

TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Phytopathologie

The impact of transgenic expression of barley (*Hordeum vulgare*)
RHO-like GTPases on plant development and disease susceptibility

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:

Univ.-Prof. Dr. J. Schnyder

Prüfer der Dissertation:

1. Univ.-Prof. Dr. R. Hückelhoven
2. Univ.-Prof. Dr. J. Durner

Die Dissertation wurde am 09.06.2009 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 18.08.2009 angenommen.

To my father and mother...

Parts of this work have already been published:

- **Pathuri IP, Imani J, Babaeizad V, Kogel KH, Eichmann R, Hückelhoven R** (2009) Ectopic expression of barley constitutively activated ROPs supports susceptibility to powdery mildew and bacterial wildfire in tobacco. European Journal of Plant Pathology, DOI: 10.1007/s10658-009-9484-5
- **Pathuri IP, Eichmann R, Hückelhoven R** (2009) Plant small monomeric G-proteins (RAC/ROPs) of barley are common elements of susceptibility to fungal leaf pathogens, cell expansion and stomata development. Plant Signaling & Behavior, 4: 109-110.
- **Pathuri IP, Zellerhoff N, Schaffrath U, Hensel G, Kumlehn J, Kogel KH, Eichmann R, Hückelhoven R** (2008) Constitutively activated barley ROPs modulate epidermal cell size, defense reactions and interactions with fungal leaf pathogens. Plant Cell Reports, 27: 1877-1887.

Abbreviations

BI-1	BAX INHIBITOR-1
CA	Constitutively activated
CWA	Cell wall apposition
DAB	3,3-Diaminobenzidine
DN	Dominant negative
GTP	Guanosine tri phosphate
GDP	Guanosine di phosphate
HAI	Hours after inoculation
HR	Hypersensitive response
Hv	<i>Hordeum vulgare</i> (barley)
MLA	MILDEW LOCUS A
MLO	MILDEW LOCUS O
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
Nt	<i>Nicotiana tabacum</i> (tobacco)
Os	<i>Oryza sativa</i> (rice)
PCD	Programmed cell death
<i>Pst</i>	<i>Pseudomonas syringae</i> pv. <i>tabaci</i>
RAC	Ras related C3 botulinum toxin substrate
RAR	Required for MLA specific resistance
RBOH	Respiratory burst homologue
RHO	Rat sarcome oncogene product (RAS) homologue
ROP	RHO of plants
ROR	Required for mlo specific resistance
ROS	Reactive oxygen species

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1. Introduction

Global food security is one of the major concerns of the world today. In order to ensure increased nutrition for a growing population, it will be necessary to expand food production faster than population growth. The only solution to this problem is to increase the yields of major food crops, particularly cereal grains, using currently available land and less water. In terms of food production, biotic constraints like pests and pathogens take a heavy toll by up to 30 % reduction in crop yields worldwide (Christou *et al.*, 2004) despite the large scale usage of pesticides. In this context, the scientific field of *phytopathology* can address this issue effectively by investigating the etiology and epidemiology of plant diseases to reduce crop loss for achieving future food security. As a part of evolution, both plants and pathogens have developed a multitude of mechanisms for their defense and infection strategies, respectively. Hence, to develop effective, durable, economic and environmentally sound strategies for the control of crop diseases, an improved understanding of the mechanisms of disease development and the molecular networks involved in plant susceptibility at genetic and physiological level is needed.

1.1 Host-pathogen relationship

Plant disease is the result of infection by other organism that adversely affects the growth, physiological functioning and productivity of a plant. The plant, which is getting infected, is called host and the parasitic organism that causes disease is called a pathogen. Plant pathogens are often divided into biotrophs and necrotrophs, according to their lifestyles. Biotrophs are specialized to feed on living plant tissues. They are mostly obligate parasites and cannot grow in the absence of their host plant. Biotrophs have a narrow host range, and strains of these pathogens have often adapted to a specific line of a given plant species. Many biotrophs produce haustoria as feeding structures that invaginate the plasma membrane of host cells, enabling them to create a specific microenvironment for retrieval of nutrients. Examples for obligate biotrophs are powdery mildew fungi, downy mildews and rust fungi (Lawrence *et al.*, 1994; O'Connell and Panstruga 2006). Necrotrophic pathogens destroy host tissues

and derive nutrients from dead or dying cells. They usually grow on plant tissues that are wounded, weakened or senescent and frequently produce toxins to kill host tissue prior to colonization. They are often facultative parasites and can live as saprophytes in the absence of a living host. Necrotrophs usually have a broad host range. Examples of necrotrophs are *Fusarium graminearum*, which causes head blight on many cereals, and *Botrytis cinerea* known as gray mould fungus that infects many plant species (Van Kan 2006; Osborne and Stein 2007). There is another group of pathogens that behave as both biotrophs and necrotrophs, depending on the environmental conditions and the stages of their life cycles. Such pathogens are called hemibiotrophs. Examples of hemibiotrophic plant pathogens are *Magnaporthe oryzae*, responsible for rice blast disease, and various *Colletotrichum* species that cause anthracnose diseases in many plants (Ribot *et al.*, 2008; Perfect *et al.*, 1999).

1.2 The barley-powdery mildew pathosystem

1.2.1 Barley - the host plant

Barley (*Hordeum vulgare*) is a domesticated grass family member which is regarded as the most valuable cereal grain after rice, wheat and maize. Barley is one of the most ancient crop plants and serves as a major animal feed crop, with smaller amounts used for malting and in health foods. Taxonomically, barley belongs to the kingdom Plantae, the division of Magnoliophyta, the class Liliopsida, the order of the Poales, the family Poaceae, and the genus *Hordeum*. Barley species are distributed over many parts of the world, mostly in temperate regions. Barley is a diploid, self-pollinator with seven pairs of chromosomes and has been extensively studied both genetically and cytologically (Jørgensen 1994; Nilan 1964). Barley ranks fourth in quantity produced and in area of cultivation of cereal crops in the world. Barley is widely adaptable and is currently a major crop of the temperate and tropical areas.

Like other crops, barley often suffers from various diseases. The damage caused by any disease will depend upon the genetic constitution, susceptibility of the cultivar variety and the environmental conditions during disease development. A variety of pathogens infect different organs of barley plants, which ends up in

several diseases. Common pathogens that attack barley leaves and stems are *Puccinia hordei* causing rust disease, *Blumeria graminis* f.sp. *hordei*, causing powdery mildew, *Xanthomonas* species, causing bacterial blight, and barley yellow dwarf virus, causing barley yellow dwarf disease. Barely pathogens that infect head and seeds are *Fusarium* species, causing head blight, *Ustilago* species, causing smut diseases, and *Claviceps purpurea*, causing ergot disease. Pathogens that cause barley crown and root rot are *Cochliobolus sativus* and *Pythium* species. In barley, it is estimated that foliar diseases predominantly cause up to 25 % yield losses (James 1969). Amongst all, barley powdery mildew disease is the most destructive foliar disease of barley and may cause up to 40 % yield losses in temperate regions (Jørgensen 1988).

1.2.2 Barley powdery mildew fungus

Powdery mildew appears as a dusty white to gray coating over leaf surfaces and other aerial plant parts. The disease is distributed worldwide and is most damaging in cool and wet climates. This disease is caused by a class of obligate biotrophic fungi. Powdery mildews taxonomically belong to kingdom *Fungi*, the division of the *Ascomycota*, the class of *Leotiomycetes*, the order of *Erysiphales*, and the *Erysiphaceae* family. This family currently consists of 21 genera such as *Blumeria*, *Erysiphe*, *Golovinomyces* and others. The genus *Blumeria* includes only one species *B. graminis*, commonly known as cereal powdery mildew fungus. *B. graminis* is classified into eight *formae speciales* (*ff. spp.*) that are strictly adapted to colonize individual genera of the grass family. These include *B. graminis* f. sp. *hordei* (*Bgh*) on barley, *B. graminis* f.sp. *tritici* (*Bgt*) on wheat etc.. The barley-*Bgh* interaction is compatible and results in powdery mildew disease, whereas the barley-*Bgt* interaction is incompatible due to non-host resistance (Wyand and Brown 2003; Heath 1981).

During the life cycle of *Bgh*, its haploid form prevails except for a short diploid phase after mating that includes the formation of cleistothecia. Asexual reproduction is common during the growing season, which involves the formation of conidiophores and production of asexual haploid spores called conidia. Superficial mycelium and conidia-producing conidiophores are responsible for the powdery appearance of the disease symptoms. Spores are

blown away by wind to other parts of the plant or to other plants to start a new infection cycle. Infection by conidia requires high humidity, whereas sporulation and spore dispersal favor drier conditions. Airborne spores can migrate hundreds of kilometers (Jørgensen 1994). During winters, the fungus survives on infected plant parts and in debris such as fallen leaves. It produces sexual fruiting bodies known as cleistothecia that appear as small black dots within the white powdery patches and can resist harsh winter conditions. In the following spring, sexual spores (ascospores) get released from the cleistothecia and are carried away by the wind to begin a new infection. The barley-*Bgh* pathosystem emerged as a model system for investigating the mechanisms of susceptibility and resistance of obligate fungal biotrophs in cereals.

1.2.3 The compatible interaction between barley and *Bgh*

The barley-powdery mildew disease cycle commences once asexual conidia come into contact with the leaf surface. Under favorable conditions conidia start germinating within 2 hours after inoculation (HAI). After initial contact with the host surface, the conidia form a primary germ tube (PGT), which attaches to the leaf surface and forms a short peg that penetrates the cuticle (Edwards 2002). This structure serves only for adherence, water uptake and signal perception but does not invade the cell (Eichmann and Hüchelhoven 2008). The next phase of germination includes formation of an appressorial germ tube (AGT) (Fig. 1.1c). The AGT further elongates and differentiates into a hooked, apical mature appressorium that, with a penetration peg, breaches both the host cuticle and cell wall to make contact with the plasma membrane of a leaf epidermal cell during 12-16 HAI. At 16 HAI, a small haustorial initial appears and further develops into a mature haustorium, which invaginates the plant plasma membrane and can be clearly visible at 24 HAI. The haustorium is the sole fungal structure that is in intimate physical contact with the host plasma membrane and produces finger-like appendages when mature. The haustorial complex includes haustorium, extra-haustorial membrane and extra haustorial matrix. This complex is crucial for nutrient retrieval and subsequent mycelial growth on the leaf surface (Schulze-Lefert and Vogel 2000). Subsequently at 36 HAI, elongated secondary hyphae (ESH) develop from the appressorium (Fig. 1.1d). The ESH start

branching at 36-48 HAI and penetrate other host cells by forming new appressoria and secondary haustoria. The aerial mycelium produces conidiophores, which have a cudgel shaped basal-cell with about eight conidia attached to each other, forming a chain. When the conidia mature, they detach from the conidiophores and are dispersed by wind. The entire asexual life cycle takes approximately 5-6 days. Fungal growth at the leaf surface typically results in the formation of a single powdery mildew colony (Boyd *et al.*, 1995; Thordal-Christensen *et al.*, 2000) (Fig. 1.1b).

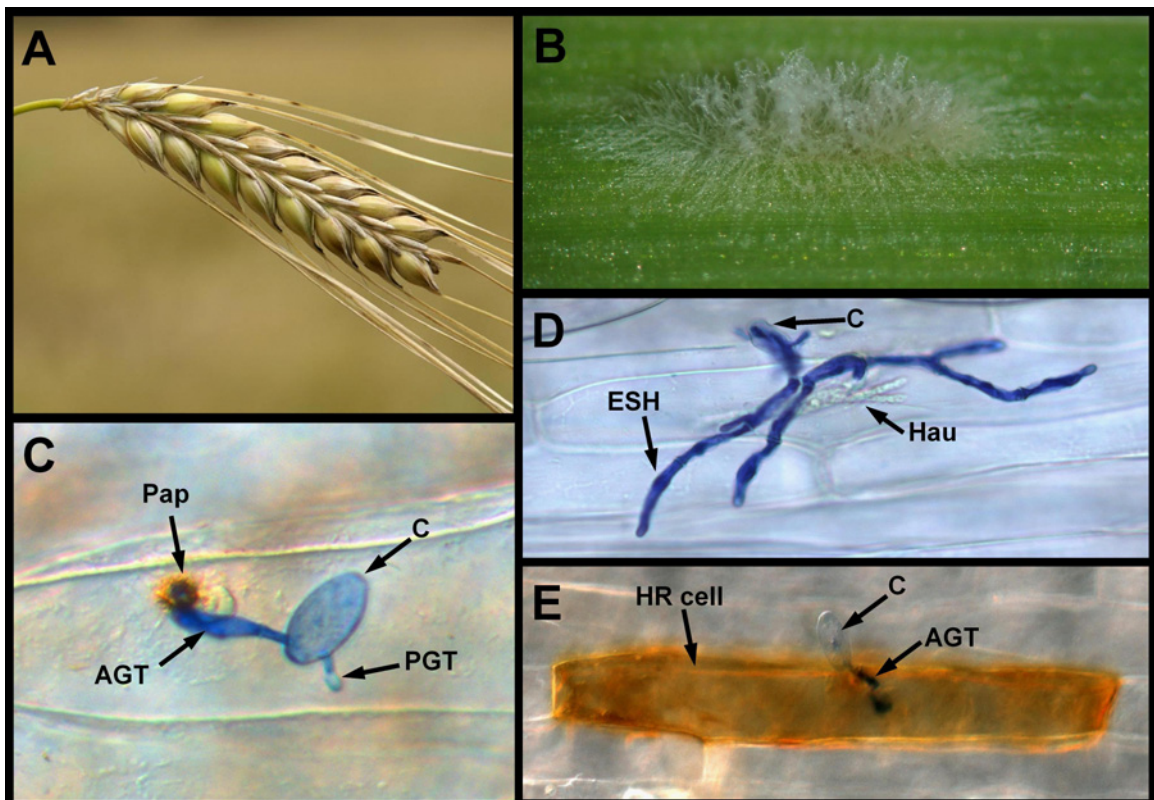


Figure 1.1 The interaction of barley with the barley powdery mildew fungus. A) Barley (*Hordeum vulgare*) ear (picture: http://www.freefoto.com/images/07/44/07_44_32_prev.jpg). B) Barley leaf surface showing a typical powdery mildew colony with mycelium and conidiophores produced by *Bgh* (picture: Ruth Eichmann, Chair of Phytopathology, TU Munich). C) *Bgh* conidium (C) germinates to form a primary germ tube (PGT) and an appressorial germ tube (AGT). A cell wall apposition, also called papilla (Pap), underneath an appressorium of *Bgh*. Brownish staining in the papilla resulted from the polymerization of diaminobenzidine, indicating the presence of hydrogen peroxide (picture: Ruth Eichmann). D) *Bgh* successfully penetrated into a barley epidermal cell by forming the intracellular feeding organ, a haustorium (Hau), and subsequently developed elongated secondary

hyphae (ESH). E) Hypersensitive Response (HR) of a barley epidermal cell attacked by *Bgh*. The whole-cell diaminobenzidine staining indicates hydrogen peroxide production. Superficial fungal structures in C-D were stained with blue ink.

1.3 Resistance

Plants are susceptible to only a limited number of pathogens. Most infections fail due to active defense mechanisms, or absence of basic compatibility of the host plant to a given pathogen (Heath 1981; Thordal-Christensen 2003). Many components of the plant immunity and defense mechanisms have been extensively studied in the past years. Resistance and susceptibility are opposite sides of the same coin. Constant efforts are being made from both plants and pathogens to succeed one over the other. As a part of this process, plants recognize pathogen-derived molecules and trigger defense responses to avoid the disease, and pathogens overcome plant defense mechanisms to make them susceptible to their infection. Plant-pathogen interactions end either in successful colonization of the host plant by the pathogen, thereby causing disease symptoms, or in an effective resistance response of the plant, preventing pathogen ingress. In the former case, the outcome of the interaction is susceptibility of the plant and in the latter case it is resistance.

1.3.1 Plant innate immunity

Plants have an innate immunity system to defend themselves against pathogens. The plant innate immune system recognizes and responds to molecules common to many classes of microbes, including non-pathogens. It operates through transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) of potential pathogens (Bittel and Robatzek 2007; Ingle *et al.*, 2006; Iriti and Faoro 2007; McDowell and Simon 2008; Zipfel 2008). Several PAMPs have been identified for plant pathogens, including flagellin, lipopolysaccharide and elongation factor Tu (EF-Tu) from gram-negative bacteria as well as chitin and β -glucans from fungi and oomycetes (Chisholm *et al.*, 2006; Gómez-Gómez and Boller 2002; Nürnberger and Brunner 2002; Nürnberger *et al.*, 2004). PRRs recognize a particular domain of a larger PAMP molecule that often possesses

structural or enzymatic functions that are crucial for the pathogen (De Wit 2007). The PRRs of plants include leucine rich repeat (LRR) receptor like kinases (RLKs), LysM receptor kinases and others (Altenbach and Robatzek 2007; Bittel and Robatzek 2007; Ingle *et al.*, 2006; Zipfel 2008). Upon PAMP recognition, PRRs induce defense responses such as cell wall alterations, deposition of callose and the accumulation of defense-related proteins including chitinases, glucanases and proteases, activation of mitogen-activated protein kinase (MAPK) signaling cascades and in some cases, hypersensitive response (HR)-like cell death (Bittel and Robatzek 2007; De Wit 2007; Ingle *et al.*, 2006). This innate immunity of plants is known as PAMP-triggered immunity (PTI), which accounts for basal disease resistance in plants (Iriti and Faoro 2007; Jones and Dangl 2006; Schwessinger and Zipfel 2008; Zipfel 2008). A paradigm for a PRR is FLS2 in *Arabidopsis*. FLS2 is a LRR-RLK that mediates flagellin (flg22)-induced primary defense response in *Arabidopsis* against the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. Presence of the FLS2 receptor in plants restricts bacterial growth, whereas lack of FLS2 makes plant more susceptible to this bacterium (Gómez-Gómez and Boller 2002; Zipfel *et al.*, 2004).

Successful pathogens overcome host immune responses with the help of effectors that interfere with defense signaling in host cells (Bent and Mackey 2007; Da Cunha *et al.*, 2007). This can be further encountered in resistant host plants, which recognize pathogen effectors and trigger defense responses resulting in so called effector triggered immunity (ETI) (Chisholm *et al.*, 2006; De Wit 2007; He *et al.*, 2007; Jones and Dangl 2006). This response to pathogen virulence factors largely operates from inside the cell. This is carried out by the polymorphic nucleotide binding and leucine-rich repeat (NB-LRR) proteins that are mostly encoded by specific disease *RESISTANCE* (*R*) genes (Bent and Mackey 2007; Dangl and Jones 2001). NB-LRR proteins recognize corresponding pathogen effectors and trigger ETI. The recognized effector is termed as *AVIRULENCE* (*AVR*) protein. Different models were reported to elucidate the mode of recognition of pathogen effectors by the NB-LRR R-proteins. The simplest form of recognition occurs via direct physical association of the pathogen effector with the R-protein, similar to a ligand binding to its receptor. (Caplan *et al.*, 2008; Deslandes *et al.*, 2003; Ellis *et al.*, 2007; Jia *et al.*, 2000). Another model,

'guard hypothesis' states that NB-LRR R-proteins indirectly recognize pathogens using an intermediary host factor which is referred to as guardee. According to this model, resistance proteins act by monitoring (guarding) the target of their corresponding pathogen effector (Caplan *et al.*, 2008; Dangl and Jones 2001; Van der Biezen and Jones 1998). Such an indirect detection mechanism allows a limited number of NB-LRR R-proteins to detect the activity of multiple pathogen effectors by monitoring the key targets of pathogenesis in the plant and respond when those targets are perturbed (DeYoung and Innes 2006; McHale *et al.*, 2006). The recent 'decoy model' states that the effector target monitored by the R-protein is a decoy that is specialized in perception of the effector by the R-protein but alone has no function either in the development of disease or resistance (Van der Hoorn and Kamoun 2008). R protein-mediated defense responses often culminate in a localized programmed cell death reaction known as hypersensitive response (HR) and additional locally induced defense responses such as expression of pathogenesis-related (PR) proteins and callose deposition at the site of infection that block further growth of the pathogen (De Wit 2007; Jones and Takemoto 2004). This would further induce the development of systemic acquired resistance (SAR) via the salicylic acid (SA) signaling pathway. SAR is a mechanism of induced defense that confers long-lasting protection against a wide range of microorganisms that often results in broad spectrum disease resistance in plants (De Wit 2007; Durrant and Dong 2004). NB-LRR mediated disease resistance is effective against biotrophic or hemibiotrophic pathogens, but not against necrotrophs (De Wit 2007; Jones and Dangl 2006). Several R-genes from monocots and dicots have been identified that are encoding R-proteins that detect a variety of bacterial, viral, fungal and oomycete effectors. An example for R-gene mediated resistance is the *MLA* (*MILDEW RESISTANCE LOCUS A*) locus of resistance specificities in barley. The polymorphic barley *MLA* locus encodes allelic receptors containing an N-terminal coiled-coil structure, a central nucleotide binding site and an LRR region. *MLA* receptors share high sequence similarities but recognize diverse AVR proteins from a different spectrum of races of the barley powdery mildew fungus, thus conferring race-specific resistance (Halterman *et al.*, 2001; Halterman and Wise 2004; Shen *et al.*, 2003) *MLA* steady-state levels are critical for effective

resistance and are subject to control by cytosolic heat-shock protein 90 (HSP90) and the co-chaperone-like proteins RAR1 and SGT1 (Bieri *et al.*, 2004; Hein *et al.*, 2005; Zhou *et al.*, 2001). The polymorphic C-terminal LRR region of MLA was identified to determine recognition specificity of the MLA receptor towards fungal avirulence effectors (Shen *et al.*, 2003), whereas the N-terminal CC domain mediate the induction of the nuclear associations between MLA receptor and WRKY transcription factors (Shen *et al.*, 2007). The effector-triggered MLA-WRKY association appears to contribute to receptor-triggered disease resistance and host cell death at attempted fungal infection sites and this association is assumed to provide a link to integrate signals generated by PRRs and R-proteins (Shen *et al.*, 2007; Shen and Schulze-Lefert 2007).

1.4 Defense mechanisms

During evolution plants have developed an armory of physical, chemical and biochemical defense mechanisms to survive in a hostile habitat and to overcome the peculiar constraints that result from a stationery way of life. Plants developed diverse defense strategies to encounter diverse plant pathogens. Defense mechanisms can generally either be preformed or induced. Preformed defenses include thick cell walls that are difficult to penetrate and a waxy cuticle that runs off water and dry plant surfaces rapidly, providing less support for the pathogens on the surface (Chrispeels and Sadava 2003; Martin 1964). In addition to physical barriers that block infection, plants also contain a diverse array of antimicrobial compounds. The co-evolution of plants with pathogens has resulted in the production of a large array of secondary metabolites that include phenolics, tannins, glycosides, flavonoids etc. and many of them function as defense compounds (Dixon 2001; Grayer and Kokubun 2001). Some antimicrobial compounds are always present in plants (so-called phytoanticipins) and many others get induced by the infection of a pathogen. Phytoalexins belongs to this class of antimicrobial compounds whose production gets triggered by pathogen infection (Chrispeels and Sadava 2003; Grayer and Kokubun 2001). However, some pathogens successfully avoid the preformed defense and start establishing an infection. Then, the induced defense mechanisms come into play, which are aimed more specifically towards

combating the pathogen. These mechanisms include formation of cell wall appositions, hypersensitive response, production of pathogenesis related proteins and others (Edreva 2005; Hüchelhoven 2007; Pontier *et al.*, 1998; Van Loon *et al.*, 2006).

