2)	>wheat|CD883519	FPF1 protein (Flowering Promoting Factor 1)	membrane traffic / signaling	78	331	8.5e-09	AAR97604

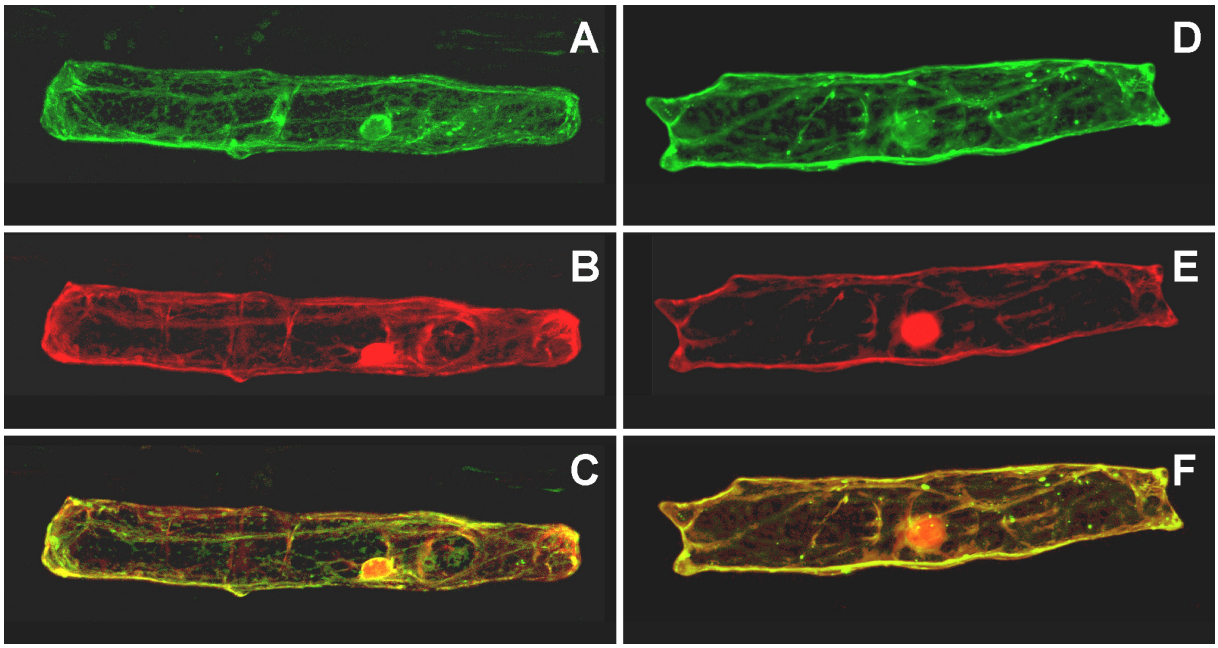
(a): Yellow shadowed boxes indicate potentially chimeric clones

(b): Differential expression at 12 or 24 HAI is indicated for ***Bgh***, ***Bgt*** or both. The number of biological and technical repetitions that gave positive results is given in brackets.
Red numbers indicate up-regulation, green numbers indicate down-regulation. Magenta colored boxes indicate ESTs that were identified only in one biological repetition but were confirmed by further ESTs of the identical unigene or by independent means such as RT-PCR

(c) TIGR barley database accessible under http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=barley. Please note that some clones matched the same TIGR entry and thus represent the same unigene.



Supplementary figure 2: BCL-X_L but not barley BI-1 protects yeast cells from BAX-induced lethal effects. A and B Yeast cells containing BAX under the control of a galactose-inducible promoter or the corresponding empty vector were co-transformed with plasmids containing either barley BI-1 (HvBI-1) or mammalian BCL-X_L. Cells were grown on SD-Leu/-Ura/glucose plates for five days. **A** Single colonies were serial 10-fold diluted (from left to right) and then transferred onto either glucose- or galactose-containing plates. Plates were photographed after 5 d of growth at 30°C. **B** Single colonies were checked for the presence of vector containing BAX, HvBI-1 or BCL-X_L by PCR with specific oligo DNA primers. Primers 5'-GGCCAGGCAACTTTAGTG-3' and 5'-TGATCTGTTCAGAGCTGGTG-3' were used to amplify a 281 bp *BAX*-specific fragment; primers 5'-GGCCAGGCAACTTTAGTG-3' and 5'- GTCGACGCGGTGACGGTATCTACATG-3' were used to amplify a 979 bp barley *BI-1*-specific fragment; and primers 5'-GGCCAGGCAACTTTAGTG-3' and 5'-TCTGGGAAAGCTTGTAGGA-3' were used to amplify a 287 bp *BCL-X_L*-specific fragment.