1.4.1 Formation of cell wall appositions

Plants successfully defend the vast majority of potential pathogens that arrive on their surface. Resistance to penetration at the epidermis is a key component of basal defense against many biotrophic fungi and critically depends on the formation of cell wall appositions (CWA), also known as papillae at the site of attempted penetration (Aist 1976; Israel *et al.*, 1980). In barley-*Bgh* interaction, the fungal spore attempt to penetrate the host epidermal cell between 12-16 hai, by driving a penetration peg through the cell wall. The first reaction observed in the host cell during the penetration attempt is cytoplasm aggregation, which later develops into CWA (Bushnell and Bergquist 1975). CWA are formed by directly apposing substances onto the inner surface of the plant cell walls. It is thought that CWA represent a penetration resistance mechanism, which constitutes a physical and chemical barrier to intercept the invading pathogens (Aist 1976; Israel *et al.*, 1980; Schmelzer 2002). In addition to normal wall constituents such as cellulose and pectin, CWA typically contain antimicrobial compounds such as phytoalexins, phenolics, callose, and hydrogen peroxide (Hüchelhoven 2007). Formation of CWA is achieved by the deposition of diverse materials through cytoskeleton-mediated cellular polarization and secretion (An *et al.*, 2006a; Brisson *et al.*, 1994; Hardham *et al.*, 2007; Schmelzer 2002). CWA formation also involves the accumulation of hydrogen peroxide and phenolics which results in the local protein cross-linking and lignification (Thordal-Christensen *et al.*, 1997; Hüchelhoven *et al.*, 1999). Callose, a β -1, 3 glucan, is a component of CWAs and is thought to play a role in preventing invasion of various pathogens. Synthesis and deposition of callose is triggered by biotic and abiotic stresses and can occur with great rapidity through the activation of plasma membrane-located callose synthases (Jacobs *et al.*, 2003). Although callose deposition can contribute to penetration resistance, loss of a callose synthase in *Arabidopsis* resulted in salicylic acid

mediated disease resistance but not susceptibility. This is because of the negative regulation of salicylic acid pathway by callose synthase (Nishimura *et al.*, 2003).

1.4.2 Hypersensitive Response

Pathogenic fungi can effectively overcome the cell wall-mediated defense and make efforts to penetrate the host cell. The next level of defense response that plants bring into play is the hypersensitive response (HR). HR is a locally triggered complex defense response in response to pathogen attack that involves host programmed cell death (PCD). This defense mechanism aims at disturbing nutrient uptake by the invader by means of rapid and localized host cell suicide, and to prevent a potential pathogen from spreading through the tissues. HR is targeted particularly to restrict biotrophic pathogens or hemibiotrophs during their biotrophic phase. It is a major defense mechanism that contributes to *R*-gene-mediated resistance in plants (Heath 2000; Koga *et al.*, 1990; Pontier *et al.*, 1998). *MLA*-mediated barley resistance to powdery mildew fungi is an example for *R*-gene mediated resistance that involves HR (Schulze-Lefert and Vogel 2000; Ayliffe and Lagudah 2004). *R*-proteins detect pathogen activity and trigger ROS-mediated defense signaling pathways, which eventually results in oxidative burst and cell death (Baker *et al.*, 1993; Hüchelhoven *et al.*, 1999; Hüchelhoven and Kogel 2003). HR is often apparent as necrotic lesions around infection sites (Heath 2000). HR and PCD in animal cells share a number of characteristics such as chromatin condensation and endonucleolytic DNA-cleavage and involvement of ROS, actin, mitochondria, caspases and proteases (An *et al.*, 2006b; Beers and McDowell 2001; Franklin-Tong and Gourlay 2008; Greenberg and Yao 2004; Heath 2000; Jabs 1999; Lam and Del Pozo 2000; Levine *et al.*, 1996; Mur *et al.*, 2008; Reape and McCabe 2008; Williams and Dickman 2008). Plant disease resistance is intimately connected with HR cell death in plants. Plant disease resistance (*R*) genes are crucial components in pathogen perception and the activation of HR cell death. Plant HR cell death mediated resistance responses involve the accumulation and signaling of ROS, nitric oxide and salicylic acid (Beers and McDowell 2001; Gilchrist 1998; Klessig *et al.*, 2000; Kotchoni and Gachomo 2006; Pontier *et al.*, 1998; Shirasu and Schulze-Lefert

2000). In the barley-*Bgh* interaction, HR is represented by the accumulation and oxidative cross-linking of phenolics compounds, resulting in autofluorescence (Koga *et al.*, 1990).

1.4.3 Pathogenesis-related (PR) proteins

Plants defend themselves against pathogens through a combination of constitutive and inducible defenses. The induced plant defense is particularly characterized by an increased accumulation of pathogenesis-related (PR) proteins both locally around infection sites and systemically. Plants possess an array of PR proteins in trace amounts. The biosynthesis and accumulation of PR proteins strongly increases in response to infection by various pathogens. PR proteins are assigned for an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment (Van Loon *et al.*, 2006). PR proteins have dual cellular localization - vacuolar and apoplastic, while apoplast being the main site of their action (Van Loon and Van Strien 1999). There are many families of PR proteins characterized (PR 1-17), whose functions are largely recognized. Most of them are antimicrobial defense proteins or enzymes. For instance PR-2 codes for β -1,3-glucanases, PR-3, PR-4, PR-8, and PR-11 families code for chitinases, PR-5 codes for thaumatin-like proteins, PR-6 codes for a proteinase inhibitor, PR-9 codes for a peroxidase (Van Loon *et al.*, 2006; Edreva 2005). In barley, many classes of PR proteins were reported. Some of them include glucanases (PR-2), lipid transfer proteins (PR-14), oxalate oxidase-like proteins (PR 15, PR-16), and amino peptidase-like proteins (PR-17) (Christensen *et al.*, 2002; Muthukrishnan *et al.*, 2001). *PR-1b* gene expression is rapidly induced upon powdery mildew infection in barley and the protein appears to contribute to penetration resistance against *Bgh* (Schultheiss *et al.*, 2003a).

1.5 Reactive oxygen species (ROS) in plants

Reactive oxygen species (ROS) derive from molecular oxygen by stepwise incomplete electron uptake, finally leading to complete oxygen reduction and production of H₂O. The family of ROS consists, in a narrow sense, of the

superoxide radical anion O_2^- , the hydroperoxyl radical $HO_2\cdot$, hydroxyl radical $HO\cdot$, and hydrogen peroxide H_2O_2 . Superoxide, its protonated form $HO_2\cdot$ and $HO\cdot$ are relatively short-lived, whereas H_2O_2 is comparatively stable and can cross membranes (Apel and Hirt 2004; Baker and Orlandi 1995; Quan *et al.*, 2008). ROS are toxic due to their extraordinary ability to react spontaneously with organic molecules such as phenols, fatty acids, nucleic acids and proteins. However, plants possess a battery of cellular antioxidant mechanisms such as superoxide dismutases, peroxidases, the ascorbate/glutathione cycle, and catalase by which a critical balance between the production and metabolism of ROS is maintained in the cell (Apel and Hirt 2004; Quan *et al.*, 2008). H_2O_2 generation is induced in plants that are exposed to a wide variety of abiotic and biotic stress stimuli like extremes of temperatures, UV irradiation, excess excitation energy, ozone exposure, dehydration, wounding, elicitor and pathogen challenge (Neill *et al.*, 2002). Many potential sources for ROS production have been identified such as nicotinamide adenine dinucleotide phosphate-oxidases (NADPH oxidases), cell wall peroxidases, amine oxidase, oxalate oxidase, and other flavin-containing oxidases (Bolwell *et al.*, 2002; Bolwell and Wojtaszek 1997). Whatever the source of ROS, they can act as signal molecules in inducing a range of molecular, biochemical and physiological responses within the plant cells (Apel and Hirt 2004; Neill *et al.*, 2002; Quan *et al.*, 2008). ROS could potentially affect many cellular processes involved in plant-pathogen interactions and ROS accumulation is closely associated with the induction of plant defense responses (Torres *et al.*, 2006; Hüchelhoven and Kogel 2003). ROS produced in response to pathogens and elicitors have been hypothesized to have direct antimicrobial effects and to play roles in other defense mechanisms such as defense gene expression, cell wall strengthening via cross-linking reactions of phenylpropanoids, lignin production, lipid peroxidation, phytoalexin production, and the hypersensitive reaction against viral, bacterial, and fungal pathogens (Grant and Loake 2000; Hüchelhoven and Kogel 2003).

1.6 Susceptibility (compatibility)

Plants are constantly exposed to a wide range of pathogens. Due to the multifaceted surveillance systems and defense mechanisms on the plant side

and a common lack of adaptation of many microbial pathogens, only very few microbes can successfully infect and colonize a host plant to form a compatible interaction. Compatibility portrays the complementary relationship between a susceptible plant species and the adapted pathogen species that ultimately results in disease. In general, resistance is the rule and compatibility is the exception for most plant-microbe combinations (O'Connell and Panstruga 2006).

In order to establish compatibility with the host plant, pathogens evolved diverse strategies. Plants recognize various pathogen-derived PAMPs and trigger primary immune responses (Ingle *et al.*, 2006; Jones and Dangl 2006). Some pathogens might attempt to mask their PAMPs, avoid recognition by plant PAMP receptors or suppress PAMP signal transduction in order to establish compatibility (Hückelhoven 2005). Pathogens also attempt to suppress activation and/or execution of defense responses or counter-defend activated defense by detoxification or sequestration of potentially harmful compounds that are produced by the host plant (Caldo *et al.*, 2004; Gjetting *et al.*, 2004; Schulze-Lefert and Panstruga 2003). On the other hand, they make efforts to reprogram the host's metabolic flow to their own benefit and make use of host proteins as 'susceptibility factors' (Panstruga 2003; Schulze-Lefert and Panstruga 2003).

1.6.1 Suppression of host immune responses by plant pathogens

Adapted plant pathogens suppress basal defense responses triggered by PAMP recognition of the host plant. This suppression is facilitated by pathogen produced effector proteins. Some plant pathogenic bacteria use the type III secretion system (TTSS) to inject multiple effector proteins into host cells. This system is essential for bacteria to multiply in the plant tissue and to promote the development of disease symptoms. The individual Type III effectors suppress plant innate immunity by interfering with resistance protein activation, MAPK signaling, and the execution of PCD (Bent and Mackey 2007; Da Cunha *et al.*, 2007; Göhre and Robatzek 2008; He *et al.*, 2007; Mudgett 2005).

In contrast to bacteria, the identity, release, uptake, mode of action and specific functions of fungal and oomycete effectors is not clearly understood. Fungal and oomycete effectors act either in the extracellular matrix or inside the

host cell (Ellis *et al.*, 2006; Kamoun 2006). Most of the biotrophic fungi form a specialized infection structure called haustorium, which is thought to be important in delivering effectors into the host apoplast region or into the host cell that play roles in the process of establishment and maintenance of compatibility (Catanzariti *et al.*, 2006; Kemen *et al.*, 2005; Voegelé and Mendgen 2003). Most of the fungal effectors identified so far are small peptides containing a signal for secretion into the apoplast. Some effectors serve structural roles in the extra-haustorial matrix and some promote nutrient leakage or pathogen dispersal. Some of the effectors of biotrophs are presumed to act intracellularly and they appear to be synthesized in haustoria or intracellular hyphae, channeled through the pathogen's secretory pathway and finally discharged into the interfacial matrix via exocytosis (Chisholm *et al.*, 2006; O'Connell and Panstruga 2006; Panstruga 2003) or sometimes the pathogen effectors are translocated into the host cells to alter host transcription and thus promotes pathogen infection (Catanzariti *et al.*, 2007). A number of fungal effectors were identified in extracellular fungal pathogens such as *Cladosporium*, *Fusarium*, *Magnaporthe*, *Rynchosporium* and haustoria forming fungal pathogens such as rusts and powdery mildews (Stergiopoulos and De Wit 2009). The transient expression of two *Avr* genes from *Bgh* (*Avr-a10* and *Avr-k1*), enhanced the successful haustorial penetration of *Bgh* in susceptible barley epidermal cells, suggesting an effector function of these proteins in promoting pathogen infection (Ridout *et al.*, 2006). Similarly, the haustoria of the flax rust fungus *Melampsora lini*, also appear to mediate the delivery of effector proteins inside host cells (Catanzariti *et al.*, 2006; Dodds *et al.*, 2004). Several of the oomycete and fungal effectors are either apoplastic, i. e. secreted into the plant extracellular space, or cytoplasmic effectors that are translocated inside the plant cell (Kamoun 2006; Kamoun 2007; Stergiopoulos and De Wit 2009).

Some of the oomycete effectors are characterized as polypeptides that are primarily localized in the host apoplast. Many of them are enzyme inhibitors or proteases which probably function in microbial counter defense to secreted plant pathogenesis-related proteins such as chitinases and endoglucanases (Kamoun 2006). Some effectors from oomycete pathogens possess a common signal peptide for secretion from the pathogen, combined with an N-terminal

conserved RXLR motif often ending in the sequence EER (Allen *et al.*, 2004; Armstrong *et al.*, 2005; Rehmany *et al.*, 2005; Shan *et al.*, 2004). The RXLR-EER motif is similar to a host-cell-targeting signal of virulence proteins of malaria causing *Plasmodium* species, suggesting that the role of RXLR-EER motifs in the delivery of oomycete AVR proteins inside plant cells (Birch *et al.*, 2006; Morgan and Kamoun 2007). Indeed, recent investigations demonstrated that the RXLR-EER motifs are required for the translocation of oomycete effectors into host plant cells, after secretion from haustoria (Birch *et al.*, 2008; Whisson *et al.*, 2007). HR is a major prevailing defense response against biotrophic pathogens. Hence, biotrophs attempt to avoid triggering of HR to keep the host cell alive in order to establish compatibility. The 'green island' effect provides evidence that host cell death is indeed suppressed during compatible biotrophic interactions. Green islands are the regions where the leaf areas around successful infection sites display delayed senescence in comparison to the rest of the leaf tissue (Scholes and Rolfe 1996; Walters *et al.*, 2008).

1.6.2 Factors contributing to host susceptibility in the barley-powdery mildew interaction

The process of compatibility is a complex interplay between the host plant and the pathogen, which involves highly specific molecular interactions at various stages of the infection. Pathogens likely require appropriate host target molecules to control the host plant effectively. Conceptually, this might be achieved by exploiting specific host molecules as susceptibility factors for pathogenesis. This would mean that the lack of essential host factors should result in failure of the pathogen, which leads to resistance against a given pathogen species. Resistance of plants to potyviruses through lack of the eukaryotic translation elongation factor isoform, eIF (iso) E4 serves as an example for this type of immunity (Robaglia and Caranta 2006). Since resistance would be brought about by the loss of function of a single gene, it is expected to be recessively inherited. A range of examples of monogenic recessively inherited and pathogen-specific disease resistance loci have been reported from both monocot and dicot plant species.

1.6.2.1 MLO

One of the best-studied examples is powdery mildew resistance mediated by loss-of-function alleles of the barley *mildew resistance locus o* (*mlo*). Barley *MLO* encodes a member of a novel plant-specific family of integral membrane proteins. Presence of wild-type *MLO* is required for the successful entry of barley powdery mildew sporelings into host epidermal cells. *MLO* is a heptahelical plasma membrane-localized protein that possibly functions in the cellular exocytotic processes. Powdery mildew fungi appear to specifically corrupt *MLO* to modulate vesicle-associated processes at the plant cell periphery for successful pathogenesis and defense suppression (Büschges *et al.*, 1997; Panstruga 2005; Hückelhoven 2005). Recessively inherited loss-of-function mutant alleles (*mlo*) of barley result in race-non-specific effective penetration resistance to the barley powdery mildew fungus. The *mlo*-mediated penetration resistance at the cell wall involves the function of actin cytoskeleton and also requires functional *ROR1* and *ROR2* (required for *mlo*-specified resistance) genes (Freialdenhoven *et al.*, 1996; Miklis *et al.*, 2007). For several decades, *mlo*-resistance has been envisaged as a unique feature of the monocot barley. But recent investigations demonstrated the conserved requirement of *MLO* proteins in powdery mildew pathogenesis in the dicot plant species like *Arabidopsis* and tomato (Bai *et al.*, 2008; Consonni *et al.*, 2006) which indicates the conservation of a common host cell entry mechanism by the powdery mildew fungi during the evolution. Like *mlo*, *Arabidopsis* powdery mildew resistant (*pmr*) mutants confer recessively inherited enhanced disease resistance to *Golovinomyces cichoracearum*, a powdery mildew of cruciferous plants that also colonizes *Arabidopsis*. Six *pmr* loci (*pmr1-6*) have been identified for loss of *G. cichoracearum* sporulation (Vogel and Somerville 2000). Another example from *Arabidopsis* is the loss of the downy mildew resistant (*dmr*) gene that results in reduced susceptibility to the downy mildew oomycete *Hyaloperonospora parasitica* (Van Damme *et al.*, 2005). Furthermore, in these three mutants (*mlo*, *pmr* and *dmr*), resistance is not associated with the constitutive expression of known defense marker genes. Thus, recessive inheritance of resistance to various pathogens in plants provides an evidence for the existence of host susceptibility

factors that are being exploited by pathogens in the process of establishing compatibility.

Besides MLO, there are a variety of host proteins which acts as host susceptibility factors in the barley-powdery mildew interaction. A potential cell death inhibitor BAX INHIBITOR-1 (HvBI-1) likely operates as host susceptibility factor during the infection of powdery mildew fungi. Over expression of HvBI-1 can breach *mlo*-mediated resistance and non-host penetration resistance to wheat powdery mildew fungus (Hückelhoven *et al.*, 2003; Eichmann *et al.*, 2004). Likewise, a barley NADPH oxidase and small G-proteins of the plant specific RAC/ROP family have been found to operate in barley susceptibility to *Bgh* (Schultheiss *et al.*, 2002, 2003b, 2005; Trujillo *et al.*, 2006).

1.6.2.2 Plant NADPH oxidases (respiratory burst oxidase homologues)

The plasma-membrane NADPH oxidase is recognized as one of the primary enzyme complexes involved in the anti-pathogen action of neutrophils and other phagocytes in mammals. This action is mediated by the production of toxic ROS by a burst of oxygen consumption known as the 'respiratory burst'. For this reason, these enzymes are also known as respiratory burst oxidase homologues (RBOHs). gp91^{phox} is the enzymatic subunit of the phagocyte NADPH oxidase that transfers electrons to molecular oxygen to generate superoxide by using NADPH as substrate. Activity of the NADPH oxidase also requires the involvement of RAC G-proteins (Jones *et al.*, 2000). Plant homologues of the mammalian gp91^{phox} respiratory burst NADPH-oxidase subunit have been identified and partially characterized in several plant species (Lightfoot *et al.*, 2008; Torres and Dangl 2005; Torres *et al.*, 1998; Trujillo *et al.*, 2006; Wong *et al.*, 2007). The mammalian gp91^{phox} and corresponding plant RBOHs share many structural and functional similarities, but differ in the inclusion of an extended N-terminal region in the plant RBOHs. This plant RBOH-specific region contains two EF-hand motifs that bind Ca²⁺, suggesting that calcium ions play a role in regulating the function of these oxidases (Keller *et al.*, 1998; Torres *et al.*, 1998). Members of the plant RBOH family are found to mediate the production of apoplastic ROS during defense reactions, and also in response to abiotic environmental and developmental cues (Torres and Dangl 2005).

The O₂⁻ production by the NADPH oxidase involves the one electron reduction of oxygen to O₂⁻ using NADPH as electron donor. NADPH oxidase-dependent ROS production is required for plant developmental processes like cell expansion during organ morphogenesis, root hair and pollen tube growth (Foreman *et al.*, 2003; Carol and Dolan 2006; Jones *et al.*, 2007; Potocký *et al.*, 2007). ROS production is associated with lignification during xylem differentiation and suberization in response to wounding (Barcelo 2005; Razem and Bernards 2003). NADPH oxidase-dependent ROS signaling is involved in abscisic acid-, methyl jasmonate-, and ethylene-mediated stomatal closure (Kwak *et al.*, 2003; Suhita *et al.*, 2004; Desikan *et al.*, 2006). Several RBOHs were identified and characterized in plant species such as *Arabidopsis*, rice and barley (Lightfoot *et al.*, 2008; Torres and Dangl 2005; Torres *et al.*, 1998; Wong *et al.*, 2007). Investigations on *Arabidopsis* AtRBOHD and AtRBOHF suggest a complex signaling network and a functional overlap between distinct RBOH family members in plants (Torres *et al.*, 2002; Kwak *et al.*, 2003).

Plant RBOHs play crucial roles in a variety of plant-microbe interactions. It has been reported that RBOH genes are transcriptionally up-regulated in tobacco and potato when treated with fungal elicitors (Simon-Plas *et al.*, 2002; Yoshioka *et al.*, 2001). It has been shown that the *Nicotiana benthamiana* genes *NbrbohA* and *NbrbohB* are required for the accumulation of H₂O₂ and for resistance to the oomycete *Phytophthora infestans* (Yoshioka *et al.*, 2003). In *Arabidopsis*, RBOHD is responsible for ROS produced in response to avirulent bacterial or oomycete pathogens, whereas RBOHF seems to be important in the spatial control of HR (Torres *et al.*, 2002). In barley, HvRBOHA was suggested to operate in susceptibility to powdery mildew disease (Trujillo *et al.*, 2006).

1.6.2.3 Small GTPases of the RAC/ROP family

G-proteins are also called GTP-binding proteins as they possess a guanine nucleotide binding domain. When bound to GDP, G-proteins are in an inactive state. Conversely, the replacement of GDP by a GTP molecule turns the protein into an active state. The shuttling between the two activity statuses allows G-proteins to transmit external signals into a functional response in the cell (Berken and Wittinghofer 2008; Yang 2002). A large number of G-proteins have been

discovered over the years, and all fall into two distinct classes known as heterotrimeric G-proteins and monomeric small GTPases. Heterotrimeric G-proteins are a class of signal transduction proteins thought to exist in all eukaryotes (New and Wong 1998). Heterotrimeric G-proteins consist of three subunits, alpha (α), beta (β) and gamma (γ), are located on the cytoplasmic side of the plasma membrane and are known to play roles in a variety of cellular processes like cell proliferation, pollen germination, organ development, biotic interactions and others (Yoshikawa *et al.*, 2000; Ullah *et al.*, 2001; Wu *et al.*, 2007; Lease *et al.*, 2001; Llorente *et al.*, 2005). Monomeric small G-proteins (often called GTPases to distinguish them from heterotrimeric G-proteins) are comprised of a single unit. They resemble the α subunit of heterotrimeric G-proteins and acts as molecular switches in a similar way. These small GTP-binding proteins exist in eukaryotes ranging from yeast to human and constitute a super family consisting of more than 100 members. The Ras (rat sarcome oncogene product) super family is structurally classified into five families: Ras, Rho, Rab, Sar1/Arf, and Ran. Rho family GTPases regulate signal transduction at the plasma membrane. Rho family is composed of the three subfamilies namely RAC (Ras related C₃ botulinum toxin substrate), RHO (Ras homologue) and Cdc42 (Cell division cycle protein 42) (Takai *et al.*, 2001). The RHO-like GTPase family in plants has no clear homologs in the RHO subfamilies of animals. Instead, they contain a unique subfamily called ROPs (Rho of plants) or RACs, because the primary amino acid sequences are most similar to animal RACs (Brembu *et al.*, 2006). ROPs share more than 70% amino acid identity with each other and 45-64% identity with other members of the Rho family (Zheng and Yang 2000a). All members of the Rho family, including the plant-specific RAC/ROP subfamily share a similar five-loop domain structure. In plant species such as maize (*Zea mays*), rice (*Oryza sativa*), *Arabidopsis thaliana* and barley (*Hordeum vulgare*), 6 to 11 ROP family members have been identified and extensively studied (Christensen *et al.*, 2003; Schultheiss *et al.*, 2003b; Vernoud *et al.*, 2003; Winge *et al.*, 2000).

In response to the extracellular signals, G-proteins get activated by GTP binding and trigger the downstream effectors in the signal transduction pathways. By hydrolyzing GTP to GDP and Pi, conformational changes are reverted and the protein becomes inactive again (Brembu *et al.*, 2006; Yang 2002). The activity

status of plant RAC/ROP proteins is adjusted most likely by receptor-like kinases, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Berken and Wittinghofer 2008). The ability of the small G-proteins to cycle between an active GTP-bound and an inactive GDP-bound state makes them an ideal “molecular switch” in the cell. ROP proteins function as key regulators in a number of cellular processes (Gu *et al.*, 2004; Nibau *et al.*, 2006). RHO and ROP GTPases in animals and plants play similar roles in certain cellular processes such as cell polarity and ROS production, whereas plant ROPs also regulate plant specific processes, such as lignin synthesis (Brembu *et al.*, 2006). ROP signaling is required for functions such as cell morphogenesis, hormone signaling, or abiotic and biotic stress responses (Yang and Fu 2007). ROPs were shown to regulate the polar growth and morphogenesis in several cell systems including pollen tubes, developing root hairs, and leaf epidermal cells (Fu *et al.*, 2005; Jones *et al.*, 2002; Gu *et al.*, 2005). RAC/ROP-regulated processes include exo- and endocytosis, cytoskeleton organization, cytosolic Ca²⁺ signaling, production of reactive oxygen species and regulation of gene expression (Berken 2006; Nibau *et al.*, 2006). ROPs also operate in disease resistance and susceptibility of plants. Certain rice ROPs play role in the plant pathogen interactions. OsRACB, for example, acts as a negative regulator of basal disease resistance, and OsRAC1 is involved in race-specific resistance to the rice blast fungus *Magnaporthe grisea* (Jung *et al.*, 2006; Ono *et al.*, 2001).