Supplementary figure 3: Comparison of the subcellular localization of GFP-BI-1 and GFP-BI-1ΔC fusion constructs. Confocal laser scanning whole cell projection of barley epidermal cells transiently expressing either *GFP-BI-1* (**A-C**) or *GFP-BI-1ΔC* (**D-F**) together with *DsRED*. The plasmids were coated onto micro-projectiles and delivered into barley leaf epidermal cells. By 72 h after transformation, distribution of GFP-BI-1 (**A**), GFP-BI-1ΔC (**D**) and DsRED fluorescence (**B, E**) was detected. **C** and **F** represent the respective merged images.

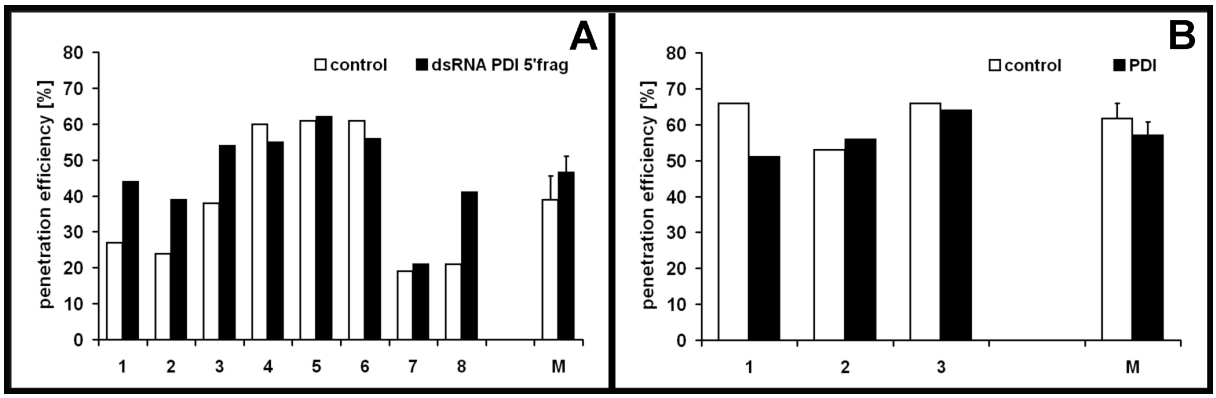
Supplementary table 2: Open reading frame of the *PROTEIN DISULFIDE ISOMERASE* gene