In barley, six different RAC/ROP homologous proteins were identified namely HvRAC1, HvRAC3, HvRACB, HvRACD, HvROP4 and HvROP6. Different barley RAC/ROPs showed up to 90% amino acid sequence identity. As most RAC/ROP proteins, all barley RAC/ROP amino acid sequences contain five functional domains (Schultheiss *et al.*, 2003b). The GDP/GTP-binding domain, GTPase domain and the effector-loop which is considered to be crucial for interactions with regulatory proteins are highly conserved among all six homologs. The special effector-loop, which is probably responsible for downstream signaling, and the C-terminal hyper variable region, which contains signals targeting the RAC/ROP proteins to membranes, do not show high similarities among various barley RAC/ROP family members. HvRACB and HvRACD possess a shorter C-

terminal sequence and belong to type I, whereas HvRAC1, HvRAC3, HvROP4 and HvROP6 possess relatively longer C-terminal hyper variable regions and belong to type II. HvRACB and HvRACD contain typical prenylation signals at their C-termini, whereas the hyper variable regions of all other RAC/ROPs contain additional cysteine residues that might be palmitoylated (Schultheiss *et al.*, 2003b). Some of the barley RAC/ROP proteins modulate susceptibility to the powdery mildew disease caused by *Bgh* (Schultheiss *et al.*, 2002; 2003b). In single cell transient transformation assays, over-expression of three out of five epidermis-expressed barley ROPs (HvRACB, HvRAC3 and HvROP6) supported fungal penetration, but only if they were in constitutively activated (CA) forms, i. e. permanently GTP-bound where as the knock-down of HvRACB led to enhanced resistance against the powdery mildew fungus (Schultheiss *et al.*, 2002; 2003b). Transient knock-down of HvRACB showed no alteration in powdery mildew resistance in *mlo5 ror1* double-mutants, which are moderately susceptible to *Bgh* (Schultheiss *et al.*, 2002). In addition, both MLO and HvRACB were shown to influence actin reorganization in barley cells under attack from *Bgh* (Opalski *et al.*, 2005), suggesting a potential link between HvRACB and MLO in supporting susceptibility to *Bgh* in barley. Stable transgenic barley plants expressing CA HvRACB-G15V showed enhanced susceptibility to powdery mildew and reduced sensitivity to abscisic acid mediated stomata closure (Schultheiss *et al.*, 2005). However, the function of HvRAC3 or HvRAC1 at the entire plant level has not been analyzed yet.

1.7 Objectives

Developing potentially resistant crop plants against major pathogens is one of the most important concerns of plant biologists. Research in the recent times has greatly increased the knowledge of underlying genetic mechanisms in plant disease resistance. Considerable efforts have been made in unraveling the signaling pathways involved in plant disease resistance. However, the knowledge on mechanisms of susceptibility is limited. In order to use crop resistance mechanisms in a durable way, it is essential to look at the 'susceptibility' side of the disease from the perspective of plant susceptibility factors. Therefore this work was focused on better understanding of barley small G-proteins (RAC/ROPs) as host susceptibility factors in association with the barley powdery mildew fungus. For this purpose, constitutively activated RAC/ROP expressing stable transgenic plants were analyzed by using various cytological and molecular tools. These investigations helped to get a deeper insight into the physiological and pathogenesis-related function of three different barley ROPs, HvRACB, HvRAC3 and HvRAC1 at the entire plant level. Additionally, the impact of heterologous expression of monocot barley ROPs in dicot tobacco was investigated to define cross-species conserved functions of RAC/ROPs.

In recent years, involvement of ROPs in cell polarity and morphogenesis is well acknowledged in dicot plants. This work gives interesting insights into ROP functions in monocot plant development. The NADPH oxidase mediated ROS production was shown to be involved in various plant functions. Another part of the work was intended to discover the interaction between barley RAC/ROPs and NADPH oxidases. To achieve this, protein-protein interaction studies were carried out by using targeted yeast two hybrid screening and Bimolecular Fluorescence complementation (BiFC) *in planta*. Furthermore, barley RAC1 was assessed for its cell death inducing ability.

Taken together, the aim of this work was to gain deeper insight into ROP mediated processes that define plant development and pathogen interactions. The understanding of the underlying determinants in ROP-mediated host susceptibility might provide the possibility to generate enduring crop resistance.

2. Materials and methods

2.1 Plants, pathogens and inoculation procedures

Stable genetic transformation of barley (*Hordeum vulgare* L.) with *pLH6000-ZmUbi::HvRACB-G15V*, *pLH6000-ZmUbi::HvRAC1-G23V* and *pLH6000-ZmUbi::HvRAC3-G17V* constructs was performed by *Agrobacterium tumefaciens*-mediated genetic transformation. Constructs were generated as described in Schultheiss *et al.*, (2005) and transgenic plants were generated as described by Hensel and Kumlehn (2004) and Hensel *et al.*, (2008). Transgenic barley seeds were provided by G. Hensel and J. Kumlehn (Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany). Transgenic plants and plants of the parental cultivar 'Golden Promise' were grown in a growth chamber at 18°C, 60% relative humidity, and a photoperiod of 16 h (150 $\mu\text{mol s}^{-1}\text{m}^{-2}$ photon flux density) up to E.C. 30. From the fifth week onwards, plants were grown in a greenhouse at 20°C. For transient transformation experiments, barley cultivar Manchuria plants were grown in a growth chamber for 7 days.

Blumeria graminis (DC) Speer f.sp. *hordei* Em. Marchal (*Bgh*), race A6 (Wiberg 1974), was maintained on 'Golden Promise' in growth chambers under the same conditions as described above. For microscopic evaluation, *Bgh* was inoculated onto second or third leaves of 14-20 days old barley plants to give a density of 30 conidia mm^{-2} . The outcome of the interaction was evaluated after 24 and 48 h by using light and fluorescence microscopy. For macroscopic analyses, detached third leaves were inoculated with a density of 5 conidia mm^{-2} and evaluated 7 days after inoculation.

Stable genetic transformation of tobacco (*Nicotiana tabacum*) cultivar 'Xanthi' with *pCAMBIA-CaMV35S::HvRACB-G15V* or *pCAMBIA-CaMV35S::HvRAC3-G17V* was performed by *Agrobacterium*-mediated genetic transformation as described by Langen *et al.*, (2006) and Horsh *et al.*, (1985). Transgenic tobacco seeds were provided by J. Imani (Institute of Phytopathology and Applied Zoology, University of Giessen, Germany) (Pathuri *et al.*, 2009a). Seeds of the T₁ generation with proven transgene expression in T₀ were sown on selective Murashige and Skoog (1962) solid medium containing 50 mg L⁻¹ hygromycin. Intact plants were transplanted onto soil and cultivated in the growth chamber.

They were grown under greenhouse conditions at 25°C, with 16 h of light and 8 h of darkness and a light intensity of 175-185 $\mu\text{mol photons s}^{-1} \text{m}^{-2}$. Analysis of tobacco resistance to the tobacco powdery mildew fungus *Golovinomyces cichoracearum* was performed using detached leaves of 6-8 weeks-old tobacco plants. Leaves were inoculated with conidia of *G. cichoracearum* to give a density of 400 conidia cm^{-2} . Inoculated leaves were kept on 0.5% water agar plates at 22°C, 100% relative humidity and 50-60 $\mu\text{mol s}^{-1}\text{m}^{-2}$ photon flux densities. Macroscopic evaluation was done 7 days after inoculation. Pure culture of *Pseudomonas syringae* pv. *tabaci* (*Pst*) (strain 50312 from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was grown for 18 h in glucose yeast extract medium (20 g L^{-1} glucose, 10 g L^{-1} yeast extract, 20 g L^{-1} calcium carbonate (light precipitate)) at 28-30°C. The bacterial culture was diluted with a solution of 0.9% NaCl to get a final inoculum concentration of 10^6 cells ml^{-1} and used for infiltration into detached tobacco leaves, which were kept on 0.5% water agar in closed plastic boxes. Mock inoculation was done by injecting 0.9% NaCl solution. Fully expanded leaves of 7 weeks old tobacco plants were used for *Pst* inoculation. To examine bacterial growth, leaf disks of 0.5 cm diameter were punched from the initially infiltrated area and the area directly adjacent to this with a cork borer at 4 days after inoculation. Leaf discs were surface sterilized in 70% ethanol and then homogenized in sterile water. Bacterial populations were measured by the standard plate-dilution method, on glucose yeast extract nutrient agar (yeast extract medium supplemented with 17 g L^{-1} agar) plates. Colony forming units (cfu) were counted after 48 h of incubation at 28°C.

2.2 Examination of transgene presence and expression in the segregating plant populations

For total RNA extraction, young leaves from the transgenic plants were harvested in liquid nitrogen. The leaf tissue was ground into fine powder in liquid nitrogen and mixed with 1 ml of RNA extraction buffer (38% phenol, 0.8 M guanidinium thiocyanate, 0.4 M ammonium thiocyanate, and 0.1 M sodium acetate (pH 5), 5% glycerol mixed in water) and 200 μl of chloroform. After

vigorous vortexing and centrifugation at 13,500 rpm for 15 min at 4°C, the supernatant was mixed again with 1 ml of chloroform. Phenol and chloroform were used to remove protein and lipid contaminants respectively. The supernatant was then mixed with 1 ml of iso-propanol and incubated overnight at -20°C to precipitate the RNA. After centrifugation at 13,500 rpm for 20 min at 4°C, the pellet was washed twice with 1 ml of 70% ethanol and dried thoroughly before dissolving it in sterile RNase-free water. RNA integrity was assessed by examining rRNA bands after electrophoresis on a denaturing agarose gel (1.2% agarose with 5% formaldehyde).

To test the T₁ segregating individuals, mRNA expression was analyzed by one-step reverse transcription (RT)-PCR using the One-step RT-PCR kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Prior to RT-PCR, DNA contaminations were removed using 1 u DNase I (Fermentas, St. Leon-Rot, Germany) per µg sample RNA. The reaction was incubated at 37°C for 30 minutes and then inactivated with 2 µl EDTA (25 mM) for 10 minutes at 70°C. PCR Primers were designed to allow amplification of the expressed transgenic *CA HvRAC* but not endogenous *RAC*. For all three constructs of *HvRACB-G15V*, *HvRAC1-G23V* and *HvRAC3-G17V*, a 5'-primer targeting a non-translated part of the maize ubiquitin promoter (UbiUTR5prime1 5'-AACCCAGATCTCCCCCAAATC-3') was used. The 3' primers used for *HvRACB-G15V*, *HvRAC1-G23V* and *HvRAC3-G17V* were *RacB3'Sal* (5'-GTCGACCTTCGCCCTTGTTCTTTGTC-3'), *Rac1-EST-3'Sal* (5'-GTCGACCCCATTTGGAGAACAACCAC-3'), and *Rop8-3'Sal* (5'-GTCGACGCAAGGAACCTTCTCTTTCATC-3'), respectively. The same primer combinations were used to test segregating individuals by genomic PCR using REExtract-N-Amp plant PCR kit (Sigma-Aldrich Chemie GmbH, Munich, Germany).

Another method of crude DNA extraction was also used for PCR check on the genomic level. In this method, 7-10 fresh leaf discs of 0.8 cm diameter were crushed in 500 µl DNA extraction solution (200 mM Tris (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS in water) and subsequently mixed with 500 µl chloroform. After mixing and centrifugation at 13000 rpm for 10 min, the clear supernatant was collected and mixed with 500 µl of iso-propanol to precipitate the DNA. This solution was centrifuged at 13000 rpm for 5 min to separate the DNA pellet. The

pellet was washed twice with 500 μ l of 70% ethanol and dried thoroughly before dissolving it in 20 μ l of sterile water. 2-3 μ l of this crude DNA suspension was directly used as template in a genomic PCR for the transgene check.

For all transgenic lines, transgene integration and expression was confirmed by genomic and RT-PCR, respectively. Additionally, visible phenotypes of transgenic plants were used to confirm the transgenic nature of individuals. In most of the experiments, more than one independent transgenic line was used. The bulk of segregants that lost the transgene due to segregation were used as azygous controls in all experiments. Average azygous controls from individual lines behaved similarly. For statistical analysis of the transgene effect, transgenic individuals were pooled from different lines and corresponding controls, respectively.

2.3 Semi-quantitative RT-PCR of CA ROP-expressing barley in response to *Bgh* infection

Semi-quantitative two-step RT-PCR was performed to detect differential gene expression patterns of CA ROP-expressing barley in response to *Bgh* infection in comparison to Golden Promise wild type plants and wild type segregants. Barley parent cultivar Golden Promise, transgenic wild type segregants, CA RACB-G15V and CA RAC1-G23V barley plants were inoculated with *Bgh* spores at a density of 100 mm⁻². At least four leaves from individual plants were harvested at time points 0, 12 and 24 HAI. Total RNA was extracted from the leaves by using the protocol described above (chapter 2.2). 5 μ g of total RNA from each sample 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 20 μ l. 0.5 μ l aliquots of the first strand cDNA were subsequently used as template for common PCR amplification with gene specific primers under stringent conditions. Information on PCR conditions and primer sequences that were used for the amplification of various genes can be taken from table 2.1.

Table 2.1 Primers and PCR conditions for gene expression analyses by semi-quantitative RT-PCR.

identity	primer name	primer sequences	annealing temp.	cycle number	product size (bp)
Ubiquitin	Ubi-kurz-5' Ubi-kurz-3'	ACCTCGCCGACTACAACAT CAGTAGTGGCGTCGAAGTG	60°C	23	263
PR-1b	HvPR1b PR1b-3'	GGTATAGAGCAGGCCCATAGAA GTGTGGAGCCGTAGTCGIAGT	60°C	23	381
Protein disulfide isomerase (PDI)	I-14:3PstIrev I-14:3fwd3'frag	CTGCAGTCTCAGAGCTCATCCTTCA GAGCCAAAGGGGCTTCCAG	57°C	25	604
Bax Inhibitor-1	Bax_b_for Bax_b_rev	GTCACACCTCAAGCTCGTTT ACCCTGTACACGAGGATGCTT	60°C	26	269
MLO	Mlo fwd1 Mlo rev2	CAGCTGCACGCTTTCATCTT AAATCTGCACTGGGATGTC	58°C	25	1199
Auxin responsive (H-11:5)	H-11:5_fwd H-11:5_rev	GCACGATCCCGACTGC GATCGCGTCCGAGCTTT	56°C	26	269
Auxin responsive (HvIAA)	HvIAAfwd HvIAArev	ACCAAGGGCCAAAGGACA TTGCTTCTGGTCCACCAAC	56°C	28	188
HvRACB	RacB-UTR-5' RacB-UTR-3'	CTTCCTCCTCCTCCCTGT CCTCGACAAAGAGACCTTGC	57°C	28	820
HvRAC1	Rac1-5'cds Rac1endo3'UTR	CAGGTTTCATCAAGTGGCT TTTTCAAGCCTGCCCAAGGA	54°C	28	876
CA HvRACB-G15V	UbiUTR5prime1 RacB3'sal1	AACCAGATCTCCCCCAAATC GTCGACCTCGCCCTGTCTTTGTC	55°C	25	696

Table 2.1 continued

identity	primer name	primer sequences	annealing temp.	cycle number	product size (bp)
CA HvRAC1-G23V	UbiUTR5prime1 Rac1-EST-3' Sal	AACCAGATCTCCCCCAAATC GTCGACCCCAATTGGAGAACAAACCAC	55°C	25	819
BCI-4 (Ca ²⁺ -binding EF-hand protein)	BCI-4fwd BCI-4rev	GGCCTATTATTCACGCITGC ACCTTCTTCGGGACAAACCT	55°C	25	190
BCI-1 (MeJA-inducible lipoxigenase)	BCI-1fwd BCI-1rev	CCCACGGACGAGAAGATG TTTGGTGCTCATCCCAIT	53°C	25	190
BCI-3 (acid phosphatase)	BCI-3fwd2 BCI-3rev2	GTCATCGACCAGGCCATC GTACGCCGTCGAAGCTCGT	58°C	24	165
cat. subunit of protein phosphatase 2A-1	HvPP2ArevSmalnoS PP2A-1fwdSmal	CCCGGGGTACAAAAGTAGTCGGGGGT CCCGGGCGCGGCAGCGACGATG	57°C	24	946
cat. subunit of protein phosphatase 2A-3	HvPP2ArevSmalnoS PP2A-3fwdSmal	CCCGGGGTACAAAAGTAGTCGGGGGT CCCGGGTGCCAGCGGCCGAGATG	57°C	24	947

BCI – barley chemically induced genes (Beßer *et al.*, 2000); MeJA – Methyl Jasmonate; cat. – catalytic.

2.4 Microscopic analysis of transgenic plants

2.4.1 Staining and microscopy of *Bgh* infection structures

After harvesting inoculated leaf samples at indicated time points, they were fixed in a fixation solution (0.15 % trichloro acetic acid (w/v) in ethyl-alcohol: chloroform (4:1; v/v)). Fungal structures were stained by ink (10% blue ink and 25% acetic acid in water) and the samples were analyzed by using bright-field and fluorescence microscopy as described by Hückelhoven and Kogel (1998). Penetration of attacked cells was ascertained by detection of haustorium formation. Whole-cell hydrogen peroxide (H_2O_2) staining with 3,3-diaminobenzidine (DAB), auto-fluorescence, cytoplasmic granulation and discontinuity of cytoplasmic strands were taken as a reliable measures of cell death (Görg *et al.*, 1993; Hückelhoven *et al.*, 2000; Koga *et al.*, 1990). Because the rate of fungal penetration into short and long epidermal cells is different, only short cells directly adjacent to stomata (cell type A) and short cells not directly adjacent to stomata (type B) were evaluated, whereas long epidermal cells covering vascular tissue (type C) were excluded (for leaf topography see Koga *et al.*, 1990). To avoid misinterpretation due to the effects of induced accessibility or induced inaccessibility in cells where penetration was successful or unsuccessful, respectively (Lyngkjaer and Carver, 1999), interaction sites where only one fungus per cell attempted to penetrate were evaluated. Detection of H_2O_2 was performed using the DAB-uptake method as described previously (Hückelhoven *et al.*, 2000; Hückelhoven *et al.*, 1999; Thordal-Christensen *et al.*, 1997).

For callose staining, green leaves were cleared in ethanol:acetic acid (6:1 v/v) overnight and washed with water. Subsequently, cleared leaves were incubated in 0.05% methyl blue (w/v) in 0.067M K_2HPO_4 buffer at pH 9.2 overnight and washed in water for microscopy. Accumulation of autofluorescent material was observed using epi-fluorescence microscopy as described by Jarosch *et al.*, (1999).

2.4.2 Microscopic evaluation of leaf epidermal cells and root hair phenotypes

Fully expanded leaves of same age from various transgenic barley lines were harvested to measure the size of epidermal cells. A 5 cm leaf tip region was removed and the next 5 cm leaf segment was taken to count the number of B-cells (see Koga *et al.*, 1990) per row in order to calculate the average B-cell length. The number of stomata per row was also counted. From each leaf, two rows of cells from both sides of the mid rib were counted and the average was taken. Each data set was derived from four to five leaf samples taken from individual plants (approximately 200 cells per transgenic line). To visualize epidermal cell shapes, sample fixation and modified pseudo-Schiff propidium iodide (mPS-PI) staining was performed exactly as described by Truernit *et al.*, (2008). Propidium iodide staining was allowed for 2 h. The samples were then transferred onto microscope slides and covered with a chloral hydrate solution (4 g chloral hydrate, 1 mL glycerol, and 2 mL water). Epidermal cells were examined by using a confocal laser scanning microscope (Leica SP5, Mannheim, Germany). Samples were excited with a 488 nm laser line. Emission was detected between 560 and 675 nm. The epidermal cell layer between two rows of stomata was scanned in xy direction in 10 z-sections at 1.5 μm increments.

In case of tobacco, leaf discs were collected from fully expanded leaves to measure the epidermal cell lengths. The leaf discs from the mid region of leaves of equal developmental stage were placed in clearance solution (0.15% trichloro acetic acid [w/v] in ethylalcohol:chloroform [4:1; v/v]) and subsequently stored in 50% watery glycerol. Cleared leaves were observed with an Axioplan microscope (Zeiss, Jena, Germany). The measurement of cell sizes of epidermal cells was conducted under the same magnification for all samples in all experiments. Each data set of a given line was derived from the measurement of at least 150 cells collected from three different leaves of three individual plants in three independent experiments.

Barley root hair phenotypes were observed in one week old barley plants grown on solid (0.6% gelrite) half-MS medium (Murashige and Skoog 1962) containing

5% sucrose. Tobacco root hair phenotypes were observed in two weeks old transgenic tobacco plants of T₁ or T₂ generations grown on selective hygromycin-containing medium and compared to both transgenic empty vector control lines and wild type lines grown on hygromycin-free medium (Murashige and Skoog 1962). Phenotypes were observed by using binoculars and light microscope (Axioplan, Zeiss, Jena, Germany).

2.5 Transient transformation and cell viability assay in barley single epidermal cells

Barley leaves were transiently transformed via ballistic delivery of expression vectors into single epidermal cells of barley (Schweizer *et al.*, 1999). All genes to be tested were subcloned into pGY-1 to be controlled by the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Hückelhoven *et al.*, 2003; Schweizer *et al.*, 1999). To examine the cell death-inducing capacity of HvRAC1, we adapted a cell viability assay previously described by Eichmann *et al.*, (2006). The assay is based upon the ballistic delivery of expression constructs into epidermal cells according to a transient transformation protocol (Schweizer *et al.*, 1999). For this assay, barley cultivar 'Manchuria' was used. Each shot delivered 312 µg of 1.1 µm size tungsten particles coated with 0.5 µg of *psGFPHdel* as reporter gene, together with 1 µg of *CA HvRAC1-G23V*, *CA HvRACB-G15V* or empty vector *pGY-1*. To test, whether *CA HvRAC1-G23V*-induced cell death could be suppressed by the barley cell death inhibitor BAX INHIBITOR-1 (HvBI-1), a barley ascorbate peroxidase (APX, accession number AJ006358; Hess and Börner 1998) or the murine cell death suppressor BCL-X_L, the respective plasmids were delivered along with *CA HvRAC1-G23V*. 0.8 µg of *pHvBI-1* or 1 µg of *pHvAPX* or 1 µg of *pBCL-X_L* (Eichmann *et al.*, 2006; Hückelhoven *et al.*, 2003) or the respective amount of empty vector *pGY-1* was delivered into barley epidermal cells. Transiently transformed leaf segments were analyzed 16-20 h after transformation. By using fluorescence microscopy, the *sGFPHdel* co-expressing cells were evaluated in terms of maintenance of cytoplasmic movement as a marker of cell viability.

2.6 Targeted yeast two hybrid screening to test the interaction between barley RAC/ROP and RBOH proteins

2.6.1 Cloning of cDNAs into yeast vectors

Wild type (WT), constitutively activated (CA) and dominant negative (DN) forms of various barley ROPs were used in the targeted yeast two-hybrid screening. WT HvRACB, CA HvRACB-G15V, CA HvRAC1-G23V and CA HvRAC3-G17V were cloned in frame with the DNA binding domain (BD) into the yeast pGBKT7 DNA-BD expression vector (Matchmaker Two-Hybrid Library Construction and Screening Kit, Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) (Schultheiss *et al.*, (2008). Additionally, DN HvRACB-T20N, DN HvRAC1-T28N and WT HvRAC3-coding sequences in pGBKT7 vector were also used. Bait plasmids were constructed by H. Schultheiss (Institute of Phytopathology and Applied Zoology, Justus-Liebig University, Giessen, Germany) and Jutta Preuss (Lehrstuhl für Phytopathologie, Technische Universität München, Germany). For all ROP proteins, prenylation and palmitoylation sites were removed to facilitate transport to the nucleus.

Table 2.2 Primer sequences for cDNA amplification and cloning of HvRBOHs into the yeast expression vector pGADT7.

Construct	Primers	Primer sequences
pGADT7-RBOHA-1-401	rboh24EcoRI+Cfwd RBOH_Y2HBamHlrev	GAATCCAAATCGTCAACCCAGTTTGG GGATCCCTAGATCGGGCTCCTATACCT
pGADT7-RBOHJ-1-292	RBOHJfwdEcoRI RBOHJrevBamHI	GAATTCATGGCGGACCGGCCAGCG GGATCCTACCGCTCCAGTTCCTCGT
pGADT7-RBOHB1-1-295	RBOHB1fwdEcoRI RBOHB1revBamHI	GAATTCATGGCTGACATTGAAGCT GGATCCTAACGCTCCAGTTCCTCT
pGADT7-RBOHB2-1-354	RBOHB2fwdEcoRI RBOHB2revBamHI	GAATTCATGGAGATGCCTGATATT GGATCCTAGCGCTCCAGTTTCCT

For the cloning of different RBOH proteins into the yeast expression vector pGADT7-Rec AD (Matchmaker Two-Hybrid Library Construction and Screening Kit, Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), only the N-terminal part of the cDNAs was used as it was assumed to interact with RAC/ROP

proteins (Wong *et al.*, 2007). cDNAs encoding the N-terminal regions of barley RBOHA (F2) (aa 1-401), RBOHJ (aa 1-292), RBOHB1 (1-295) and RBOHB2 (1-354) were amplified from a barley cDNA pool by using specific primers (see Table 2.2). The PCR fragments were ligated into the pGEM-T cloning vector (Promega, Mannheim, Germany) according to the manufacturer's instructions. After sequence confirmation, RBOH fragments were subcloned in frame with the activation domain of the yeast expression vector pGADT7 by using EcoRI and BamHI restriction sites.

2.6.2 Yeast transformations and drop-assay

Yeast cells were co-transformed with the pGBKT7 and pGADT7 vectors containing respective HvROP and HvRBOH cDNA sequences. Different combinations of barley ROPs and RBOHs were transformed into yeast cells including the empty vector controls. The transformation was done with the lithium acetate (LiAc) method for small-scale yeast transformation as described in the Yeast Protocol Handbook (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). *Saccharomyces cerevisiae* strain AH109 was pre-cultured at 30°C in YPDA (2 % peptone, 1 % yeast extract, 0.003 % adenine and 2 % glucose in water at pH 6.5) broth to give a density of OD₆₀₀ 0.4-0.6. After centrifugation, competent cells were washed with sterile water and re-suspended in freshly prepared, sterile 1x TE/1x LiAc solution. For simultaneous co-transformation, 0.1 µg of each plasmid (in the respective combinations) and 0.1 mg of salmon testes carrier DNA (Sigma-Aldrich Chemie GmbH, München, Germany) were added to 100 µl of yeast competent cells. To this, 600 µl of PEG/LiAc solution (40 % polyethylene glycol in 1x TE/1x LiAc solution) was added and incubated at 30°C for 30 min. After adding 70 µl of dimethyl sulfoxide (DMSO), cells were transferred into a 42°C water bath for 15 min and then put on ice for brief chilling. The suspensions were centrifuged and the pellet were re-suspended in sterile 1x TE buffer and spread on synthetic dropout (SD) medium with selection markers. In this case, double dropout medium lacking amino acids tryptophan and leucine (-TL) was used to select for the presence of both pGADT7 and pBKT7 plasmids. After about 5 days of incubation at 30°C, single colonies of transformed yeast appeared on the plates.

To test for protein-protein interactions, all combinations of transformed yeast cells were grown on the selective quadruple dropout medium, lacking four amino acids (tryptophan, leucine, adenine and histidine (-TLAH)). Since adenine and histidine biosynthesis requires transcriptional activation after bait and pray interaction, growth on (-TLAH) medium indicates the interaction between the two respective proteins in the yeast cells. For this purpose, a qualitative drop assay was performed. Single colonies of each co-transformation event were picked-up from the (-TL) medium plates and diluted in YPDA broth. For the yeast drop assay, yeast suspensions were adjusted to a concentration of about 400 cells/ μL . 10 μl of each combination of yeast transformants were dropped onto the (-TL) and (-TLAH) media plates. The cells were grown at 30°C for about 7 days before they were photographed. A qualitative β -galactosidase assay was also done by growing yeast cells on (-TLAH) medium with X- α -gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside) at a concentration of 40 mg L⁻¹.

- **YPDA medium**

- 2 % peptone
- 1 % yeast extract
- 0.003 % adenine
- 2 % glucose
- 2 % agar (for plates only) in distilled water
- adjust pH to 6.5 and autoclave

- **Double dropout (-TL) medium**

- 0.17 % yeast nitrogen base without amino acids
- 0.5 % ammonium sulfate
- 0.059 % amino acids from 10x Dropout stock without tryptophan and leucine
- 2 % glucose
- 2 % agar (for plates only) in distilled water
- adjust pH to 5.8 and autoclave

- **Quadruple dropout (-TLAH) medium**

- 0.17 % yeast nitrogen base without amino acids
- 0.5 % ammonium sulfate

0.055 % amino acids from 10x Dropout stock without
tryptophan, leucine, adenine and histidine
2 % glucose
2 % agar (for plates only) in distilled water
adjust pH to 5.8 and autoclave

- **10x Dropout stock per L**

200 mg L-adenine hemisulfate salt
200 mg L-arginine HCl
200 mg L-histidine HCl monohydrate
300 mg L-isoleucine
1000 mg L-leucine
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
200mg L-uracil
1500 mg L-valine

- **10x TE buffer**

0.1 M Tris-HCl
10 mM EDTA in distilled water
adjust pH to 7.5 and autoclave

- **10x LiAc solution**

1 M lithium acetate in distilled water
adjust pH to 7.5 and autoclave

- **50% Polyethylene Glycol (PEG)**

50g of PEG mol. wt. 3,300 in 100mL distilled water
sterilized by autoclaving

2.7 Bimolecular fluorescence complementation (BiFC)

Split YFP-mediated BiFC was performed to verify *in planta* protein-protein interactions of barley RAC1 with RBOHs (Walter *et al.*, 2004). For this, RAC1 variants (WT RAC1, CA RAC1-G23V, and DN RAC1-T28N) were fused with the C-terminal part of yellow fluorescing YFP, and RBOHB1 and RBOHB2 were fused with the N-terminal part of YFP. WT RAC1-, CA RAC1-G23V-, and DN RAC1-T28N-coding fragments were amplified from of the respective pGY1 expression constructs (Caroline Höfle, Lehrstuhl für Phytopathologie, Technische Universität München, Germany, personal communication) by using hvRac1-5'BsrGI (5'-TGTACAGCTGGAGAGGAGAGGAGAGG-3') and hvRac1-3'SacI (5'-GAGCTCCCCATTGGAGAACAACCAC-3') primers. The PCR fragments were ligated into the pGEM-T cloning vector (Promega, Mannheim, Germany) according to the manufacturer's instructions. After sequence confirmation, the fragments were subcloned into the pUC-SPYCE vector (Walter *et al.*, 2004), in frame with the C-terminal fragment of YFP using BsrGI and SacI restriction sites. The resulting fusion constructs were pUC-SPYCE-WT RAC1, pUC-SPYCE-CA RAC1-G23V and pUC-SPYCE-DN RAC1-T28N.

Table 2.3 Primer sequences for cDNA amplification and cloning of HvrBOHs into the BiFC vector pUC-SPYNE.

Construct	Primers	5'-and 3' primer sequences
pUC-SPYNE-RBOHA-1-401	rboh24EcoRI+Cfwd RBOHArevBamHIInoStop	GAATCCAAATCGTCAACCCAGTTTGG GGATCCTCGGATCGGGCTCCTATACCT
pUC-SPYNE-RBOHJ-1-292	RBOHJfwdEcoRI RBOHJrevBamHIInoStop	GAATTCATGGCGGACCGGCCAGCG GGATCCTATCCGCTTCCAGTTCTCGT
pUC-SPYNE-RBOHB1-1-295	RBOHB1fwdEcoRI RBOHB1revBamHIInoStop	GAATTCATGGCTGACATTGAAGCT GGATCCTATACGCTTCCAGTTCTCCT
pUC-SPYNE-RBOHB2-1-354	RBOHB2fwdEcoRI RBOHB2revBamHIInoStop	GAATTCATGGAGATGCCTGATATT GGATCCTATGCGCTTCCAGTTTTCCT

The N-terminal regions of different RBOH (A, J, B1 and B2) genes were amplified from respective pGEMT constructs (see chapter 2.6.1) using respective primers (see Table 2.3) and cloned into pGEM-T (Promega, Mannheim, Germany). After sequence confirmation, the RBOH coding sequences were subcloned into the pUC-SPYNE vector (Walter *et al.*, 2004) in frame with the YFP N-terminus using SpeI and BamHI restriction sites, resulting in pUC-SPYNE-RBOHA-1-401, pUC-SPYNE-RBOHJ-1-292, pUC-SPYNE-RBOHB1-1-295 and pUC-SPYNE-RBOHB2-1-354 constructs.

Particle bombardment (see chapter 2.5) technique was to co-transform fusion constructs in various combinations into barley epidermal cells together with red fluorescent protein (RFP) as soluble protein marker. Each shot delivered 0.5 µg of RFP plasmid (Jutta Preuss, Lehrstuhl für Phytopathologie, Technische Universität München, Germany, personal communication) and 1 µg of each BiFC construct (pUC-SPYCE and pUC-SPYNE). pUC-SPYCE-CA HvRACB-G15V and pUC-SPYNE-HvRIC171 were used as a positive control (Schultheiss *et al.*, 2008) and empty BiFC vectors were used as negative controls. Transformed short epidermal cells were checked for RFP and YFP fluorescence by a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems, Mannheim, Germany) 48 h after transformation. RFP was excited by a 561 nm laser line and detected at 585-635 nm while YFP was sequentially excited at 514 nm and detected at 525-556 nm. For excitation and detection of BiFC, identical microscope settings were used for all samples. Argon laser was set to 20% laser intensity, and the 514 nm laser line was set to 15% of the maximum.

2.8 Statistical analyses

Statistical analyses of the data were carried out by using either Student's t-tests or ANOVA (analysis of variance) and Tukey's multiple comparison tests. The details of the statistical analyses were mentioned in the respective figure legends.

3. Results

3.1 Phenotypic characterization of CA HvRAC/ROP-expressing transgenic barley and tobacco plants

Constitutively activated (CA) forms of three barley RAC/ROP proteins (CA HvRACB-G15V, CA HvRAC3-G17V and CA HvRAC1-G23V) were generated by mutational exchange of the amino acid glycine to valine in the GTPase domain of the respective proteins. CA *HvRACB-G15V*, CA *HvRAC3-G17V* and CA *HvRAC1-G23V* coding sequences were expressed under the maize ubiquitin 1 promoter (*ZmUbi1*) in barley, while CA *HvRACB-G15V* and CA *HvRAC3-G17V* were expressed under control of the cauliflower mosaic virus 35s promoter (*CaMV35S*) in tobacco. Stable transgenic plants of barley and tobacco were produced by *Agrobacterium tumefaciens*-mediated transformation. T₁ and T₂ generations of transgenic plants from several independent lines were used in the experiments. All three genotypes of barley segregated in a ratio of about 0.8:1 (CA *HvRACB-G15V* (125:154); CA *HvRAC1-G23V* (186:252); CA *HvRAC3-G23V* (87:103)) instead of 3:1 for transgene expression, which was also confirmed by a χ^2 test. This might indicate male sterility of the CA ROP-expressing barley (see also Schultheiss *et al.*, 2005). The presence and expression of the transgenes in individuals was confirmed by genomic and one step RT-PCR before using them for experiments. In all experiments, a subpopulation of all individuals checked by PCR had lost the transgene due to segregation. These individuals had a wild type genotype combined with a tissue-culture background. These azygous individuals constitute ideal genetic controls. In order to confirm that T-DNA insertion or somatic mutations during regeneration would not be cause of potential CA HvROP-induced phenotypes, at least three independent transgenic lines for each construct were used in all experiments. According to Student's t-tests or χ^2 tests, respectively, independent transgenic lines behaved similarly, and thus data from different lines with the same HvROP construct were pooled and treated as a metapopulation.

3.1.1 Macroscopic analysis of plant phenotypes

All CA HvRACB-G15V, CA HvRAC3-G17V and CA HvRAC1-G23V-expressing barley plants were macroscopically indistinguishable from the wild type until they reached the three-leaf stage. The following leaves often remained twisted after leaf unfolding and resulted in a downward rolled phenotype (Schultheiss et al., 2005). Later, plants consistently showed shorter internodes, stunted shoots, less tillering, and strongly reduced formation of ears demonstrating developmental failure. In contrast, the azygous sibling plants, which had lost the transgene due to segregation, set ears and developed normally like that of wild type plants (Fig. 3.1).

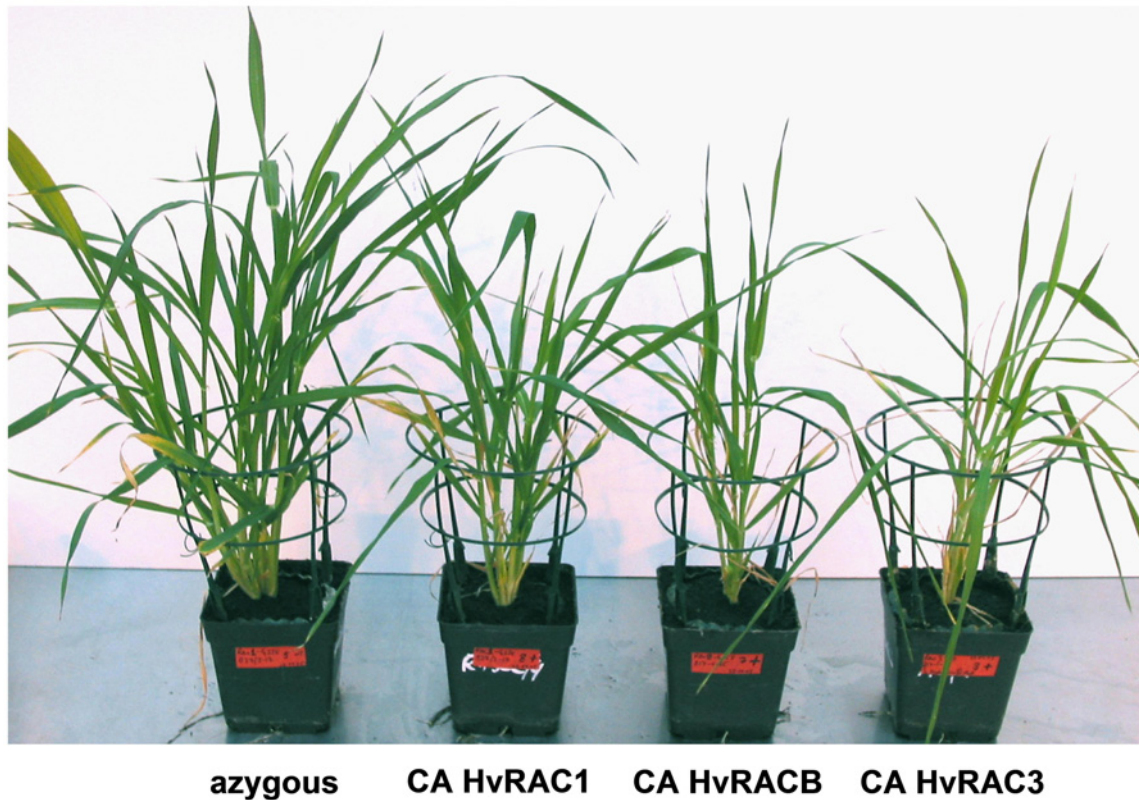


Figure 3.1: Phenotypes of CA HvROP-expressing barley plants. Shoot development of 7 weeks old wild type segregant, CA HvRACB-G15V (line 18-1-6), CA HvRAC3-G17V (line 17-3-16) or CA HvRAC1-G23V (line 27/2-29) barley plants.

CA HvRACB-G15V and CA HvRAC3-G17V-expressing transgenic tobacco plants exhibited no obvious phenotypic aberrations in terms of plant height and growth

rate when compared to either empty vector control or wild type plants. However, CA HvROPs induced an obvious leaf phenotype in tobacco. CA HvRACB-G15V tobacco leaves were often more lancet-like and the proportion of the leaf length to width axis is greater than wild type or vector controls (Fig. 3.2a). CA HvRAC3-G17V tobacco leaves appeared wavy, leathery and showed an irregular leaf texture (Fig. 3.2a). The leaf surface was uneven and hairy and could be clearly distinguished from wild type (Fig. 3.2b). In CA HvRAC3-G17V tobacco, minor veins were not clearly visible on the leaf surface, which might be due to the disproportionate growth of the leaf tissues or mesophyll hypertrophy.

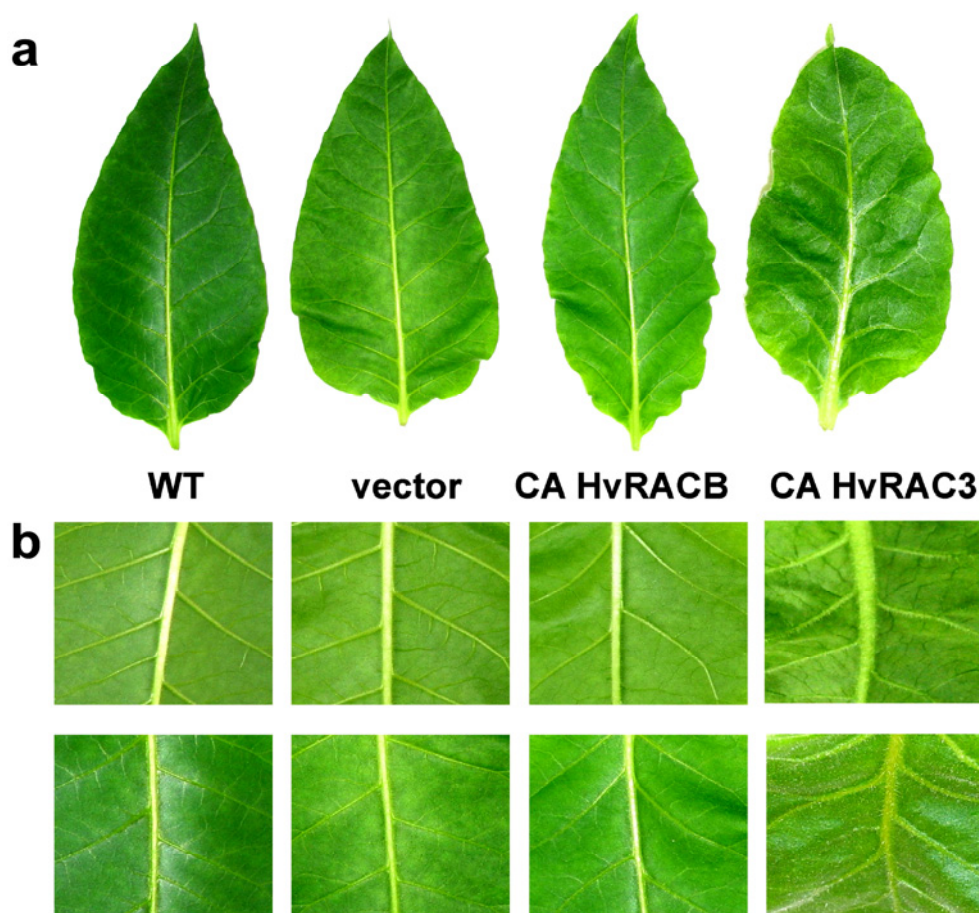


Figure 3.2: Leaf phenotypes of tobacco plants expressing CA HvRACB-G15V or CA HvRAC3-G17V. a) Fully expanded leaves of 8 weeks old tobacco plants of wild type, empty vector control, *CaMV35S::CA HvRACB-G15V* (Line 11L1) or *CaMV35S::CA HvRAC3-G17V* (12L3). b) Comparison of leaf lamina morphology on both abaxial and adaxial planes in higher magnification. All leaves were photographed at the same age and magnification.

3.1.2 Microscopic analysis of root hair phenotypes

When barley seedlings grew on artificial solid medium containing 5% sucrose (w/v), which appears to enhance root hair defects (Yang *et al.*, 2007), root hairs of both CA HvRACB-G15V and CA HvRAC3-G17V were short, swollen and irregular when compared to those of azygous controls or wild type barley. CA HvRAC1-G23V barley had also deformed root hairs but the phenotype was less obvious when compared to CA HvRACB-G15V or CA HvRAC3-G17V (Fig. 3.3).

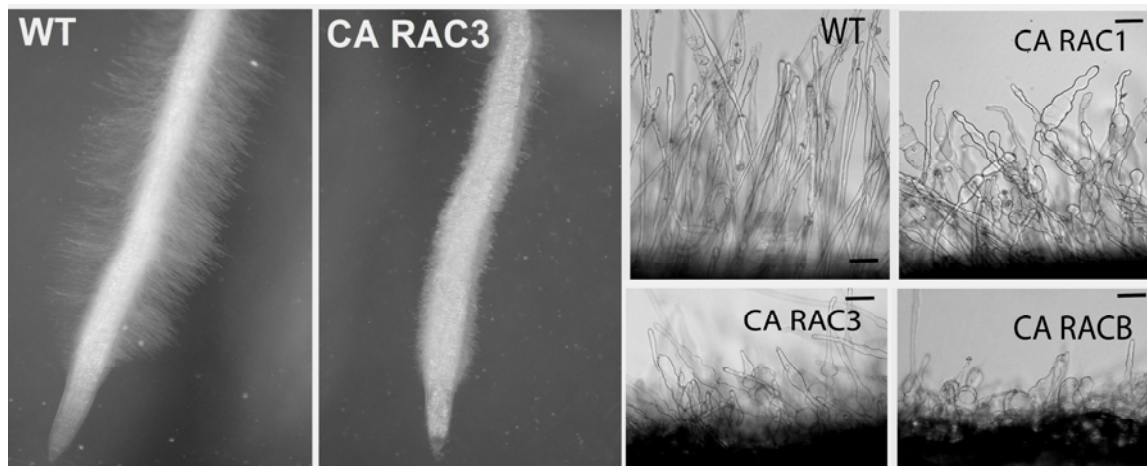


Figure 3.3: Root and root hair phenotypes of CA HvROP expressing barley. Root phenotype of 1-week-old barley grown on Murashige-Skoog medium with 5% sucrose. Pictures show representative sectors of the differentiation zone of barley roots and details of wild type or aberrant root hair development in CA HvROP barley. Bar 30 μm . Repetition of the experiment led to similar results (Lines used: CA HvRACB-G15V 18-1-6-14; CA HvRAC1-G23V 27-2-29; and CA HvRAC3-G17V 17-3-16).

In tobacco, root hairs of CA HvRACB-G15V and CA HvRAC3-G17V-expressing plants showed aberrant phenotypes when grown on Murashige and Skoog agar plates. Root hairs were stunted and swollen, or had balloon-like tips (Fig. 3.4). This was observed irrespective of whether CA HvRACB-G15V or CA HvRAC3-G17V was expressed. Together, results shows that CA HvRACB-G15V and CA HvRAC3-G17V disturbed root hair tip growth in barley and tobacco.

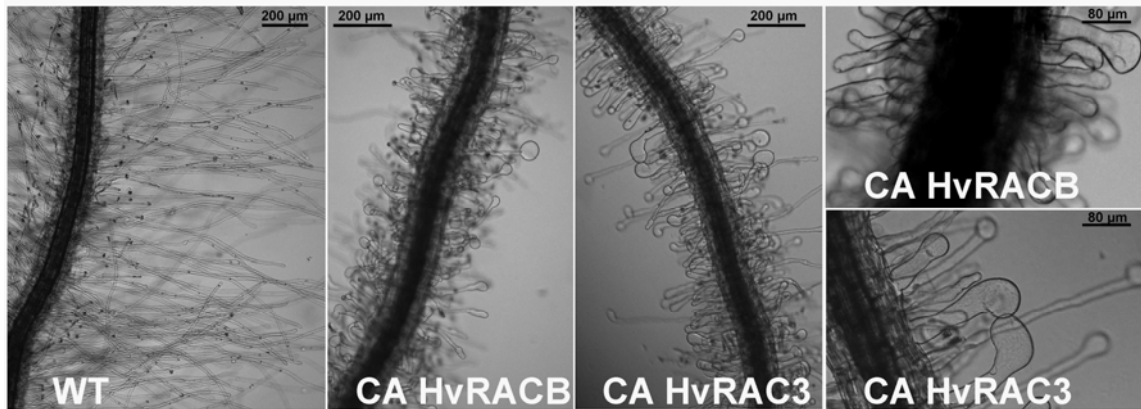


Figure 3.4: Root hair phenotypes of CA HvROP expressing tobacco. Root hair phenotypes of 2 weeks old tobacco plantlets of wild type, CAMV35S::CA *HvRACB*-G15V (11L1) or CAMV35S::CA *HvRAC3*-G17V (12L3).