name	sequence	
PROTEIN DISULFIDE ISOMERASE (PDI) **GenBank accession number: L33250**	CAGTTCCGCC **ATG**GCGATCT CCAAGGTCTG GATCTCGCTG CTGCTCGCGC TTGCCGTCGT	60
	CCTGTCCGCC CCGGCGGCCA GGGCGGAGGA GGCCGCCGCC GCGGAGGAGG CCGCGGCCCC	120
	CGAGGCCGTG CTCACCCTGC ACGCCGACAA CTTCGACGAC GCCATCGGCC AGCACCCCTT	180
	CATCCTCGTC GAGTTCTACG CCCCATGGTG TGGACACTGC AAGAGCCTGG CACCGGAGTA	240
	TGAGAAGGCG GCCCAGCTGT TGAGCAAGCA CGACCCAGCG ATTGTTCTTG CTAAGGTTGA	300
	TGCCAACGAT GAGAAGAACA AGCCGCTTGC GGGCAAGTAC GAGGTCCAGG GCTTCCCTAC	360
	CCTTAAGATC TTCAGGAACG GAGGAAAGAG CATTCAGGAA TACAAGGGTC CCAGGGAGGC	420
	CGAGGGAATT GTTGAATACT TGAAGAAGCA GGTTGGCCCT GCTTCCAAGG AGATCAAGGC	480
	ACCTGAAGAT GCCACTTACC TTGAAGACGG CAAGATCCAC ATTGTTGGTG TTTTCACGGA	540
	ATTCAGTGGC CCTGAGTTTA CGAACTTCCT TGAGGTTGCT GAGAAGCTGC GGTCTTATTA	600
	TGACTTTGGC CACACTGTGC ATGCCAACCA TCTCCCACGT GGTGATGCAG CAGTGGAGAG	660
	GCCAGTGGTC AGGCTATTCA AGCCATTTGA TGAGCTCGTT GTTGATAGCA AGGATTTTGA	720
	TGTTTCTGCT TTGGAGAAAT TCATTGATGC TAGCAGCACC CCGAAAGTTG TTATTTTTGA	780
	TAAGAACCCT GACAACCATC CGTACCTCTT GAAATTCTTC CAGAGCAATG CTCCCAAGGC	840
	CATGCTCTTT TTGAACTTCT CCACTGGACC GTTTGAGTCC TTCAAATCAG CCTACTATGG	900
	TGCTGTAGAG GAGTTCAGTG GCAAGGATGT CAAGTTCCTA ATTGGTGACA TTGAATCGAG	960
	CCAAGGGGCT TTCCAGTACT TTGGGCTGAA AGTCGACCAG GCACCACTTA TCCTCATTCA	1020
	AGACGGTGAC TCCAAGAAGT TTTTGAAGGA ACATGTTGAG GCTGGCCAAA TCGTTGCTTG	1080
	GTTGAAGGAT TACTTTGATG GTAAATTGAC TCCATTCAGG AAGTCCGAGC CTATTCCTGA	1140
	GGCGAACAAT GAGCCTGTGA AGGTAGTTGT GGCTGACAAC GTTCATGACG TGGTCTTCAA	1200
	ATCTGGCAAA AATGTTCTTA TTGAGTTCTA TGCGCCCTGG TGCGGACACT GCAAGAAGCT	1260
	AGCACCCATC TTGGACGAGG CAGCTGCCAC TCTTCAAAGC GAAGAGGACG TTGTGATCGC	1320
	GAAGATGGAC GCCACCGAGA ATGACGTGCC GGGCGAGTTT GATGTCCAGG GTTACCCGAC	1380
	CCTGTACTTC GTCACTCCCA GCGGGAAGAA GGTCTCTTAT GAGGGTGGCA GGACGGCCGA	1440
	CGAGATCGTT GACTACATCA GGAAGAACAA GGAGACTGCT GGGCAGGCGG CGGCGGCGAC	1500
	CGAGAAGGCG GCGGAACCGG CTGCCACCGA GCCTTTGAAG GATGAGCTC**T GA**GCAACAGT	1560
	CTTTCTAGCA GCAGACAGGT AGAGGATGGG GAAACATGTT TTGGCAAGGC AGATTCCAAC	1620
	GCCAGATTTT GCGAGGGGGG TCGAGAGTTG GTTGTTGGAT GTGTTGGCCC CGGTTTTGCC	1680
	TGATACTGTA TCCCGTTGCG AGAAATACTG TAACAAATCT TCAGTCGTGT ACTTAGTTAA	1740
	ATTATCGAGA AGTGACAGTT GAAATGTTTG GTGGAGAGCT CTGGTAATAA ATGGCACTTC	1800
	TGTTCCTGGC AGAATC	1816

Bold: Assumed start (ATG) and stop (TGA) codons of the *PDI* open reading frame.

Shaded in black: Annealing sites of the primers, which comprise the 997 bp PDI 5' fragment, which was used as template for *in vitro* synthesis of double stranded RNA for the RNA interference experiments (see Supplementary figure 4A).

Arrows: Annealing sites of the primers used for the amplification of the *PDI* open reading frame, which was used in the *PDI* overexpression experiments (see Supplementary figure 4B).