3.1.3 Microscopic analysis of leaf epidermal cell phenotypes

Although CA HvROP-barley plants showed some developmental failure, the shape of epidermal cells was not dramatically changed when compared to wild type. However, the size of the epidermal cells appeared irregular (Fig. 3.5a). Hence, the length of epidermal B-cells covering mesophyll tissue between longitudinal vessels was measured in all the three HvROP expressing genotypes of barley. Expression of CA *HvRACB*-G15V and CA *HvRAC1*-G23V significantly enhanced B-cell length by about 20%, whereas CA *HvRAC3*-G17V enhanced cell length non-significantly by 8% when compared to the azygous controls (Fig. 3.5b).

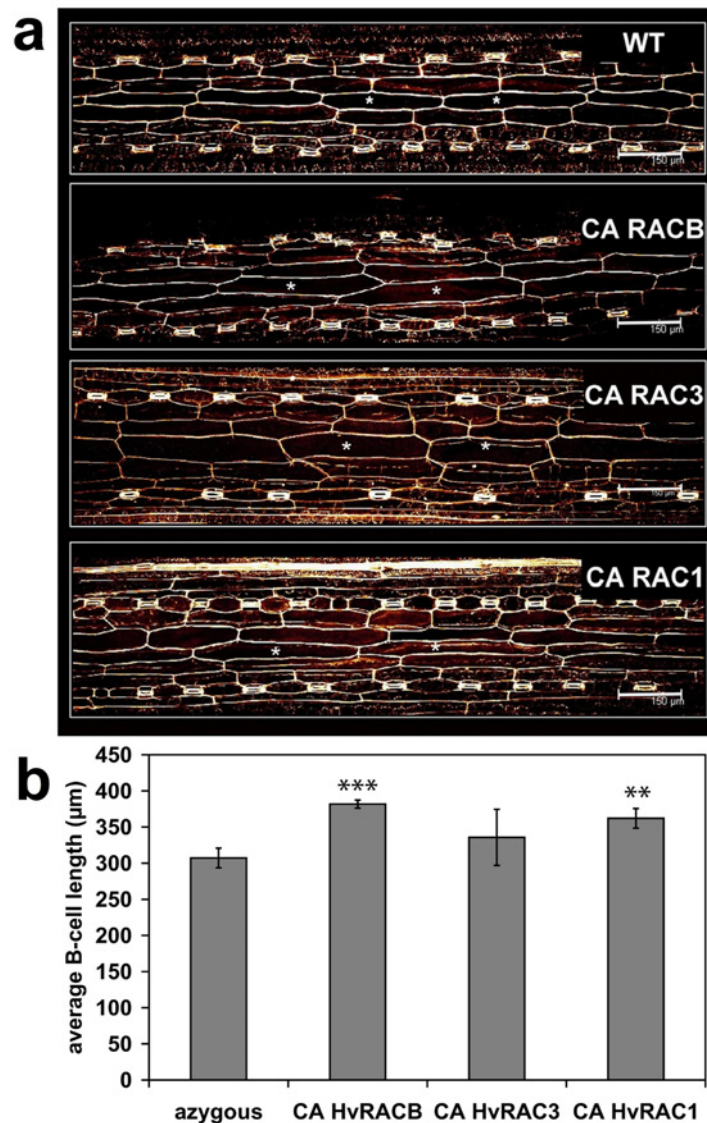


Figure 3.5: Phenotype and size of barley epidermal cells of transgenic plants expressing distinct CA HvROPs. a) Confocal laser scanning micrographs of cell walls of the adaxial epidermis of fourth leaves of indicated barley genotypes after modified pseudo-Schiff propidium iodide (mPS-PI) staining. Middle row of B-cells is marked with asterisks. Bar = 150 μm. b) Average cell sizes were measured in fully expanded leaves of 3 weeks old barley plants. Data show average size of epidermal cells covering mesophyll tissue in between two rows of longitudinal main vessels without having direct contact to stomata complexes. Data were pooled from three independent segregating transgenic lines for each CA HvROP construct. Error bars show 95% confidence intervals. **, ***; Student's t-test:

$p < 0.01$; $p < 0.001$ when compared to azygous control. Repetition of the experiments led to similar results (lines used: CA HvRACB-G15V 18-1-10, 17-1-11, 18-1-6; CA HvRAC1-G23V 27-3-17, 27-2-2; and CA HvRAC3-G17V 14-1-8, 17-3-16).

Furthermore, reduced or abnormal development of stomata was observed as an effect of CA HvROP expression. In barley, stomata and short epidermal cells alternate in a row of leaf epidermal cells (Fig. 3.6a). The number of stomata was significantly reduced in all three CA HvROP-expressing barley genotypes when compared to azygous controls (Fig. 3.6e). Apart from the stomata number, developmental abnormalities were observed in the arrangement of epidermal cells such as the presence of more than one short epidermal cell in between two stomata (Fig. 3.6b) or two stomata lacking an intercalated short epidermal cell

(Fig. 3.6c), or stomata failed to develop, which ended up in an abnormally short epidermal cell (Fig. 3.6d). Although such abnormalities were also rarely observed in wild type plants, leaves of all three CA HvROP genotypes exhibited a clearly higher frequency of abnormalities in a given length of a stomata row (see supplemental fig. 2).

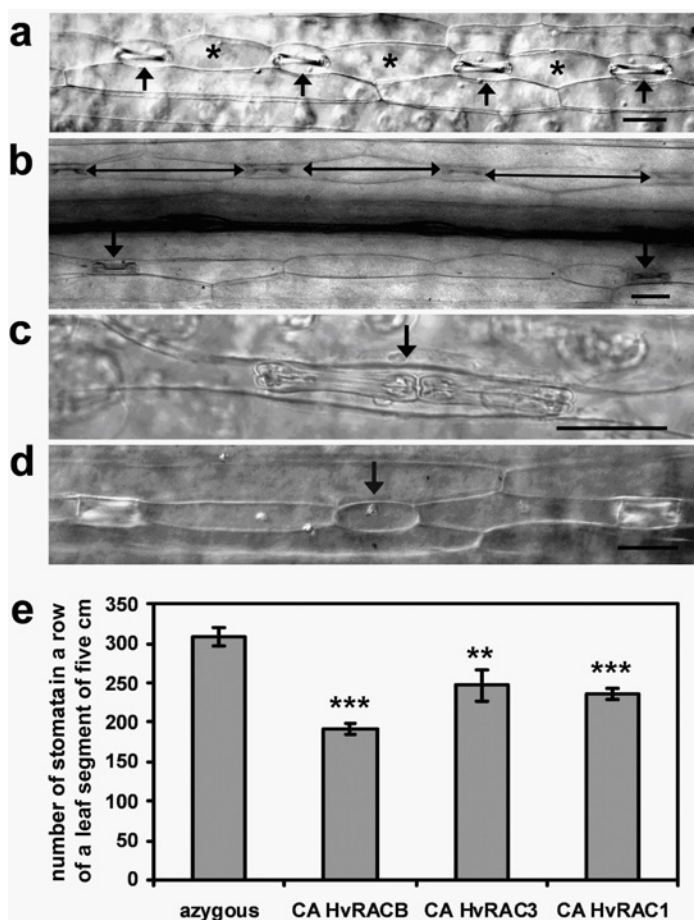


Figure 3.6: Stomatal abnormalities observed in CA HvROP-expressing transgenic barley leaves. a) Wild type adaxial leaf epidermis with alternating stomata complexes (arrows) and short epidermal cells (asterisks). b) Presence of more than one short epidermal cell in-between two stomata. Arrows point at stomata. Double headed arrows highlight intercalated cells with enhanced cell length. c) Two stomata lacking an intercalated short epidermal cell. d) Stoma failed to develop and left an abnormal blank cell. Scale bars = 50 μ m. e) Average number of stomata present in 5 cm of a stomatal row in transgenic plants expressing distinct CA HvROPs. For

all samples, stomatal rows present on either side of the mid rib were counted in the leaf upper epidermis. Fully expanded leaves of 3 weeks old barley plants were used for counting stomata. Error bars show 95% confidence intervals. **, ***; Student's t-test: $p < 0.01$; < 0.001 when compared to azygous control. Repetition of the experiments led to similar results. Repetition of the experiment led to similar results. (Lines used: CA HvRACB-G15V 18-1-10, 17-1-11, 18-1-6; CA HvRAC1-G23V 27-3-17, 27-2-2; and CA HvRAC3-G17V 14-1-8, 17-3-16).

In tobacco, epidermal pavement cell size was dramatically increased by the expression of either CA HvRACB-G15V or CA HvRAC3-G17V (Fig. 3.7a), suggesting enhanced cell expansion. Out of four independent CA HvRAC3-G17V transgenic lines, 12L3 and 12L4 showed the highest increase in epidermal

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cell size (up to 97% relative to the wild type), whereas in the other two lines (12L1 and 12L6) cell expansion was weaker (up to 29% increase) in comparison to wild type leaves. In case of CA HvRACB-G15V transgenic tobacco lines, 11L3 displayed the highest increase (up to 66%) in epidermal cell size, while the other three lines (11L1, 11L2 and 11L4) showed an increase in cell expansion of up to 31% when compared to the wild type leaves (Fig. 3.7b). Taken together, in three independent experiments all independent transgenic tobacco lines expressing CA HvROPs showed a significant increase in epidermal cell size (Fig. 3.7a).

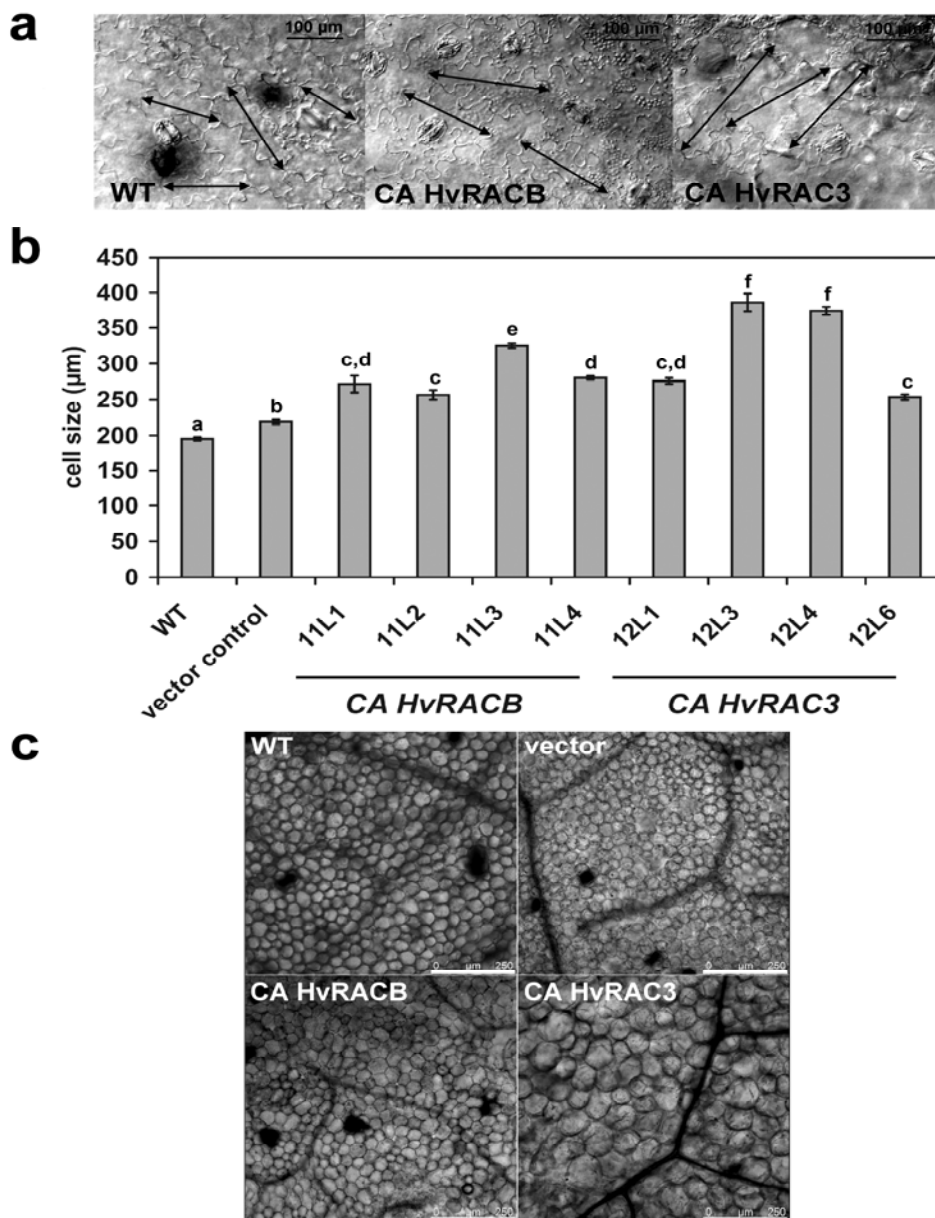


Figure 3.7: Epidermal and mesophyll cell sizes of tobacco plants expressing CA HvRACB-G15V or CA HvRAC3-G17V. a) Epidermal cell phenotype of fully expanded leaves of 8 weeks old tobacco plants of wild type, *CaMV35S::CA HvRACB-G15V* (11L1) or *CaMV35S::CA HvRAC3-G17V* (12L3). Bars = 100 μm . b) Average cell sizes of fully expanded leaves of 8 weeks old tobacco plants of wild type, empty vector control, *CaMV35S::CA HvRACB-G15V* (4 lines transformed with binary vector construct 11) or *CaMV35S::CA HvRAC3-G17V* (4 lines transformed with binary vector construct 12). Data were analyzed by ANOVA (analysis of variance) and Tukey's multiple comparison test. Error bars show standard errors of the means of three independent experiments. Averages highlighted with the same letter are not statistically different at $p < 0.001$. c) Mesophyll cell phenotype of fully expanded leaves of 8 weeks old tobacco plants of wild type, empty vector control, *CaMV35S::CA HvRACB* or *CaMV35S::CA HvRAC3-G17V*. All pictures were taken with the same magnification. Bars = 250 μm .

In both CA HvRACB-G15V and CA HvRAC3-G17V-expressing tobacco, interdigitation was less sawtooth-like when compared to wild type but rounder. In some CA HvRACB-G15V tobacco lines, patches of pavement cells had less prominent lobes and appeared brick-like. HvROPs also affected trichome development in tobacco leaves. In particular, CA HvRAC3-G17V-expressing plants showed bigger trichomes (Fig. 3.2b). To observe a potential hypertrophy of the mesophyll, mesophyll cell size was observed under the bright field microscope. This revealed that mesophyll cells of CA HvRAC3-G17V tobacco were much bigger than that of wild type or vector controls, whereas CA HvRACB-G15V effect on mesophyll cell size was less clear (Fig. 3.7c).

3.2 Evaluation of disease development in CA HvROP-expressing barley and tobacco challenged by leaf pathogens

3.2.1 CA HvROPs induce susceptibility to biotrophic *Golovinomyces cichoracearum* in tobacco

CA HvROP-expressing tobacco leaves were inoculated with spores of the compatible powdery mildew fungus *G. cichoracearum*. Macroscopic inspection of leaves 7-9 days after inoculation revealed typical powdery mildew symptoms on both wild type and empty vector control leaves (Fig. 3.8a). However, both CA HvRACB-G15V and CA HvRAC3-G17V tobacco lines showed strongly enhanced

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disease symptoms with mycelium sometimes covering the whole leaf area (Fig. 3.8a). Susceptibility to *G. cichoracearum* was quantified by estimating diseased leaf areas at seven days after inoculation. In tobacco lines expressing either of the CA HvROPs, the diseased leaf area was increased by up to 200% when compared to wild type leaves and leaves of the empty vector control (Fig. 3.8b). However, only single lines proved statistically different from the respective controls. To uncouple the CA HvROP effects from potential tissue culture or transgene-position effects in individual lines, data from each four lines bearing either CA HvRACB-G15V or CA HvRAC3-G17V were averaged for an independent statistical analysis. This revealed a significant impact of the CA HvROP transgenes on susceptibility when compared to either wild type or empty vector controls (Table 3.1).

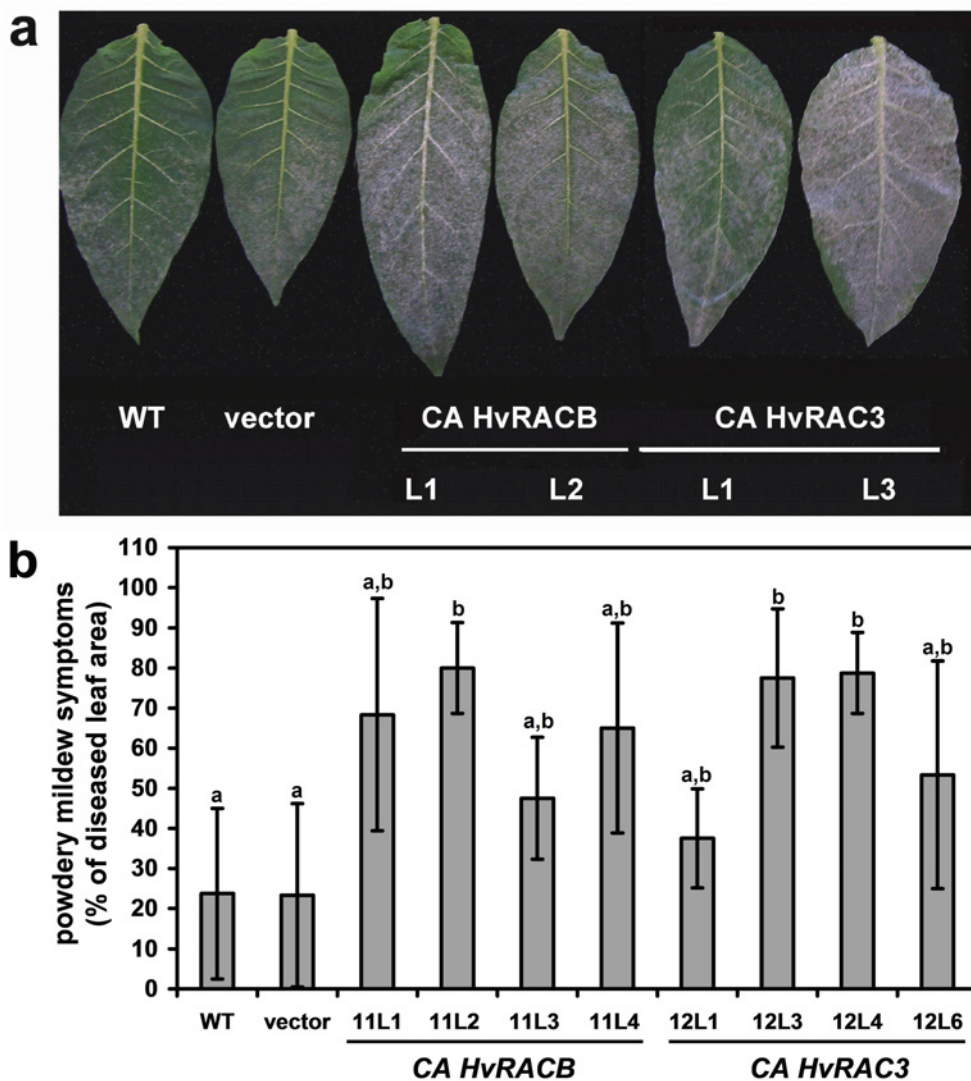


Figure 3.8: Powdery mildew disease ratings of tobacco plants expressing CA HvrACB-G15V or CA HvrAC3-G17V. a) Fully expanded leaves of 8 weeks old tobacco plants of wild type, empty vector control, *CaMV35S::CA HvrACB-G15V* or *CaMV35S::CA HvrAC3-G17V*. Leaves were inoculated with *Golovinomyces cichoracearum* and photographed 9 days later. b) Powdery mildew symptom rating on fully expanded leaves of 8 weeks old tobacco plants of wild type, empty vector control, *CaMV35S::CA HvrACB-G15V* (4 lines transformed with binary vector construct 11) or *CaMV35S::CA HvrAC3-G17V* (4 lines transformed with binary vector construct 12). Leaves were inoculated with *G. cichoracearum* and rated 7 days later. Error bars show 95% confidence intervals. Data were analyzed by ANOVA (analysis of variance) and Tukey's multiple comparison test. Averages highlighted with the same letter are not statistically different at $p < 0.05$. Repetition of the experiments led to similar results.

Table 3.1 CA HvrROP effects on tobacco powdery mildew symptoms

Genotype	Wild type	Empty vector	CA HvrACB-G15V	CA HvrAC3-G17V
Average percentage of leaf area covered with powdery mildew symptoms	18,3 ^a	23,3 ^a	62,3 ^b	63, 9 ^b

^{a,b} Averages highlighted with the same letter are not statistically different at $p < 0.05$. Data were analyzed by ANOVA (analysis of variance) and Tukey's multiple comparison test.

3.2.2 CA HvrAC3-G17V, but not CA HvrACB-G15V enhances susceptibility to *Pseudomonas syringae* pv. *tabaci*

To test the specificity of CA HvrROPs on the influence of plant-pathogen interactions, we inoculated CA HvrROP-expressing tobacco plants with a virulent strain of *P. syringae* pv. *tabaci*, the causal agent of the tobacco wildfire disease (Wolf and Foster 1917). Seven days after inoculation, wild type, empty vector control and CA HvrACB-G15V tobacco leaves appeared similar in terms of disease development after injecting a *Pst* suspension. The injected area turned necrotic and the directly adjacent tissue appeared chlorotic. In contrast, CA HvrAC3-G17V tobacco leaves injected with bacterial suspensions developed heavy disease symptoms along with tissue maceration and liquid oozing from the infected area (Fig. 3.9a).

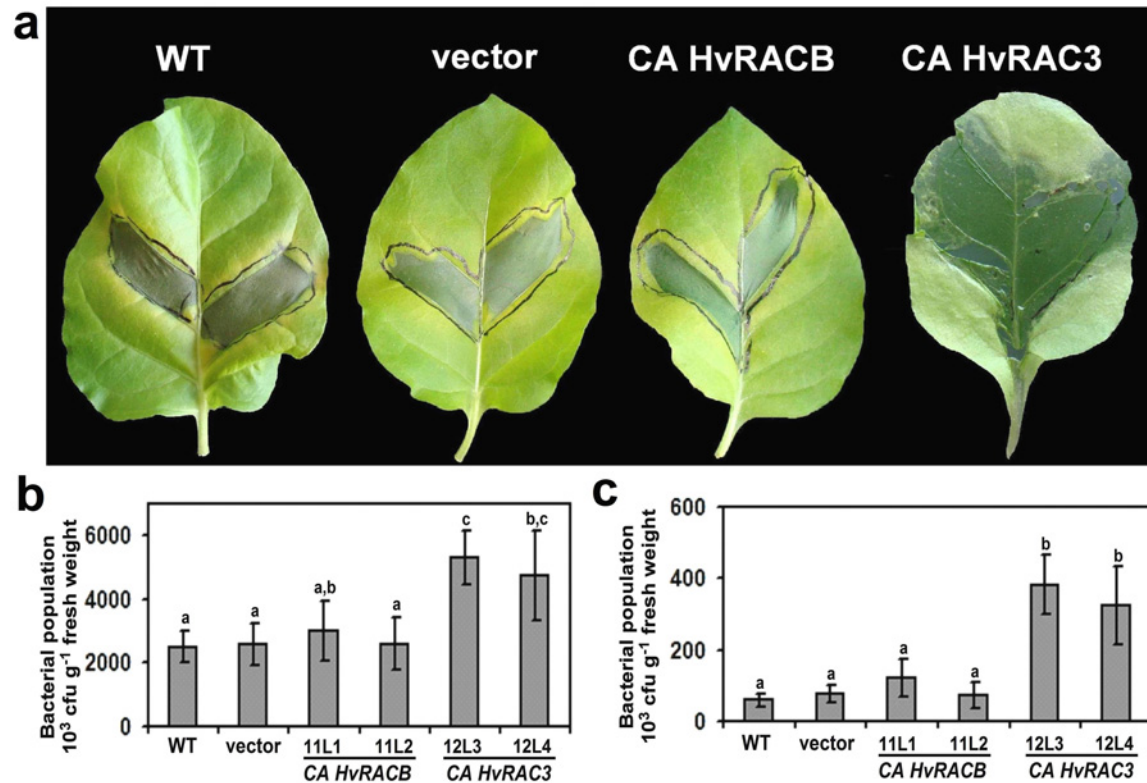


Figure 3.9: Infection of CA HvROP tobacco leaves with the wild fire bacterium *Pseudomonas syringae* pv. *tabaci*. Leaves were infiltrated with bacterial suspension at a concentration of 10⁶ cfu ml⁻¹. a) Wildfire disease symptoms on leaves of seven weeks old tobacco plants of wild type, empty vector control, CaMV35S::CA HvRACB-G15V (11L1) or CaMV35S::CA HvRAC3-G17V (12L3) photo-graphed 7 days after inoculation. The infiltrated areas are indicated by black marker ink. Symptom development assay was repeated with each four lines of CA HvRACB-G15V (11L1, 11L2, 11L3, 11L4) and CA HvRAC3-G17V (12L1, 12L3, 12L4, 12L6) and similar results were observed. b) Bacterial growth in the *Pst*-infiltrated areas of tobacco leaves four days after inoculation. c) Bacterial growth in the surrounding leaf area of the *Pst*-infiltrated zone in tobacco leaves four days after inoculation. Error bars show 95% confidence intervals. Repetition of the experiments led to similar results. Data were analyzed by ANOVA (analysis of variance) and Tukey's multiple comparison test. Averages highlighted with the same letter are not statistically different at p<0.05.