Supplementary figure 4: Impact of *PROTEIN DISULFIDE ISOMERASE* (*PDI*) RNA interference and overexpression on the interaction of barley with *Bgh*. A Ballistic transfer of double-stranded RNA (dsRNA) into barley epidermal cells initiates the onset of a process called RNA interference, leading to gene silencing (RNAi; Schweizer *et al.* 2000b). *In vitro* synthesized dsRNA corresponding to a 1 kb *PDI* 5' fragment (see Supplementary table 2) was delivered into epidermal cells of barley cv. Ingrid together with GFP as transformation marker. In control experiments, GFP together with dsRNA of a human thyroid hormone receptor, which shows no homology to plant sequences, was used. 4 h after transformation, barley leaf segments were inoculated with spores of *Bgh*. In 8 independent experiments, penetration efficiency, i.e. the number of penetrated cells divided by number of attacked cells multiplied by 100, was assessed. **B** Leaf segments of barley cv. Ingrid were co-transformed with *PDI* and *GFP* expression constructs, in which both genes were under the control of the CaMV 35S promoter. Leaf segments were inoculated with *Bgh* 4 h after transformation and penetration efficiency was assessed. *PDI* plasmids deriving from two different clones out of one transformation event were used for these experiments. For information on the *PDI* full-length sequence see Supplementary table 2. M represents average values of independent experiments. Bars represent standard errors.

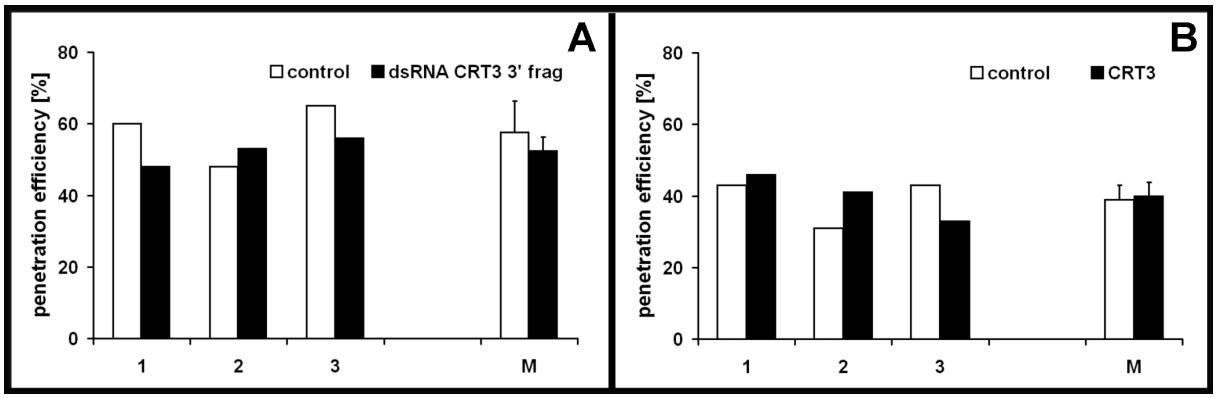
Supplementary table 3: Putative open reading frame of the *CALRETICULIN 3* gene

name	sequence
putative *CALRETICULIN3* (*CRT3*) TIGR database accession TC131592	CGGCACGAGG GCAGCCACCA CCTACTCTTC GTCTCCGGCG **ATG**TGTAGCA GCCGCCGGCG 60 GGGCGACCGC CAGCTCCAGC TCCTGCACCG CCTCCTCGCG CTCTCCTCGC TGCTCCTGCT 120 CGCCTCCGGG GAGGTCATCT TCGAGGAGCG GTTCGAAGAT GGTTGGGAGA CACGTTGGGT 180 GAAATCCGAT TGGAAAAGGA GCGAAGGGAA AGCCGGTACA TTCAAGCACA CGGCAGGGAA 240 ATATTCTGGG GATCCTGATG ACAAAGGCAT TCAAACAACG ATAGATGCTA GGCATTTTGC 300 CATCTCAGCC AAGATACCGG AGTTCAGTAA CAAGGGCCGA ACATTGGTGG TCCAGTACTC 360 CATAAAGTTT GAGCAGGAAA TTGAATGTGG CGGCGGCTAT ATTAAGCTAA TGTCTGGTTA 420 TGTCAATCAG AAGAAATATA GTGGAGACAC TCCATACAGC TTGATGTTTG GGCCAGATAT 480 ATGCGGGACT CAAACAAAGA AGCTGCATCT TATACTCTCT TACCAGGGGC AGAACTATCC 540 TATCAAAAAA GATCTACAAT GCGAAACCGA CAGGCTCACG CATGTTTACA CATTCATTCT 600 TAGGCCCGAT GCATCTTATA GTTTACTTGT TGATAACCGT GAAAGAGAAT CTGGGAGCAT 660 GTACACTGAT TGGGACATCT TGCCTCCTCG TAAAATCAAG GATGTTGGTG CCAAAAAGCC 720 TAAGGATTGG GATGACAGAG AGTATATTGA GGATCCCGAT GCGGTTAAAC CTGAGGGCTA 780 TGATTCTATT CCAAGAGAGA TTCCTGACCC AAAGGATAAG AAGCCCGACA CGTGGGACGA 840 TGATGATGAT GGCATATGGA AACCTAGGAG GATACCGAAT CCAGCATACA AAGGACAATG 900 GAAGCGCAAG AAAATTAAGA ACCCTAACTA CAAGGGTAAA TGGAAGATCC CATGGATTGA 960 TAATCCAGAG TTTGAGGATG ATCCAGATTT ATATGTACTG AAACCTCTGA AGTATATTGG 1020 AATTGAAGTT TGGCAGGTAA AAGCCGGTTC TGTTTTTGAC AACATCCTCA TTTGCGATGA 1080 CCCGGAATAT GCAAAACAGG TTGCCGATGA AACGTGGGGT GCAAATAAGG AGGCTGAAAA 1140 GGAGGCTTTT GAAGAAGCTG AAAAGGAGAG GAAAGCTAGA GAAGATAAGG AAGCTCAACA 1200 AGCAAGGGAG GAAGGAGAGC GACGGAGGAG AGAGAGGGGT GATCGACACC GTGGCAGGGA 1260 CCACTACAAG GACAGATACA AAAGACGCAA CAGGGATCAC TGGGATGACT ACCATGATGA 1320 GCTT**TGA**GGC CCCAAAAATC TTCCATTATT CGAAGGAGAA GCTGGGATTT GACATGAACA 1380 TTGCGTTATA GTTGCTGCCA ATAATTGAAG TGTTCCTTGT CTCGTGTGAG GGAAACTGAT 1440 TGAATTGACC ACGAAGATCA TTCCTATGTG ACAGCAGGGC AGACAATTCC ACCTATCCTC 1500 AAAAGTCAAA AACTTGGCCT TGGCCCTGAA ATCTGATGTT GTGAGTGTAA TGTACATGGT 1560 CAGCGATGTG CTTCCTTTTG TAAACAGCTG TTGCTAATAC AAATAAAGAG ATTTTCAAGC 1620 ATAATTCAAA TGACTATTGT TGC 1680

Bold: Assumed start (ATG) and stop (TGA) codons of the *CRT3* open reading frame.

Shaded in black: Annealing sites of the primers, which comprise the 775 bp *CRT3* 3' fragment, which was used as template for *in vitro* synthesis of double stranded RNA for the RNA interference experiments (Supplementary figure 5A).

Arrows: Annealing sites of the primers used for the amplification of the *CRT3* open reading frame, which was used in the *CRT3* overexpression experiments (Supplementary figure 4B).