Four days after inoculation, bacterial populations in the infiltrated areas were measured in all tobacco lines. In tobacco lines expressing CA HvRAC3-G17V, the number of cfu g⁻¹ fresh weight was increased by up to 100% when compared to wild type, empty vector control and CA HvRACB-G15V-expressing plants (Fig. 3.9b). Bacterial populations were also quantified in the leaf area surrounding the

infiltrated site. CA HvRAC3-G17V leaves showed up to five times the number of cfu when compared to controls (Fig. 3.9c).

3.2.3 CA HvROPs induce susceptibility to powdery mildew in barley

To test the susceptibility of CA HvROP-expressing barley plants, three weeks old leaves were inoculated with *Bgh* spores. Seven days after inoculation, disease severity was macroscopically evaluated by rating the infected leaf area. All three CA HvROP barley plants were more susceptible to powdery mildew with CA HvRACB-G15V having the strongest and CA HvRAC3-G17V having the weakest effect (Fig. 3.10a). In CA HvRACB-G15V and CA HvRAC1-G23V barley, diseased leaf area was significantly increased by up to 84% and 72% respectively, whereas CA HvRAC3-G17V barley showed 27% increase, which was not statistically different when compared to azygous control leaves (Fig. 3.10b).

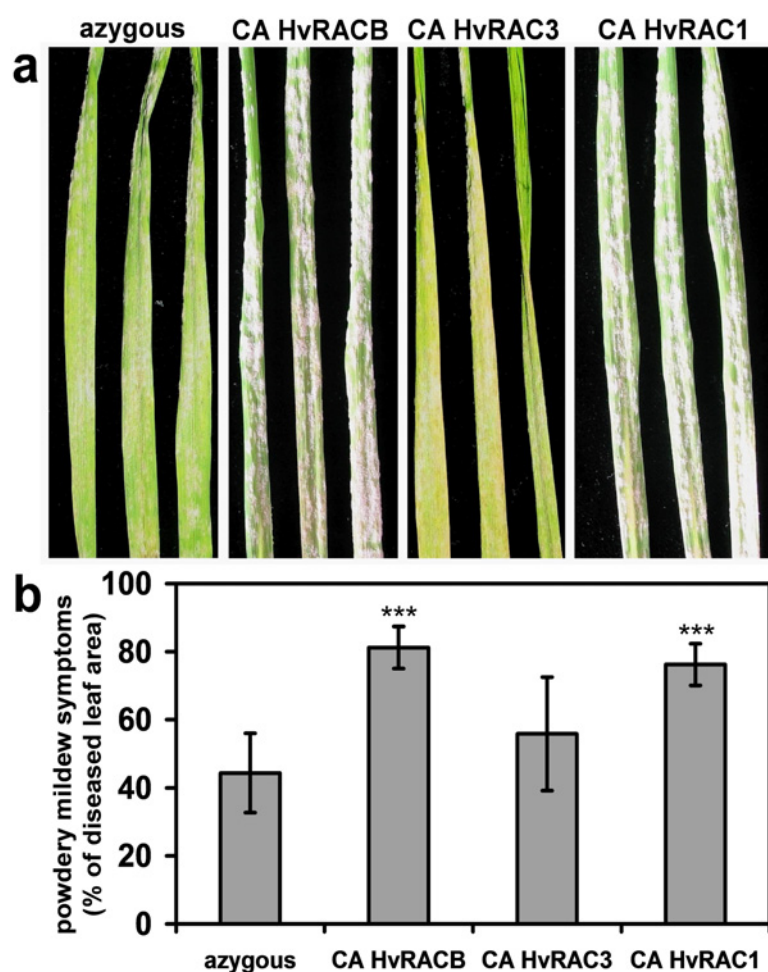


Figure 3.10: Powdery mildew infection of barley plants expressing CA HvROPs. a) Fully expanded leaves of 3 weeks old barley plants were inoculated with *Bgh* and the disease symptoms were photographed 7 days later. b) Powdery mildew symptom rating on inoculated leaves. Error bars show 95% confidence intervals. ***, Student's t-test: $p < 0.001$ when compared to azygous control. Repetition of the experiment led to similar results. (Lines used: CA HvRACB-G15V 18-1-10; CA HvRAC1-G23V 27-3-17; and CA HvRAC3-G17V 14-1-8).

3.3 Cyto-histochemical stainings and microscopic investigations of the interaction of CA HvROP-barley genotypes with *Bgh*

3.3.1 CA HvROPs support penetration by *Bgh* or the hypersensitive reaction or both

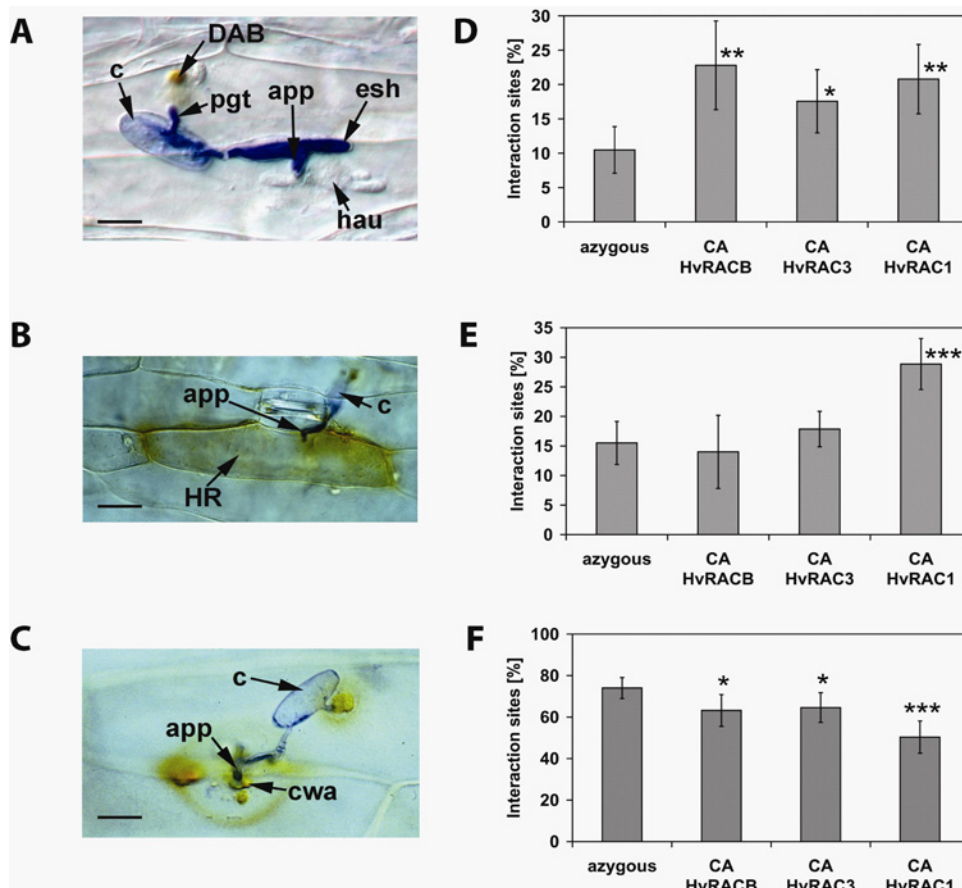


Figure 3.11: Microscopic inspection of phenotypes related to the interaction of transgenic barley lines expressing distinct CA barley ROPs with *Bgh*. a) Penetration site at 24 HAI on wild type barley. The fungus produced a primary germ tube (pgt), an appressorium (app), a haustorium (hau) and small elongated secondary hyphae (esh) from its conidium (c). Plant reacted with H_2O_2 production as indicated by DAB staining in association with the pgst. Bar = 10 μ m. b) Non-penetrated cell shows HR associated with whole cell DAB staining at 24 HAI on wild type barley. Bar = 30 μ m. c) DAB staining in association with formation of a CWA at a site where the fungus failed in penetration on wild type barley. Bar = 20 μ m. d) Average fungal penetration success displayed as percentage of all interaction sites where *Bgh* was able to establish a visible haustorium in epidermal cells of barley at 24 HAI (see a). e) Average frequency of interaction sites where the fungus failed in penetration and the attacked cell

responded with hypersensitive cell death accompanied by whole cell DAB staining (see b). f) Average frequency of interaction sites where the fungus failed in penetration, the attacked cell survived and displayed local DAB staining beneath the fungal appressorium (see c). Error bars show 95% confidence intervals. *, **, ***; Student's t-test: $p < 0.05$, < 0.01 ; < 0.001 when compared to azygous control. Each column represents data summarized from a metapopulation of three to four independent transgenic lines for each CA barley ROP construct (lines used: CA HvRACB-G15V 18-1-10, 18-1-6, 17-1-15, 17-1-22; CA HvRAC3-G17V 14-1-8, 16-1-1, B19-3-3; CA HvRAC1-G23V 27-3-17, 27-3-7, 27-2-32). Repetition of the experiments led to similar results.

To study the interaction of CA HvROP-expressing barley with *Bgh* in detail, two weeks old leaves were inoculated with *Bgh* and a microscopic analysis of the interaction outcome was performed at 24 and 48 hours after inoculation (HAI). All three CA HvROP-expressing barley genotypes were significantly more susceptible to fungal penetration than azygous controls (Fig. 3.11a, d). Attacked but non penetrated cells were also inspected for the frequency of H_2O_2 accumulation as evidenced by 3,3-diaminobenzidine (DAB) staining (Thordal-Christensen *et al.*, 1997). Whole cell DAB staining is indicative of a hypersensitive cell death reaction (HR), which is part of basal resistance in barley (Hückelhoven *et al.*, 1999; Koga *et al.*, 1990; Thordal-Christensen *et al.*, 1997). CA HvRAC1-G23V significantly supported whole cell H_2O_2 accumulation. However, this was observed only in cells where a fungal penetration attempt failed, i.e. where neither a fungal haustorium initial nor elongated secondary hyphae of the fungus developed (Fig. 3.11b, e). In contrast, neither CA HvRACB-G15V nor CA HvRAC3-G17V influenced the frequency of whole cell DAB staining. However, no spontaneous cell death was observed on non inoculated CA HvRAC1-G23V barley.

The number of living cells showing localized DAB staining beneath the fungal appressorium (Fig. 3.11c, f), which is typically associated with the formation of non penetrated cell wall appositions (CWAs, Thordal-Christensen *et al.*, 1997; Hückelhoven *et al.*, 1999) was also evaluated. This revealed that all three CA HvROPs reduced the frequency of local DAB staining at sites of fungal attack with CA HvRAC1-G23V having the strongest effect. This relates to enhanced fungal penetration, which was not accompanied by local DAB staining in CWAs.

The particular strength of this effect in CA HvRAC1-G23V barley is additionally explained by frequent whole cell DAB staining. Since independent transgenic lines behaved similarly, the data from different lines with the same HvROP construct were pooled and treated as a metapopulation for statistical analysis.

3.3.2 Comparison of whole plant and detached leaf inoculation methods of *Bgh* in barley

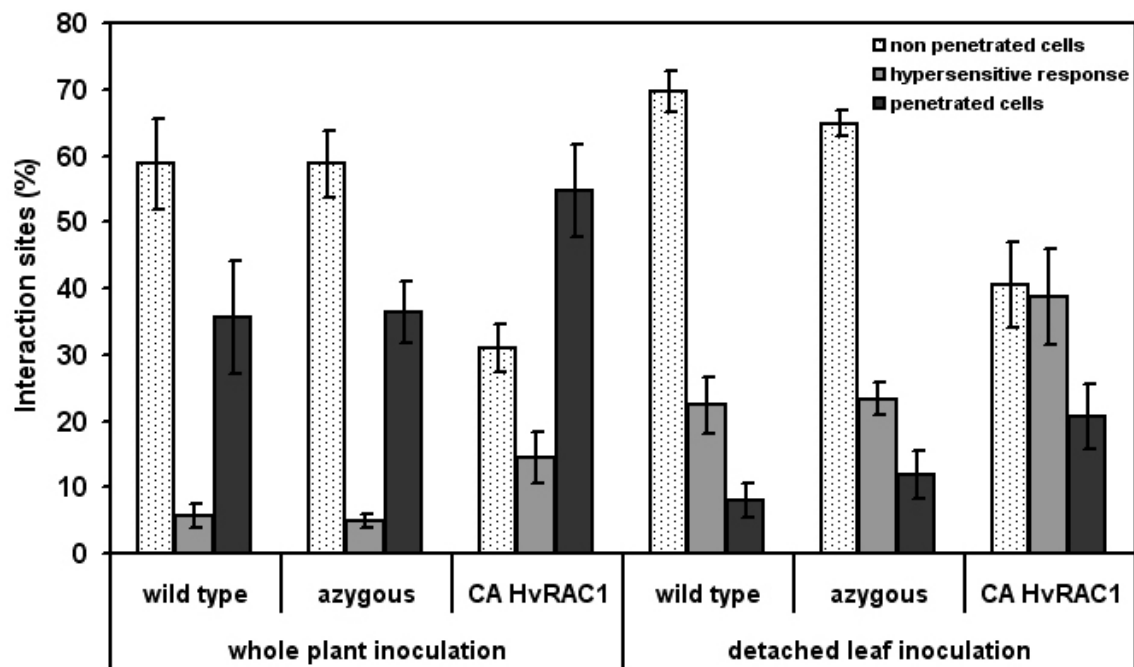


Figure 3.12: Comparison of whole plant and detached leaf inoculation of barley with *Bgh*. Leaves from wild type parent 'Golden Promise', azygous controls and CA HvRAC1-G23V barley plants were inoculated with *Bgh*. After 32 HAI, all *Bgh* interaction sites were evaluated to calculate the average frequency of non-penetrated, penetrated and the hypersensitive response (whole cell DAB staining) cells. Error bars show 95% confidence intervals. Student's t-test revealed significant differences in the frequency of interaction phenotypes in CA HvRAC1-G23V plants ($P < 0.001$) when compared to either wild type or azygous controls in both inoculation methods. Repetition of the experiment led to similar results. Lines used: CA HvRAC1-G23V 27-3-33, 27-2-29.

Inoculation experiments on detached leaves can yield quantitative or qualitative differences in interaction outcome when compared to attached leaf assays (Liu *et al.*, 2007). To test if the inoculation method would influence the

outcome of the barley-*Bgh* interaction, whole plant inoculation and detached leaf inoculation was done simultaneously on wild type Golden Promise, azygous controls and CA HvRAC1-G23V plants. Leaf samples were microscopically analyzed at 32 HAI. The rate of fungal penetration success was higher in leaves that were inoculated when attached to the plant when compared to detached leaves, whereas the frequency of whole cell DAB staining was lower on intact plants when compared to detached leaves (Fig. 3.12). However, in both cases, CA HvRAC1-G23V significantly supported fungal penetration and whole cell H₂O₂ accumulation in the epidermal cells of barley when compared to either wild type Golden Promise or azygous control plants (Fig. 3.12).

3.3.3 Frequent secondary HR observed in the CA HvRAC1-G23V barley - *Bgh* interaction

During the barley - *Bgh* interaction, when the fungal spore makes an attempt to infect the epidermal cells starting from the appressorium, the host cells tries to defend the attack either under formation of CWAs or by execution of host cell HR (Thordal-Christensen *et al.*, 1997; Hüchelhoven *et al.*, 1999). CWAs allow the fungus to make a second attempt to penetrate the host cell. Host cell HR can be executed before or after haustorium establishment by the powdery mildew fungus. Post-penetration HR supposedly kills the fungus as fungal development is arrested. Pre-penetration HR mostly results in restriction of the fungus before it can start a second penetration attempt. However, in some cases it can be observed that HR occurs in response to the second penetration attempt by the pathogen which is visible as second appressorial lobe. Thus, in barley-*Bgh* interaction, HR can be seen in response to either first or second attempt of the pathogen. Based on this qualitative difference in HR, the occurrence of one (Fig. 3.13a) or two (Fig. 3.13b) appressorial lobes of *Bgh* spores on HR cells was evaluated in different genotypes of barley. Irrespective of whether whole plants or leaf segments were inoculated, there was no significant difference in frequency of first and second lobe formation on HR cells in wild type 'Golden Promise' and azygous control plants. In whole plants, primary HR occurred in about 75% and secondary HR in about 25% of the cases. In detached leaves,

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frequency of HR, which allowed a second appressorial lobe formation, was a little lower (about 15-20%). In contrast, HR that allowed a second penetration attempt by the fungus occurred more frequently in CA HvRAC1-G23V plants. 43% of the HRs in whole plants and 38% in detached leaves allowed a second penetration attempt of *Bgh* spores (Fig. 3.13c). Thus, irrespective of the inoculation method, HR cells on CA HvRAC1-G23V plants allowed a second appressorial lobe formation more frequently when compared to either wild type or azygous controls.

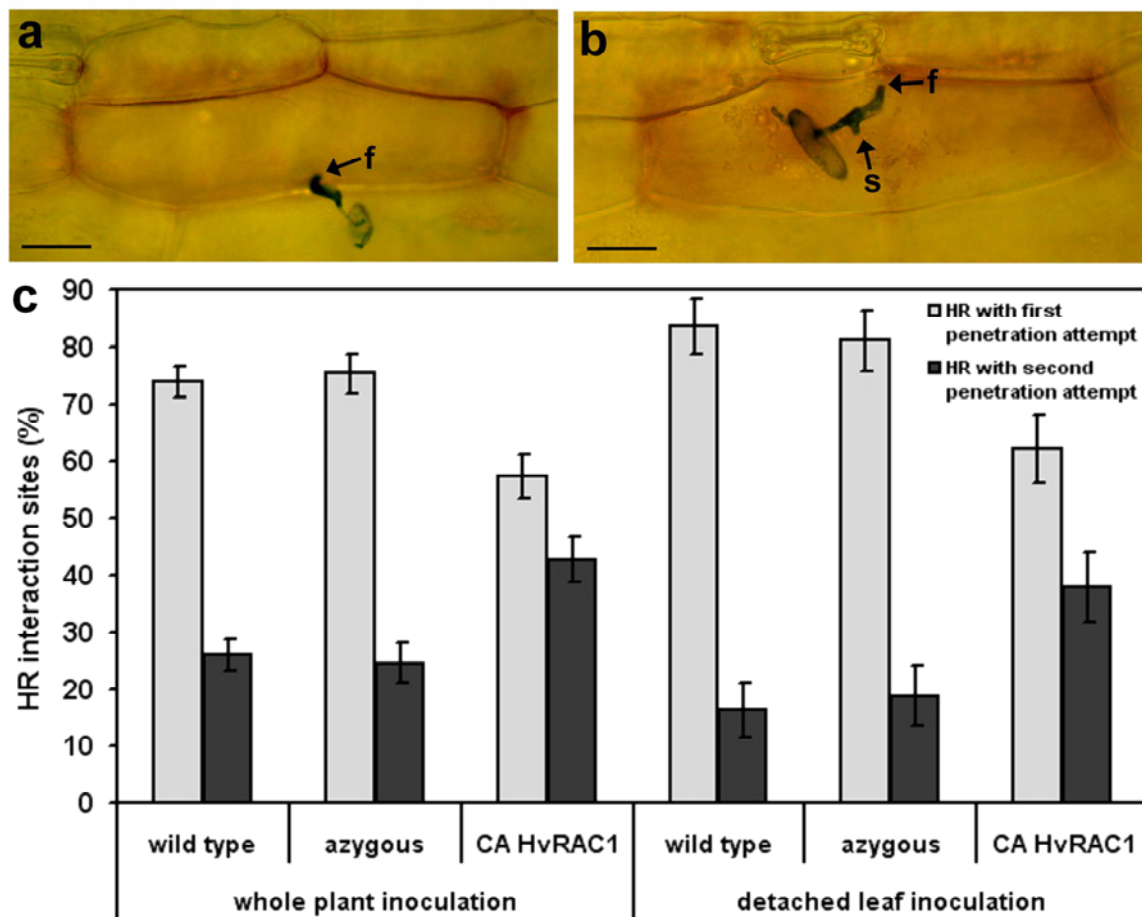


Figure 3.13: CA HvRAC1-G23V-dependent differences in appressorial lobe formation after HR. Leaves of wild type 'Golden Promise', azygous and CA HvRAC1-G23V barley plants were inoculated with spores of *Bgh* and the HR (as indicated by whole-cell DAB staining) interaction sites were microscopically analyzed. a) HR reaction that restricted fungal development at the first penetration attempt (f) by the pathogen. Bar = 30 μ m. b) HR reaction that allowed a second penetration attempt (s) by the pathogen. Bar = 20 μ m. c) Average percentages of HR phenotypes a) and b) in wild type, azygous and CA HvRAC1-G23V barley

leaves. Error bars show 95% confidence intervals. According to Student's t-test, differences in the occurrence of HR phenotypes were statistically significant ($P < 0.001$) in CA HvRAC1-G23V when compared to either wild type or azygous controls, irrespective of the inoculation method. (Lines used: CA HvRAC1-G23V 27-3-33, 27-2-29).

3.3.4 CA HvRAC1-G23V supports pathogen-induced callose deposition

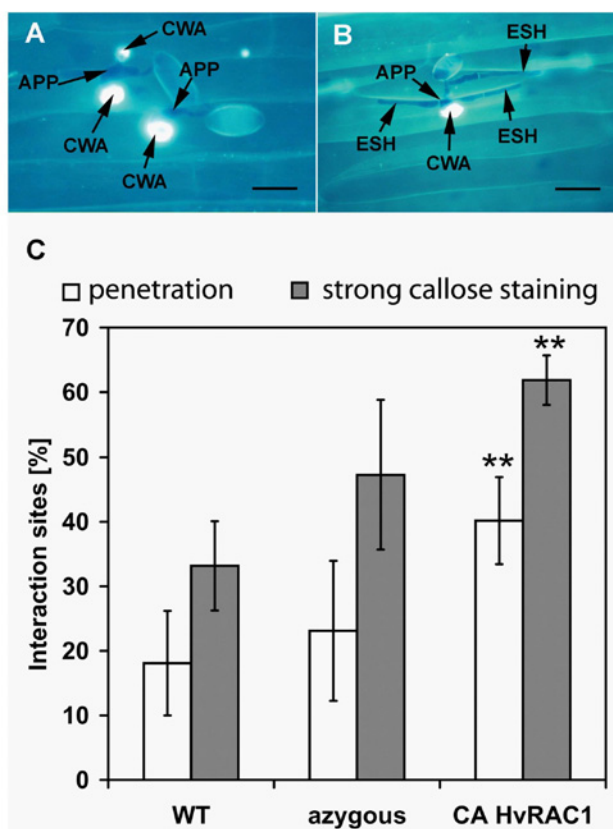


Figure 3.14: CA HvRAC1-G23V supports pathogen-induced callose deposition. a)

Strong callose depositions in cell wall appositions beneath sites of attempted fungal penetration. CWA: cell wall apposition; APP: appressorium. Bar = 30 μm . b) Strong callose depositions beneath a site of fungal penetration. ESH: elongated secondary hyphae; Bar = 45 μm . c) Columns represent the average frequency of fungal penetration success and total interaction sites with strong callose staining in CWAs beneath fungal appressoria at 40 HAI. **, Student's t-test: $p < 0.01$ when compared to either wild type or azygous control. Error bars show 95% confidence intervals. Repetition of the experiment led to

similar results (lines used: CA HvRAC1-G23V 27-3-17, 27-2-32).