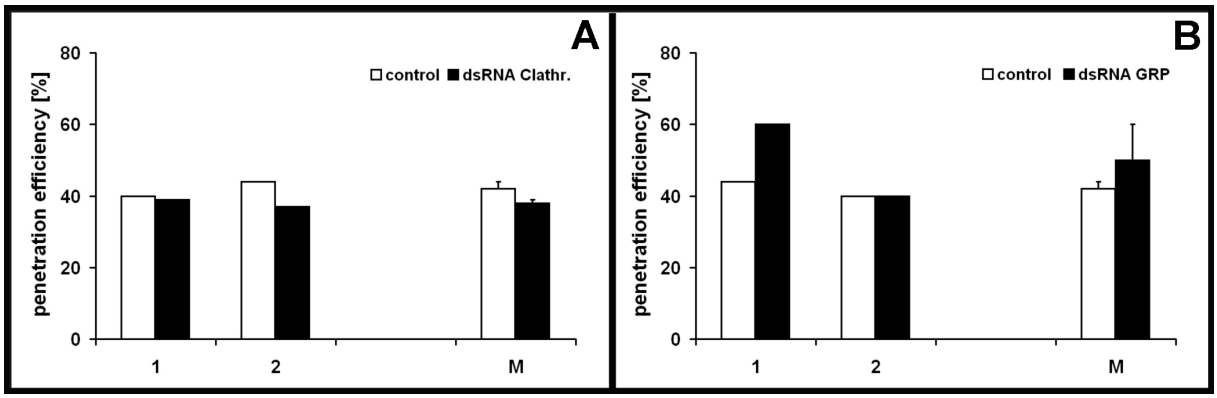


Supplementary figure 5: Impact of *CALRETICULIN 3* (*CRT3*) RNA interference and overexpression on the interaction of barley with *Bgh*. A and B *In vitro* synthesized dsRNA corresponding to a 775 kb *CRT3* 3' fragment (Supplementary table 3) was delivered into epidermal cells of barley cv. Ingrid together with GFP as transformation marker. In control experiments, GFP together with dsRNA of a human thyroid hormone receptor, which shows no homology to plant sequences, was used. 4 h after transformation, barley leaf segments were inoculated with spores of *Bgh*. In 8 independent experiments, penetration efficiency, i.e. the number of penetrated cells divided by number of attacked cells multiplied by 100, was assessed. **B** Leaf segments of barley cv. Ingrid were co-transformed with *CRT3* and *GFP* expression constructs, in which both genes were under the control of the CaMV 35S promoter. Leaf segments were inoculated with *Bgh* 4 h after transformation and penetration efficiency was assessed. For information on the *CRT3* full-length sequence see Supplementary table 3. M represents average values of independent experiments. Bars represent standard errors.

Supplementary table 4: Sequence fragments of a *CLATHRIN COAT ASSEMBLY LIKE PROTEIN* gene and a *GLYCINE-RICH CELL WALL STRUCTURAL PROTEIN* gene, which served as templates for *in vitro* synthesis of double stranded RNA.

name	sequence
CLATHRIN COAT ASSEMBLY LIKE PROTEIN **TIGR database accession TC133627**	1 CGGCACGAGG GAACCATCCA AATGAAAAGT TACCTTAGTG GGAATCCAGA 51 AATCCGTCTA GCTCTGAATG AGGATTTGGG CATTGGGAAA GAATAGCTCT 101 TCTACACATG ATTACAGAAG TTCTTCTGGA GGAGGATCCG TCGTTCTTGA 151 TGATTGTAAC TTCCATGAGT CAGTGCAGCT CGACAGTTTT GACATTGACA 201 GAACTCTGCA TTTAATACCA CCTGATGGAG AATTTCCAGT GATGAACTAC 251 CGGATGACTC AAGAATTTAA GCCACCCTTC CGCGTTACTG CACTAATTGA 301 AGAAGCTGGC CCATCTAGGG CTGAAGTTCT ATTGAAAATA CGGGCAGACT 351 TTCCTGCGAA TGTCACTGCT AACACAATCA CAGTACAGAT GCCAGTACCC 401 TCTTACACTA TGAGGGCAAG TTTTGAACTG GAAGCTGGGG CAGTTGGACA 451 GACAACGGAT TTTAAAGAAG GAACTAGGAG ACTGGAGTGG AATCTAAAGA 501 AGATTGTTGG TGGTTCCGAG CATACCCTTC GTGCAAAGCT CACATTTTCT 551 CAGGAGACAC ATGGAAACCT CACAAAGGAA GCCGGACCAG TAAACATGAA 601 CTTCACTATA CCTATGTACA ATGCTTCAAA GTTACAGGTC AGATATCTTC 651 AGATAGCGAA GAAATCCAAA ACATACAATC CATACAGGTG GGTCAGATAT 701 GTGACACAGG CCAACTCGTA TGTAGCCCGG CTATGAGGAC ATTGTTTGTA 751 CAATTCTTCC AAAGGCCCTT GTGCAACTTT CTTTTAGTGT TAATTTTTTC 801 TTCTGTAATA TGCAGAAAAT GCATGATGGT TGTTTGTATT CTGAGCAGTG 851 AATAAATTTC ATGTTCGTCT GGGA
GLYCINE-RICH CELL WALL STRUCTURAL PROTEIN (GRP) **TIGR database accession TC132933**	1 ATCAAACTGA TCATCGCCAA TGGCGGTCAA GTCTCTGGTT CTTCTTGGTG 51 TCGTACTAGC CTCGCTCCTG CTTCTCTCCG AGGATGTAGC AGATGCTAGA 101 GAACTTACTG ATGCTAAAGA GTCAGAGGAG AAGAATGTGA AACCTACAAG 151 AGGGCCGGGC TTGACTAAGG ATGAGAAGTG GGGAGGTGGA AACAAGCATG 201 ATGGAGGATA CGGAAACGGT GGAGGGTATG GAAACGGTGG TGGATATGGT 251 AACGGTGGGG GATATGGAAA CAATGGCGGG GGCGGTGGAA ACGGTGGGGG 301 GTACGGGAAC GGCGGTGGAG GCTATGGAAA CGGTGGATAC CGGAACAATG 351 GTGGAGGTTA TGGCAATGGA GGTTATGGAA ACAATGGTGG CGGGTATGGT 401 GGAGGATACG GCAATTCTGG ACATGGTGGC GGTTATGGGC GTGGTTATGG 451 CGGTGGAGGC TATGGACCTG GATATGGTGG CGGGTATGGC AATGGTGGTG 501 GAAATGGTGG GTTTGGTGGG GGATATGGTG GTGGCGGTGG ATACGGTGGC 551 GGTGGTGGGT ATGGTGGAGG TTACGGTGGA GGATCTGGTG GCGGTGGCTA 601 TCCTTGAGAT GGATAGGTGC AAGATGTCAC AATGGTTGGA ACTATGGAAA 651 CAACCACTAG ATATGGGTTT ATGTTTTAAC TAAGAATCTG TTGGGTACGT 701 CATGTGGTTA AATAAAAAAA TGTTCCTTAT GCTCGTTATA GGAGCAAGGT 751 CCGTAAGGTT GAGAAGTCAG ACGAGCAAGC CAGCCTTGTA TTATCTCCTA 801 TGTATGGAAG AGAAGATGAC TTCTAAATTG TAAGTGGGCT TTGGTTATTG 851 ATTTTCATTT TCAAGAACAT GAGAAGATCT GTGTGCCTAT ATATTATGAT 901 AGGAAACATT CATGATTTAC AAGAATAGCT CCATCCCACA AGCAAAGGAC 951 TTATGGTACT ACTCTTCTT

Shaded in black: Annealing sites of the primers, which comprise the 356 bp *CLATHRIN COAT ASSEMBLY LIKE PROTEIN gene* fragment, and the 651 bp *GRP* gene fragment which were used as template for *in vitro* synthesis of double stranded RNA for the RNA interference experiments (Supplementary figure 6).



Supplementary figure 6: Impact of RNA interference with a *CLATHRIN COAT ASSEMBLY LIKE PROTEIN* gene and a *GLYCINE-RICH CELL WALL STRUCTURAL PROTEIN* gene, respectively, on the interaction of barley with *Bgh*. *In vitro* synthesized dsRNA corresponding to a 356 kb *CLATHRIN COAT ASSEMBLY LIKE PROTEIN* gene fragment (**A**) and a 651 bp *GLYCINE-RICH CELL WALL STRUCTURAL PROTEIN* gene fragment (**B**) was delivered into epidermal cells of barley cv. Ingrid together with GFP as transformation marker. In control experiments, GFP together with dsRNA of a human thyroid hormone receptor, which shows no homology to plant sequences, was used. 4 h after transformation, barley leaf segments were inoculated with spores of *Bgh* and the penetration efficiency, i.e. the number of penetrated cells divided by number of attacked cells multiplied by 100, was assessed. M represents average values of independent experiments. Bars represent standard errors. For fragment sequences see Supplementary table 4.