Callose, a β -1, 3 glucan, is a major component of CWAs and callose deposition is a characteristic of cell wall-associated plant defense (Hückelhoven 2007; Schmelzer 2002). Since CA HvRAC1-G23V barley displayed little H_2O_2 accumulation at CWAs (Fig. 3.11c, f), callose deposition patterns in CWAs was also observed. Leaf samples inoculated with *Bgh* were stained with aniline blue at 40 HAI and the callose deposition patterns were evaluated by fluorescence microscopy. In wild type parent 'Golden Promise' and azygous control leaves, little penetration and differently strong callose deposits at sites of fungal attack were observed. However, any spatial association of strength or pattern of callose

depositions and local penetration resistance were not found (Fig. 3.14a, b). Consistent with the previous results (chapter 3.3.1), CA HvRAC1-G23V barley allowed for more penetration than wild type and azygous controls. Additionally, CA HvRAC1-G23V barley showed significantly more CWAs with strong callose deposits when compared to either control (Fig. 3.14c). Together, CA HvRAC1-G23V appeared to support local callose deposition.

3.4 Transient over-expression of CA HvRAC1-G23V reduces cytoplasmic movement in barley epidermal cells

In transient transformation assays of single epidermal cells, CA HvRACB-G15V and CA HvRAC3-G17V promoted fungal penetration success, whereas CA HvRAC1-G23V had no clear effect (Schultheiss *et al.*, 2003b). However, in stable transgenic CA HvRAC1-G23V-expressing plants, higher penetration rates of *Bgh* were observed when compared to control plants. Moreover, CA HvRAC1-G23V induced hypersensitive response in barley epidermal cells attacked but non-penetrated by *Bgh* (Chapter 3.3.1). Hence, the effect of transient over-expression of CA HvRAC1-G23V on cell death was checked in barley epidermal cells. Barley leaf epidermal cells were ballistically transformed with expression plasmids containing CA HvRAC1-G23V and sGFP-HDEL, as a marker for transgene expression (both in pGY-1 under control of the CaMV35S promoter). The sGFP-HDEL expression construct was made by replacing a part of the barley CALRETICULIN 3 (CRT3) cDNA by the GFP coding fragment. Barley CRT3 is an ER resident calcium storage protein (Kaufman 1999) that possesses an N-terminal signal peptide and a C-terminal endoplasmic reticulum (ER) retention signal (HDEL). The sGFP-HDEL fusion protein likely accumulates in mobile elements of ER or Golgi that rapidly traverse the cell along with cytoplasmic streaming (R. Eichmann, Lehrstuhl für Phytopathologie, Technische Universität München, Germany, personal communication, (Fig. 3.15a)). The cell death assay was based on the evaluation of movement of sGFP-HDEL-containing particles in the cytoplasm after transient transformation, assuming that cytoplasmic movement indicates cellular viability. Delivery of CA HvRAC1-G23V plasmid into epidermal cells of barley induced cessation of cytoplasmic streaming. At 16-20 h after

transformation, cytoplasmic movement had largely stopped in the CA HvRAC1-G23V-expressing cells. The percentage of living cells with cytoplasmic streaming was reduced up to 40% when compared to the empty vector control (Fig 3.15b, c). On the other hand, CA HvRACB-G15V had no significant effect on the vital movement of the transformed cells (Fig. 3.15b).

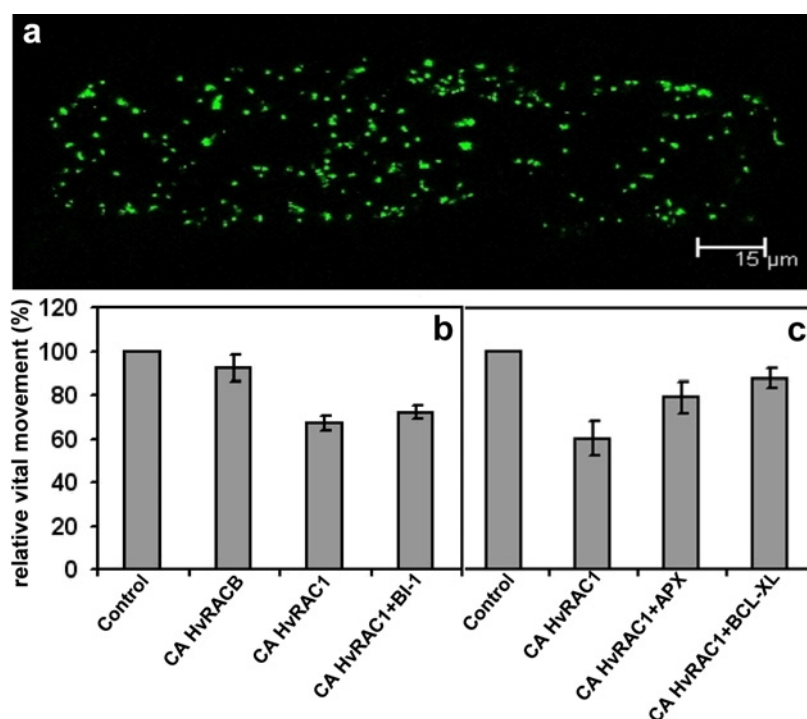


Figure 3.15: CA HvRAC1-G23V induces cessation of cytoplasmic movement in epidermal cells of barley. a) Confocal laser scanning micrograph of a single barley epidermal cell transiently expressing sGFP-HDEL. b) and c) Various cDNA expression constructs along with marker sGFP-HDEL were transiently transformed into barley leaf epidermal cells. Movement of sGFP-HDEL-containing particles was taken as a measure for cellular viability and was examined 16-20 h after transformation. Columns indicate the relative percentage of cells that retained cytoplasmic streaming after transformation with different plasmid combinations when compared to the empty vector control, which was set as 100%. Columns represent mean values from three independent experiments (approximately 150 cells evaluated per construct per experiment), error bars represent the standard errors of the means. Analysis of variance (ANOVA) and Tukey's multiple comparison test revealed that CA HvRACB-G15V expression is not statistically significant ($P > 0.05$) whereas HvRAC1-G23V is statistically significant ($P < 0.001$) when compared to the control. ASCORBATE PEROXIDASE (APX) ($P < 0.05$) and BCL-X_L ($P < 0.05$) were statistically significant from the CA HvRAC1-G23V (see c), whereas HvBI-1 expression was not statistically significant from CA HvRAC1-G23V ($P > 0.05$) (see b).

To assess whether simultaneous over-expression of barley *BAX INHIBITOR-1 (BI-1)*, a conserved suppressor of programmed cell death (Hückelhoven 2004; Eichmann *et al.*, 2006), the mammalian cell death inhibitor *BCL-X_L* (Cory and Adams 2002) or an *ASCORBATE PEROXIDASE (APX)* from barley (Hess and Börner 1998) would prevent CA HvRAC1-G23V-induced cessation of cytoplasmic streaming, the respective expression constructs together with sGFP-HDEL as marker were simultaneously delivered into barley epidermal cells by means of ballistic transformation. Simultaneous expression of *BI-1* could not suppress the effect of CA HvRAC1-G23V-induced cell death (Fig. 3.15b), while APX co-expression significantly increased the percentage of cells with cytoplasmic movement by up to 18% when compared to CA HvRAC1-G23V expression alone (Fig. 3.15c). Mammalian cell death inhibitor *BCL-X_L* could significantly rescue the CA HvRAC1-G23V effect on cell death up to 25% (Fig 3.15c). Hence, over-expression of CA HvRAC1-G23V induced epidermal cell death in barley, which could be partially rescued by the antioxidant, ASCORBATE PEROXIDASE and the mammalian cell death inhibitor *BCL-X_L*.

3.5 Amino acid sequence alignment of the N-terminal regions of barley RBOHs

Plant RBOHs have been found to play prominent roles in ROS production during several plant processes. The barley RBOH gene family consists of six members: HvrBOHB1, B2, E, F1, F2 (A), and J (Lightfoot *et al.*, 2008). The C-terminal regions of the barley RBOH proteins contain conserved binding sites for flavin adenine dinucleotide (FAD) and NADPH, whereas the N-terminal region contains two EF-hand motifs that are predicted to bind to Ca²⁺ (Lightfoot *et al.*, 2008; Trujillo *et al.*, 2006).

In rice, OsRBOHB is regulated by binding the N-terminal region of the small G-protein OsRAC1 (Wong *et al.*, 2007). Hence, the N-terminal regions of barley RBOHs were tested for an assumed interaction with barley RAC/ROPs by targeted yeast-two-hybrid analysis. For this, barley RBOH family members B1 and B2 were selected as they were phylogenetically similar to OsRACB (Fig 4.2). In addition, HvrBOHA (HvrBOHF2) and HvrBOHJ from diverse phylogenetic clades were also used (Fig 4.2).

An amino acid sequence alignment was made of the N-terminal regions of HvRBOHB1 (aa 1-295), HvRBOHB2 (aa 1-354), HvRBOHF2 (A) (aa 1-401) and HvRBOHJ (aa 1-292). All four RBOH homologues of barley possess diverse as well as conserved amino acid stretches in the N-terminal region (Fig 3.16). Amino acid conservation was especially pronounced in the predicted Ca²⁺-binding EF hand motifs. cDNAs encoding these N-terminal fragments of HvRBOHs (Fig 3.16) were isolated from barley leaf RNA-pool by reverse transcription PCR and then cloned into yeast expression vectors as preys to analyze protein-protein interactions with HvROPs.

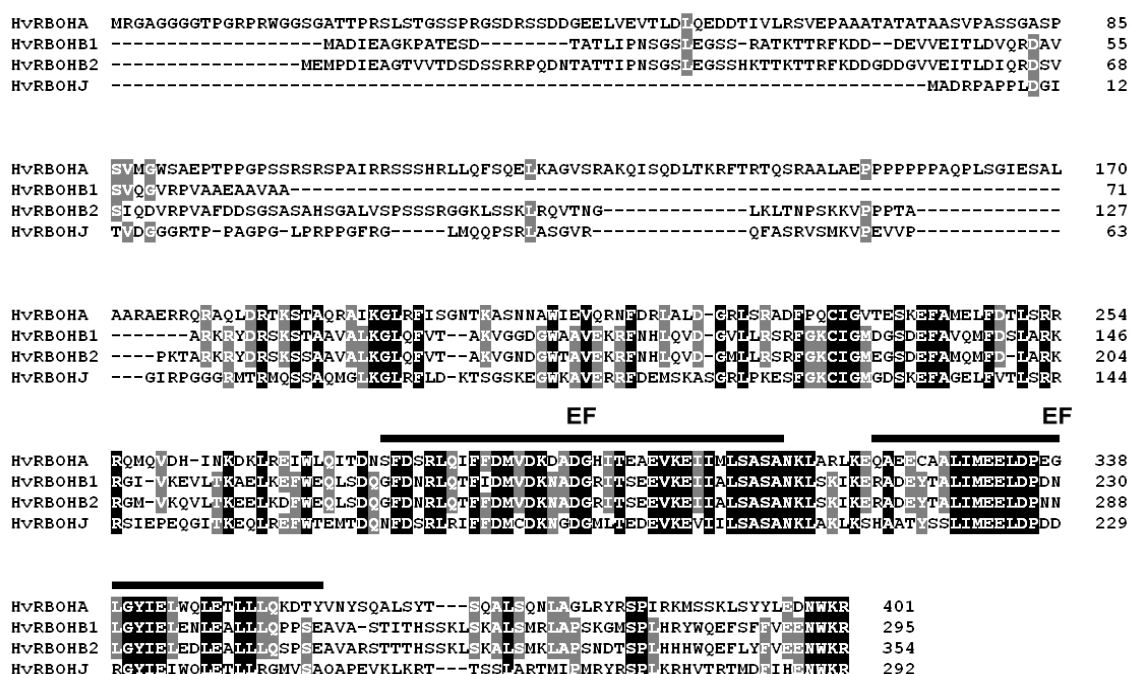


Figure 3.16: Alignment of deduced amino acid sequences of the N-terminal regions of barley RBOHA (F2), RBOHB1, RBOHB2 and RBOHJ proteins. The dark shading indicates residues that are conserved in at least three. The amino acid stretch labeled as EF indicates the two EF-hand motifs.

3.6 Targeted yeast two two hybrid screening to detect a possible interaction between barley RAC/ROP and RBOH proteins

To investigate the protein-protein interactions between barley RAC/ROPs and RBOHs, a targeted yeast two-hybrid assay was performed. Wild type (WT),

RESULTS

constitutively activated (CA), and dominant negative (DN) mutants of barley ROPs, (HvRACB, HvRAC1 and HvRAC3) and the N-terminal parts of four barley RBOHs (HvRBOHA (F2), HvRBOHJ, HvRBOHB1 and HvRBOHB2) were used in the screening (see chapter 2.6.2 for cloning procedure and transformation into yeast). All HvROPs were cloned into the yeast expression vector pGBKT7 in frame with the DNA binding domain (BD) of the GAL4 transcription factor. Presence of the pGBKT7 vector allows yeast cells to grow on tryptophan-deficient medium. The N-terminal regions of the HvRBOHs were cloned into the yeast expression vector pGADT7 in frame with the activation domain (AD) of the GAL4 transcription factor. Presence of the pGADT7 vector allows the yeast to grow on leucine-deficient medium. Hence, yeast growth on double drop out medium without amino acids tryptophan and leucine (-TL) served as a transformation marker.

-TL	pGADT7	HvRBOH A	HvRBOH J	HvRBOH B1	HvRBOH B2	-TLAH	pGADT7	HvRBOH A	HvRBOH J	HvRBOH B1	HvRBOH B2
pGBKT7						pGBKT7					
HvRACB WT						HvRACB WT					
HvRACB CA						HvRACB CA					
HvRACB DN						HvRACB DN					
HvRAC1 CA						HvRAC1 CA					
HvRAC1 DN						HvRAC1 DN					
HvRAC3 WT						HvRAC3 WT					
HvRAC3 CA						HvRAC3 CA					

Figure 3.17: Targeted yeast two hybrid assay with different HvROPs and HvRBOHs. Picture shows the result of a qualitative yeast drop assay on two fold (-TL) and four fold (-TLAH) selective media seven days after dropping. Equal amounts of yeast cells from the same suspension were dropped simultaneously on both -TL and -TLAH selective media. -TL: without

amino acids tryptophan and leucine; -TLAH: without amino acids tryptophan, leucine, adenine and histidine; WT: wild type; CA: constitutively activated; DN: dominant negative.

Interaction between two proteins that were expressed from pGADT7 and pGBKT7 yeast vectors would result in the interaction of their respective DNA activation and binding domains and the restoration of GAL4 transcription factor function. This would be followed by transcriptional activation of reporter genes and thus synthesis of the two amino acids adenine and histidine in the yeast cells. Hence, yeast growth on quadruple drop out medium without amino acids tryptophan, leucine, adenine and histidine (-TLAH) indicates the interaction between the two respective proteins. A positive interaction also induces the expression of reporter gene *LacZ* (codes for β -galactosidase), which produces a visible blue color after hydrolysis of X-a-gal in the medium.

A qualitative yeast drop assay was carried out on selective double and quadruple dropout media plates. Yeast growth on the double dropout (-TL) medium indicates the successful transformation and expression of marker genes in yeast cells (Fig. 3.17). Yeast growth on the quadruple dropout (-TLAH) medium indicates the interaction between the two respective proteins (Fig. 3.17). Out of all combinations, growth on (-TLAH) medium indicated an interaction of RBOHB1 and B2 with CA HvRAC1-G23V. A slight growth was observed for yeast cells that expressed RBOHB1 or B2 in combination with CA HvRACB-G15V (Fig. 3.17). However, these cells did not grow on (-TLAH) + X-a-gal medium. Growth was observed but the blue color was not produced by yeast cells expressing CA HvRAC1-G23V together with either RBOHB1 or RBOHB2 on (-TLAH) + X-a-gal medium, which might indicate a rather weak interaction between the respective proteins. In summary, only HvRBOH B1 and RBOHB2 but not HvRBOHA or RBOHJ interacted with CA HvRAC1-G23V but not with either WT HvRAC1 or DN HvRAC1-T28N. CA HvRACB-G15V showed a very weak interaction with HvRBOHB1 and RBOHB2, whereas neither WT HvRAC3 nor CA HvRAC3-G17V showed an interaction with any RBOH of barley.

4. Discussion

Small molecular weight GTP-binding proteins (RAC/ROPs) are known to act as 'molecular switches' in various cellular processes across dicot and monocot plant species. Several ROP family members were identified in various plant species (Christensen *et al.*, 2003; Schultheiss *et al.*, 2003b; Vernoud *et al.*, 2003). The presence of several ROP family members in plants generates a complex network of functions, because of potential functional redundancy. Therefore constitutively activated (CA) and dominant negative (DN) mutants of RAC/ROP proteins offer indispensable tool in understanding the physiological roles of the ROP-GTPases in plants. The CA and DN mutants are blocked in the cycling between GTP and GDP forms respectively, by the replacements of specific amino acid residues. These mutants have been instrumental in investigating the roles of RAC/ROPs in epidermal cell morphogenesis, pollen tube and root hair polar growth and in plant-pathogen interactions (Fu *et al.*, 2002; Jones *et al.*, 2002; Kawasaki *et al.*, 1999; Li *et al.*, 1999; Li *et al.*, 2001; Molendijk *et al.*, 2001; Schultheiss *et al.*, 2003b; Yang 2002; Zheng and Yang 2000a; 2000b). Since ROps share more than 70% amino acid identity with each other (Zheng and Yang 2000a), the heterologous expression of RAC/ROPs is useful in understanding their evolutionarily conserved functions across different plant species (Luo *et al.*, 2006; Moeder *et al.*, 2005; Sano *et al.*, 1994; Schiene *et al.*, 2000). The present study focused on the elucidation of the involvement of constitutively activated mutants of three barley RAC/ROP proteins (CA HvRACB-G15V, CA HvRAC3-G17V and CA HvRAC1-G23V, referred to as HvROPs, where more than one of them is meant) in plant development and pathogen interactions in barley and tobacco. This was carried out by using stable transgenic barley and tobacco plants expressing CA HvROPs. Furthermore, various molecular and cytological tools were applied to characterize the functions of RAC/ROP proteins in barley-powdery mildew interaction.

4.1 Phylogenetic analysis of ROP family members from various plant species

RAC/ROP proteins from different plant species share more than 70% amino acid identity with each other (Zheng and Yang 2000a). A phylogenetic analysis was carried out by comparing the deduced amino acid sequences of barley ROP family members to various ROPs from *Arabidopsis*, rice and tobacco to infer the evolutionary relationships among divergent plant ROPs. Phylogenetic analysis showed significant homologies between various ROPs from *Arabidopsis*, barley, rice and tobacco. However, being monocot plants, barley and rice share relatively high degree of similarity when compared to *Arabidopsis* and tobacco (Fig. 4.1). The phylogenetic analysis divides barley ROPs, HvRACB, HvRAC1 and HvRAC3 into three different phylogenetic clades together with closely related ROPs from *Arabidopsis*, rice and tobacco (Fig. 4.1).

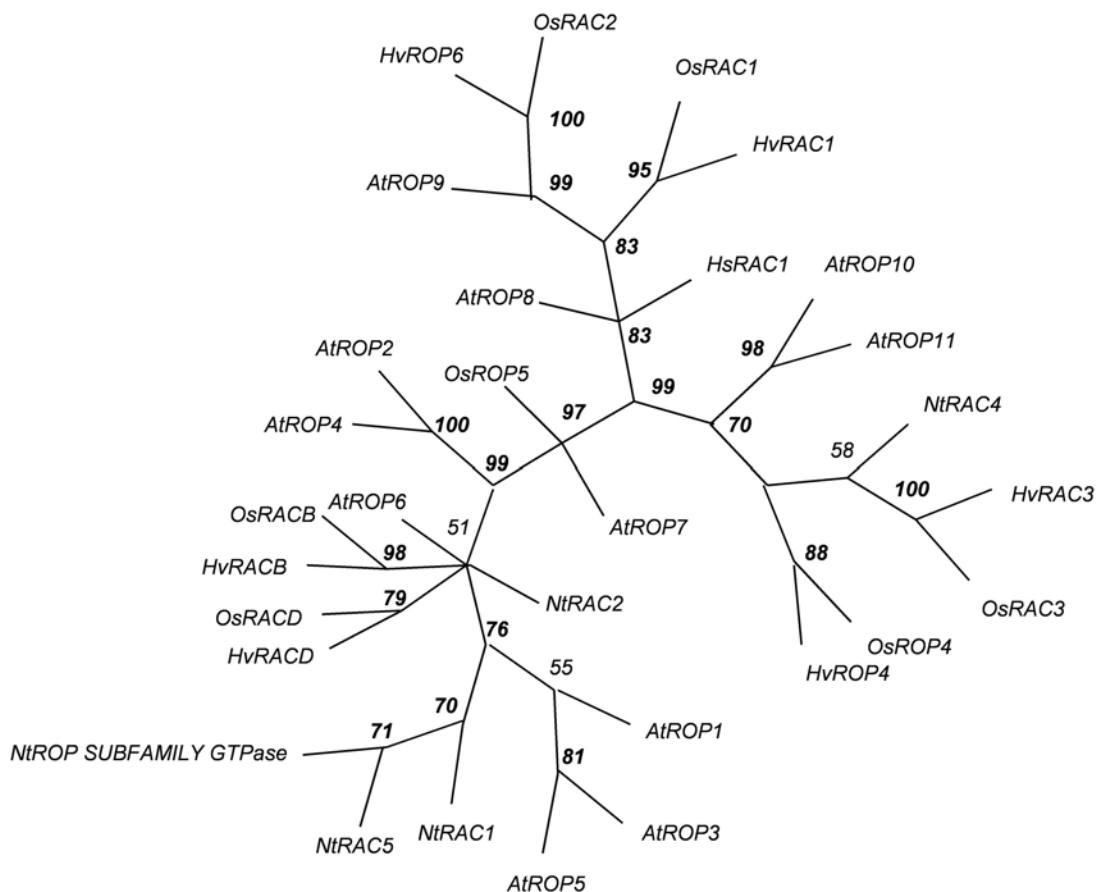


Figure 4.1: Unrooted phylogenetic tree of RAC/ROP proteins from barley, rice, tobacco and *Arabidopsis*. The tree was constructed by alignment of deduced amino acid sequences using PAUP 4.0 (Phylogenetic Analysis Using Parsimony, <http://paup.csit.fsu.edu/about.html>). Phylogenetic analysis was carried out by A. Zuccaro (Institute of Phytopathology and Applied

Zoology, University of Giessen, Germany). Neighbor-joining (NJ) and unweighted pair group method with arithmetic averages (UPGMA) algorithms were used for the phylogenetic analysis. Sequences were obtained from GenBank. Abbreviations: *Arabidopsis thaliana* (At), *Hordeum vulgare* (Hv), *Homo sapiens* (Hs), *Oryza sativa* (Os), and *Nicotiana tabacum* (Nt). GenBank accession numbers: HvRAC3 (AJ518932.1), HvROP4 (AJ439335.1), HvRAC1 (AJ518933.1), HvROP6 (AJ439333.1), HvRACB (AJ344223.2), HvRACD (AJ439334.1), OsRAC3 (NM_001054623.1), OsRAC2 (NM_001062565.1), OsRAC1 (NM_001049021.1), OsRACB (NM_001052247.1), OsRACD (AF218381.1), OsROP4 (NM_001063771.1), OsROP5 (NM_001053188.1), AtROP1 (NM_114989.3), AtROP2 (NM_101863.3), AtROP3 (NM_127334.3), AtROP4 (NM_106234.2), AtROP5 (NM_119762.3), AtROP6 (NM_119668.5), AtROP7 (NM_123965.1), AtROP8 (NM_130033.2), AtROP9 (NM_119039.2), AtROP10 (NM_114673.2), AtROP11 (NM_125682.4), NtRAC1 (AY029330.1), NtRAC2 (U64924.1), NtRAC4 (AJ496228.1), NtRAC5 (U64923.1), NtROP_SUBFAMILY_GTPase (AJ222545.2), HsRAC1 (NM_006908.4).

4.2 Barley RAC/ROP proteins function in polar growth and morphogenesis

RAC/ROP proteins get activated in response to developmental cues and trigger downstream effectors in signal transduction during plant growth processes (Yang 2002; Yang and Fu 2007). RAC/ROPs are key regulators in cell polarity, development and polar growth in various cell systems in plants, including pollen tubes, developing root hairs, and leaf epidermal cells (Fu *et al.*, 2005; Fu and Yang 2001; Gu *et al.*, 2005; Molendijk *et al.*, 2001). Coordinated spatio-temporal action of several intracellular factors such as RAC/ROP proteins, ROP-interacting proteins, actin cytoskeleton, Ca²⁺ ion gradient, membrane trafficking and vesicle transport is necessary for cell polarity in shaping plant cells (Bannigan and Baskin 2005; Basu *et al.*, 2008; Campanoni and Blatt 2007; Cole and Fowler 2006; Mathur 2006; Molendijk *et al.*, 2004; Lavy *et al.*, 2007; Lee *et al.*, 2008; Xu and Scheres 2005; Yalovsky *et al.*, 2008).