Ich erkläre:

Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Danksagung

Zunächst möchte ich Herrn Prof. Karl-Heinz Kogel für die Überlassung des Themas und für sein Vertrauen in meine wissenschaftliche Arbeit danken. Ich freue mich, dass er mir über Jahre hinweg die Möglichkeit gegeben hat, in diesem ausgesprochen angenehmen Umfeld zu arbeiten.

Herrn Prof. Wolfgang Friedt danke ich herzlich für die bereitwillige Übernahme des Koreferats.

Eine Menge Menschen haben mich im Labor tatkräftig unterstützt und dabei durch ihr positives Wesen dafür gesorgt, dass ich mich an die Zeit am Institut immer mit Freude erinnern werde. Bei ihnen möchte ich mich an dieser Stelle herzlich bedanken:
Zu meinem Glück wurde die Betreuung der Arbeit in die Hände von Ralph Hückelhoven gelegt. Mit seinen Ideen, seiner kompetenten Beratung zur Durchführung der Versuche sowie seiner geduldigen Bereitschaft zur Diskussion hatte er den größten Anteil am Gelingen dieses Projekts. Für seine große Unterstützung bin ich mehr als dankbar und äußere mich daher an dieser Stelle auch nicht zum Thema „Blaue Stutzen“. Jutta Preuss hat im Rahmen ihrer Masterarbeit zu den Ergebnissen beigetragen, Cornelia Dechert leistete wertvolle technische Hilfe und Rebekka Fensch hat für die Verschlüsselung meiner Schälchen oft alles stehen und liegen gelassen – vielen Dank dafür! Holger Schultheiss danke ich, weil er mir mehr als einmal mit seinen technischen Tipps geholfen hat und auch sonst ein guter Ratgeber war und ist. Katrin Humbroich, Dagmar Biedenkopf und Christina Neumann danke ich für die Begleitung und gute Unterhaltung während des Joggens und für ihre Freundschaft. Ich freue mich, dass ich auch meine (ehemaligen) Kollegen Christina Eckey, Marco Trujillo und Carin Jansen heute zu meinen besten Freunden zählen darf – für all die guten Gespräche, für Hilfe und kulinarische Fürsorge herzlichen Dank! Allen anderen aktuellen und ehemaligen Angehörigen des Instituts für Phytopathologie und Angewandte Zoologie danke ich ebenfalls dafür, dass Sie mir mit Rat und Tat zur Seite standen, und für die gute Unterhaltung.

Mein besonderer Dank gilt Walter und Margret Schäfer sowie Carin Jansen für die gewissenhafte Durchsicht des Manuskripts und ihre kritischen und hilfreichen Anmerkungen dazu.

Meine lieben Eltern und Geschwister haben meiner Arbeit oft mehr Interesse entgegengebracht, als ich bereit war zu stillen. Für ihre liebevolle Unterstützung bis heute kann ich mich gar nicht genug bedanken.

Schließlich meinem lieben Patrick herzlichen Dank für seine unendliche Geduld mit mir, seine Unterstützung und gute Laune, mit denen er mich in den letzten Jahren begleitet hat.

Curriculum vitae

Persönliche Angaben

Name:	Ruth Maria Eichmann
Geburtsdatum:	04. März 1977
Geburtsort:	Usingen
Familienstand:	ledig

Schulausbildung

1983 – 1987	Besuch der Grundschule in Usingen
1987 – 1989	Besuch der Förderstufe in Usingen
1989 – 1997	Besuch der Christian-Wirth-Schule in Usingen

Studium und Beruf

Oktober 1997	Beginn des Studiums der Agrarwissenschaften an der Justus-Liebig-Universität Gießen
November 2000 – November 2001	Diplomarbeit bei Prof. K.-H. Kogel am Institut für Phytopathologie und Angewandte Zoologie der Justus-Liebig-Universität Gießen
April 2002	Erhalt des akademischen Grades einer Diplom-Agraringenieurin
Mai 2002 – Mai 2005	Wissenschaftliche Mitarbeiterin am Institut für Phytopathologie und Angewandte Zoologie der Justus-Liebig-Universität Gießen