4.2.1 Barley RAC/ROP proteins operate in leaf morphogenesis and epidermal cell expansion in barley

All CA HvRACB-G15V-, CA HvRAC3-G17V- and CA HvRAC1-G23V-expressing barley plants show stunted growth and reduced plant height (Fig. 3.1), whereas CA HvRACB-G15V and CA HvRAC3-G17V did not interfere with plant height

when expressed in heterologous dicot tobacco (see chapter 3.1.1). HvRACB-homologous *Arabidopsis* CA ROP2 expression did not alter plant height (Fig. 4.1; Li *et al.*, 2001). This might indicate that HvRACB-like ROPs operate in plant height in monocots but not in dicot plants like *Arabidopsis* and tobacco. CA HvRACB-G15V-expressing tobacco leaves showed an enhanced ratio of leaf length to width axis (Fig. 3.2), which is also observed in CA AtROP2-expressing *Arabidopsis* leaves (Li *et al.*, 2001). In CA HvRAC3-G17V-expressing tobacco, leaf texture was irregular and leaf surface appeared uneven, likely due to disproportionate growth of the leaf tissues or mesophyll hypertrophy (Fig. 3.2; 3.7c). In CA HvROP-expressing barley, downward rolling of the leaves was observed (Chapter 3.1.1; Schultheiss *et al.*, 2005), which is similar to the phenotype of HvRACB-homologous CA AtROP2 and HvRAC3-homologous CA AtRAC10-expressing *Arabidopsis* leaves (Fig. 4.1; Li *et al.*, 2001; Bloch *et al.*, 2005). It is interesting that the leaf roll phenotype along the long axis is also found in *Arabidopsis* transgenic plants over-producing indole acetic acid (Li *et al.*, 2001; Romano *et al.*, 1995), and expression of CA NtRAC1, a tobacco homolog of HvRACB, which activates auxin signaling in plant growth and development (Fig. 4.1; Tao *et al.*, 2002). ROP-GTPases are known to play a key role during the auxin-mediated execution of cell polarity (Boutté *et al.*, 2007). Hence, the CA HvROP-induced altered leaf polarity might suggest the involvement of HvROPs in auxin-mediated plant developmental processes. However, preliminary data of gene-expression analysis of CA HvRACB-G15V and CA HvRAC1-G23V barley displayed no variation in the expression levels of two auxin-responsive genes (*HvIAA* and *H-11:5*, see supplemental fig. 1).

HvRACB-homologous *Arabidopsis* ROP2 as well as ROP4- and ROP2-interacting proteins operate in pavement cell development, thereby organizing both actin and microtubule cytoskeleton in lobe and neck formation. CA AtROP2 causes aberrant cell size and cell shape effects in epidermal pavement cells of *Arabidopsis* (Fu *et al.*, 2002; 2005). Similarly, ectopic expression of CA AtRAC10, a homolog of CA HvRAC3-G17V (Fig. 4.1), causes elimination of epidermal cell lobes in *Arabidopsis* (Bloch *et al.*, 2005). In contrast, barley plants expressing CA HvROPs had no dramatic cell shape phenotype but slightly more irregular cell shapes (Fig. 3.5a). Apparently, the brick-like shape of barley epidermal cells did

not allow dramatically aberrant cell shapes as were observed in CA AtROP2-expressing *Arabidopsis* plants. However, CA HvROPs induced epidermal cell expansion in barley (Fig. 3.5b). In cotton, HvRACB-like GhRAC1 is highly expressed during early phases of fiber elongation (Kim and Triplett 2004), and CA ROP2 induces lateral and radial cell expansion in *Arabidopsis* (Fu *et al.*, 2002). However, in contrast to HvRACB and HvRAC1, AtROP2 does not significantly affect cell length in *Arabidopsis* leaf longitudinal orientation (Fu *et al.*, 2002). Hence, barley and *Arabidopsis* ROPs might share conserved functions in polar growth but also differ in the kind of growth processes they are involved in.

The synchronized action of *Arabidopsis* ROP2, ROP4, actin filaments and microtubules is crucial for pavement cell morphogenesis (Fu *et al.*, 2002; 2005), and the tobacco epidermis pavement cell pattern resembles that of *Arabidopsis*. Hence, CA HvROPs were expressed in tobacco, to see if HvROPs can influence epidermal cell development similar to related dicot ROPs. Heterologous expression of CA HvROPs induced leaf epidermis cell size aberrations in tobacco (Fig. 3.7). This supports the notion that CA HvROPs might affect processes that are normally under control of tobacco ROPs. HvRACB is phylogenetically closely related to tobacco NtRAC1 (see fig. 4.1) that operates in auxin-dependent growth processes and in actin polymerization (Tao *et al.*, 2002; Chen *et al.*, 2003). HvRAC3 is similar to NtRAC4 (Fig. 4.1; Morel *et al.*, 2004), which has not yet been characterized. However, expression of CA HvRAC3-G17V-like CA AtRAC10 in *Arabidopsis* has an effect on membrane dynamics and on epidermal cell shape and size (Bloch *et al.*, 2005). Expression of CA HvROPs enhanced tobacco epidermal cell size but did not strongly affect formation of cell lobes and necks (Fig. 3.7). In contrast, *Arabidopsis* CA AtROP2 affects more strongly the cell shape than the cell size (Fu *et al.*, 2002; 2005). This might be explained by the fact that barley epidermal cells lack interdigitation as it is typically seen in dicot pavement cells. Therefore, it can be speculated that barley ROPs do not target dicot effectors of cell lobe formation but rather generally supported cell expansion in tobacco. Together, barley RAC/ROP proteins appear to operate in evolutionarily conserved determination of epidermal cell size and/or abundance.

CA HvROP expression significantly reduced the number of stomata in barley leaf epidermis (Fig. 3.6e). In part, this could be explained by enhanced length of epidermal cells intercalated between stomata (Fig. 3.6b). The reduced number of stomata explains the lower transpiration rates and less CO₂ assimilation in non-stressed CA HvRACB-G15V barley when compared to azygous wild type segregants (Schultheiss *et al.*, 2005; Pathuri *et al.*, 2009b). Apart from the stomata number, developmental abnormalities were observed in the arrangement of stomata and epidermal cells of CA HvROP-expressing barley leaves (Fig. 3.6b, c, d and supplemental fig. 2). Together, CA HvROPs have an effect on both the number and development of stomata. These observations suggest that RAC/ROPs are not only operating in cell expansion but also in barley cell differentiation for stomata development.

4.2.2 Barley RAC/ROPs control polar tip growth of root hairs in barley and tobacco

To address the question whether ROPs might affect polar tip growth, root hairs of CA HvROP-expressing barley and tobacco plants cultivated on a solid medium were observed. CA HvROPs abolished root hair tip growth and induced shortening and ballooning of root hairs in barley and tobacco (Fig. 3.3; 3.4). This supports the possibility that HvROPs operate in polar membrane transport similar to dicot ROPs (Bloch *et al.*, 2005; Campanoni and Blatt 2007; Kost 2008; Lee and Yang 2008; Yalovsky *et al.*, 2008). It had been demonstrated before that proper localization and strength of ROP activity is required for initiation and maintenance of tip growth in root hairs and pollen tubes (Carol *et al.*, 2005; Cole and Fowler 2006; Hwang *et al.*, 2005; Jones *et al.*, 2007; Klahre and Kost 2006; Lee *et al.*, 2008; Tao *et al.*, 2002; Yang 2002). *Arabidopsis* loses polarity of root hairs, when CA AtRAC10 (homolog of HvRAC3), CA AtROP2 or CA AtROP6 (homologs of HvRACB) is expressed (Fig. 4.1; Molendijk *et al.*, 2001; Jones *et al.*, 2002; Bloch *et al.*, 2005). Tobacco loses polarity of root hairs, when CA NtRAC1 (a homolog of HvRACB) is expressed, whereas NtRAC1 RNA interference abolishes root hair growth (Fig. 4.1; Tao *et al.*, 2002). Ectopic over-activation of ROPs apparently leads to loss of spatial control and of polarity resulting in isotropic growth instead

of tip growth. This may explain developmental aberrations in CA HvROP tobacco root hairs. CA HvROPs might have over-activated endogenous tobacco ROP effectors and dominated antagonistic processes, which are responsible for root hair polarity in the wild type. The fact that CA HvROPs can interfere with root hair tip growth in barley and tobacco indicates an evolutionarily conserved function of ROPs in polar growth processes. This further support that ROPs not only share high sequence similarity (Zheng and Yang 2000a; Schultheiss *et al.*, 2003b) but also conserved functions between monocots and dicots. Altogether, data demonstrate a cross-species conservation of ROP-family GTPase functions in distinct developmental processes such as plant height, leaf polarity, epidermal cell differentiation, cell expansion and root hair polar growth in barley and tobacco.

4.3 Barley RAC/ROP proteins support disease susceptibility to leaf pathogens in barley and tobacco

RAC/ROP proteins are known to act as key players in defense signaling pathways that are linked to disease resistance (Agrawal *et al.*, 2003; Moeder *et al.*, 2005; Sano and Ohashi 1995; Ono *et al.*, 2001) and susceptibility (Jung *et al.*, 2006; Schultheiss *et al.*, 2003b; 2005) against various pathogens. To investigate the role of barley RAC/ROP proteins in the outcome of various plant-pathogen interactions, inoculation tests were carried out in CA HvROP-expressing barley and tobacco plants. Whole plant inoculation and detached leaf inoculation methods were used to inoculate *Bgh* in barley, which illustrated variations in the HR and fungal penetration frequencies. Interestingly, higher rates of HR were observed after *Bgh* inoculation of detached barley leaves (Fig. 3.12). This could be because of an onset of senescence-related processes in detached leaves, or alternatively, because detached leaves are more stressed. This idea is supported because it is known that pathogenicity and disease symptom development in detached leaves can be associated with leaf senescence (Liu *et al.*, 2007). On the other hand, high penetration rates were observed when leaves of whole plants were inoculated, when compared to the detached leaves (Fig. 3.12). This seems to be plausible since *Bgh* is a biotrophic fungus and hence, the intact

leaves might offer more suitable conditions for the fungus than detached leaves during infection. However, the relation of both penetration and HR frequencies in wild type and CA HvRAC1-G23V plants remained same in either type of inoculation methods. All three CA HvROPs induced susceptibility to *Bgh* (Fig. 3.10) and supported fungal penetration and haustoria formation in barley epidermal cells (Fig. 3.11). Similarly, heterologous expression of CA HvRACB-G15V and CA HvRAC3-G17V (CAHvRAC1-G23V was not tested) induced susceptibility to the virulent powdery mildew fungus *G. cichoracearum* (Fig. 3.8). Since powdery mildew fungi are biotrophic, they produce haustoria in host epidermal cells for nutrient uptake. Cells in which a haustorial complex is established have to rapidly enlarge their membrane surface (Green *et al.*, 2002; Perfect and Green 2001), which is similar to that of tip-growing plant cells. The tip-focused growth involving ROP-GTPases is a process that takes place during germination of dicot pollen tubes and fern spores. It is suggested that tip growth is an evolutionarily ancient process, which might be conserved among plants and fungi (Bushart and Roux 2007). It has been speculated that haustorium establishment mimics inward-directed tip growth observed in pollen tube or root hair elongation (Schultheiss *et al.*, 2003b). This is supported by the observation that filamentous host actin envelopes immature haustoria of *Bgh* during invasion (Opalski *et al.*, 2005). Polarized ROP activity at sites of fungal penetration is also indicated by spatial accumulation of HvRIC171, a protein which both interacts with and is recruited to the cell periphery by activated HvRACB (Schultheiss *et al.*, 2008).

Polar actin filament orientation and local vesicle secretion are crucial for penetration resistance to powdery mildew fungi (An *et al.*, 2006a; Collins *et al.*, 2003; Kobayashi *et al.*, 1997; Kwon *et al.*, 2008; Miklis *et al.*, 2007; Opalski *et al.*, 2005; Schmelzer 2002; Underwood and Somerville 2008). The fact that CA HvROPs can interfere with root hair tip growth supports the possibility that fine-tuned endogenous HvROPs operate in polar membrane growth during formation of the haustorial complex. This view is supported because knock-down of HvRACB limits haustorium formation in barley (Schultheiss *et al.*, 2002, 2003b).

Both leaf epidermal cell expansion and enhanced susceptibility effects were strong in CA HvRACB-G15V- and CA HvRAC1-G23V- and weaker in CA HvRAC3-G17V-expressing barley plants (Fig. 3.5; 3.10), whereas in case of tobacco, both

CA HvRACB-G15V and CA HvRAC3-G17V had stronger effects on epidermal cell expansion and enhanced powdery mildew susceptibility (Fig. 3.7; 3.8). This association of cell size and susceptibility might suggest that cell expansion might antagonize cell wall-associated polarized defense reactions required for penetration resistance. However, there are also indications from experiments in barley that cell expansion can be uncoupled from susceptibility to powdery mildew. For instance, HvRACB effects can be established after ballistic transformation of fully expanded leaf cells (Schultheiss *et al.*, 2002, 2003b). Hence, the cell size does not directly determine susceptibility to powdery mildew. However, this does not exclude the possibility that cell growth and susceptibility to powdery mildew share common elements in ROP up- or down-stream signaling. It might be possible that non-self activation of ROPs by powdery mildew fungi mimics growth stimuli and induces either depolarization of epidermal cells or cell wall loosening for accessibility to fungal penetration.

The induction of powdery mildew susceptibility in tobacco by the heterologous expression of HvROPs can be explained in the following way. CA HvRACB-G15V was reported to weaken filamentous actin-based cell polarity in the interaction of barley with *Bgh* (Opalski *et al.*, 2005). Similarly, it might be possible that CA HvROPs inhibited polarization in epidermal cells of tobacco, thereby supporting penetration by *G. cichoracearum*. This is supported by the regulatory function of HvRACB-like NtRAC1 on tobacco actin depolymerization factor 1 (Chen *et al.*, 2003). It seems also possible that CA HvRACB-G15V mimicked the closely related CA NtRAC5 in that it attenuated an elicitor-activated oxidative burst. NtRAC5 negatively regulates tobacco NADPH oxidase NtRBOHD at the transcriptional and post-transcriptional level (Morel *et al.*, 2004). Ectopic activation of ROP signaling in tobacco might thus have paved the way for powdery mildew infection. Since ROPs are crucial signal transduction partners in many important cellular processes in plants, powdery mildew fungi, which undergo intimate interactions with living host cells, might target ROPs as a part of their virulence strategy. In view of the fact that CA HvROPs can negatively regulate basal resistance to powdery mildew fungi in barley and tobacco, a potential function of ROP signaling in disease susceptibility of monocot and dicot plants is suggested.

It was surprising that CA HvRAC3-G17V but not CA HvRACB-G15V promoted wildfire symptoms and multiplication of *Pst* in tobacco leaves (Fig. 3.9). Only CA HvRAC3-G17V tobacco leaves showed the irregular leaf texture, which might have supported susceptibility to *Pst*. However, phenotypes were observed as post-invasion susceptibility, because bacteria were injected. Hence, leaf invasion via stomata was not studied here. Polar host defense is also involved in post-invasive defense against bacterial pathogens (Kwon *et al.*, 2008). We speculate that polar defense in mesophyll cells might have been particularly disturbed by CA HvRAC3-G17V. This is supported because gene expression analysis in powdery mildew-inoculated barley showed that HvRACB is strongly expressed in epidermis rather than mesophyll cells, while HvRAC3 is equally expressed in mesophyll and epidermis (Schulthies *et al.*, 2003b), and mesophyll cell expansion was obvious only in CA HvRAC3-G17V tobacco (Fig. 3.7c). The latter was reminiscent of mesophyll hypertrophy, which is induced by the bacterial type III virulence effector AVRBs3 of *Xanthomonas campestris* pv. *vesicatoria* and the pepper effector target UPA20 in *Nicotiana benthamiana* (Kay *et al.*, 2007; Marois *et al.*, 2002). Again this supports the idea of common elements of cell size determination and plant disease susceptibility.

Together, the fact that ectopic expression of CA HvROPs in tobacco massively supported disease symptoms and development of leaf pathogens suggests that ROPs either supported susceptibility or hindered function of basal resistance. In context of the impact on growth processes this supports that barley ROPs interfered with endogenous ROP signaling in tobacco. Hence, data suggest a role for tobacco ROP signaling in the outcome of interactions with different leaf pathogens. This could involve HvRACB-like NtRAC5 or NtRAC1 (Tao *et al.*, 2002; Chen *et al.*, 2003; Morel *et al.*, 2004) or HvRAC3-like NtRAC4 (Morel *et al.*, 2004). Since solanaceous plants are prominent models in plant pathology and important crops, this might encourage further investigations on ROP signaling in this plant family.

4.4 HvRAC1 activity support local callose deposition and hypersensitive response during barley-*Bgh* interaction

Plant resistance to powdery mildew can be executed at pre- or post-penetration stages of the interaction. For example, race-specific resistance in barley is associated with hypersensitive cell death reaction (HR) that can be triggered at two stages of the interaction. Attacked cells undergo HR before penetration as seen in *Mlg*-mediated resistance or by undergoing HR after the fungus penetrated the epidermal host cell, which is seen in *Mla12*-mediated resistance (Görg *et al.*, 1993; Hüchelhoven *et al.*, 1999). Pre-penetration resistance is prominent in *mlo*-mediated race non-specific resistance, where the powdery mildew fungus is arrested at the penetration stage under production of CWAs, leaving the attacked cell alive. In CA HvROP-expressing barley, enhanced susceptibility could be evident from the decreased number of non penetrated CWAs, suggesting the disruption of basal resistance to powdery mildew fungus by CA HvROPs. In contrast to CA HvRACB-G15V and CA HvRAC3-G17V, CA HvRAC1-G23V supported HR-like whole cell H₂O₂ accumulation in stable transgenic barley plants when attacked but not penetrated by *Bgh* (Fig. 3.11b, e). Phylogenetic analysis of plant ROPs revealed that HvRACB, HvRAC3 and HvRAC1 belong to three different subclades of barley ROPs (Fig. 4.1; Schultheiss *et al.*, 2003b). Since all three activated barley ROPs promoted penetration success of *Bgh* but only CA HvRAC1-G23V promoted whole cell H₂O₂ accumulation, these barley ROPs might have overlapping and distinct functions in plant-microbe interactions.

In transgenic barley plants, CA HvRAC1-G23V enhanced local callose deposition, reduced the number of CWAs with H₂O₂ accumulation and supported susceptibility in barley-*Bgh* interaction (Fig. 3.11; 3.14). This shows that glucan synthase-dependent callose deposition can be uncoupled from other wall-associated defenses, and that strong callose deposition is not indicative of penetration resistance to *Bgh*. *Arabidopsis* CA ROP1 binds UDP-glucose transferase, which is part of the callose forming complex at the cell plate (Hong *et al.*, 2001). The *Arabidopsis pmr4* mutant, which is defective in CWA-associated callose deposition due to mutation of glucan synthase 5, displays enhanced

salicylic acid-dependent resistance to powdery mildew. This indicates that plant glucan synthases can negatively control callose-independent plant defense (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). Interestingly, CA HvRAC1-G23V also supported callose deposition at sites of fungal attack and resistance to penetration by *Magnaporthe oryzae* in barley (Pathuri *et al.*, 2008). Callose seems also to be involved in resistance of *Brachypodium distachyon* to adapted *Magnaporthe* (Routledge *et al.*, 2004). Hence, CA HvRAC1-G23V-induced callose deposition at CWAs could be a cause for penetration resistance specifically with *M. oryzae* infection in barley.

CA HvRAC1-G23V-induced HR occurs prior to penetration of *Bgh*, which is similar to the HR in *Mlg*-mediated resistance. CA HvRAC1-G23V-induced HR failed to offer resistance to *Bgh*. Interestingly, in CA HvRAC1-G23V plants, HR cells are often accompanied by the second penetration attempt of *Bgh* spores (Fig. 3.13), which might indicate the delayed or reduced efficiency in the execution of HR. It is possible that the high rates of delayed HR in CA HvRAC1-G23V-expressing barley plants might not be sufficient to impede fungal penetration efficiently. Similarly, HR in *Mlg*-resistant barley plants was discussed to be a secondary consequence of resistance and not necessarily required for the arrest of fungal growth (Görg *et al.*, 1993). Moreover, susceptibility in CA HvRAC1-G23V-expressing barley plants could also be a consequence of the delayed execution of defense responses rather than the complete absence of the defense reactions. It has been suggested that host cell death can be uncoupled from resistance responses. Although *mlo*-mediated resistance operates through CWAs, *mlo*-resistant plants exhibit spontaneous cell death in the absence of *Bgh*, which is spatially and temporally separated from the *mlo* resistance (Peterhänsel *et al.*, 1997; Piffanelli *et al.*, 2002). *Arabidopsis acd5* and *dll1* mutant studies showed that pathogen induced cell death can be uncoupled from the disease resistance. In *acd5* plants, cell death and defense-related responses were elicited in response to *Pseudomonas syringae* infection but, failed to confer disease resistance. These studies also showed the involvement of salicylic acid and ethylene in the regulation of cell death during some susceptible pathogen infections (Greenberg *et al.*, 2000; Pilloff *et al.*, 2002). During plant-microbe interactions, hormone production and signaling pathways are fundamental

targets for both plants and pathogens to prevail one over the other. The action of resistance or susceptibility is likely influenced by the hormonal balance that regulates and modulates complex interacting signaling networks during plant-pathogen interactions (López *et al.*, 2008). There are reports indicating the potential effects of phytohormones like auxin, salicylic acid and ethylene in ROP-activity and ROP-associated plant functions (Fischer *et al.*, 2006; Luo *et al.*, 2006). Hence, it could be possible that the discrete outcome of both susceptibility and cell death induction by CA HvRAC1-G23V during barley-*Bgh* interaction is a consequence of several complex signaling events involving various interacting partners.

All three CA HvROPs induced a drop in local CWA-associated DAB staining beneath fungal appressoria (Fig. 3.11f). Therefore, the total frequency of interaction sites with H₂O₂ production did not dramatically change in CA HvRAC1-G23V barley, whereas the spatial pattern of H₂O₂ production did (Fig. 3.11e, f). Transient knock-down of a barley NADPH oxidase, HvRBOHA (F2) did not limit the cell wall associated production of H₂O₂ (Trujillo *et al.*, 2006). This might indicate different sources of ROS during HR and during formation of CWAs, respectively. Amine oxidases, peroxidases and oxalate oxidases have been suggested as alternative sources of ROS during plant-microbe interactions (Bolwell *et al.*, 2001). ROS other than H₂O₂ might also have different functions in interaction with *Bgh* (reviewed in Hüchelhoven 2007). Alternatively, CA HvROPs might cause depolarization of attacked cells inhibiting local secretion for cell wall-associated H₂O₂ production. This is supported because CA HvRACB-G15V barley inhibits strong polarization of filamentous actin towards the site of *Bgh*-attack in barley (Opalski *et al.*, 2005). Accordingly, inhibition of actin polymerization or depolymerization of actin by over-expression of actin depolymerization factor 3 in barley induces susceptibility to *Bgh* (Miklis *et al.*, 2007). The actin polymerization inhibitor cytochalasin also inhibits local DAB staining and penetration resistance of cowpea to plantain powdery mildew fungus (Mellersh *et al.*, 2002).

The different outcomes of HvRAC1 activity during the interaction with virulent *Bgh* could be due to the various roles of ROPs in polarity, cell wall modeling and in cell death (Kawasaki *et al.*, 1999; Potikha *et al.*, 1999). Whole cell H₂O₂

accumulation was exclusively observed in cells where *Bgh* failed to penetrate. HR of non penetrated cells occurs at a low to medium frequency in basal resistance to virulent *Bgh* (Fig. 3.11; Hüchelhoven and Kogel 1998; Hüchelhoven *et al.*, 1999; Koga *et al.*, 1990). However, once virulent *Bgh* succeeds in penetrating an epidermal cell of barley, such a cell extremely rarely undergoes HR. Hence, biotrophic *Bgh* may be able to suppress host cell death after penetration. Therefore, a potential influence of CA HvRAC1-G23V on the threshold for HR-like cell death might become obvious only in non penetrated cells (Fig. 3.11e).

The closest relative of HvRAC1 in rice, OsRAC1, promotes production of ROS and HR-like cell death in response to *M. oryzae*. Additionally, DN OsRAC1 or *Medicago sativa* RAC1 antisense RNA suppress hypersensitive cell death in tobacco and rice (Kawasaki *et al.*, 1999; Moeder *et al.*, 2005; Ono *et al.*, 2001; Schiene *et al.*, 2000). OsRAC1 supports HR by interaction with and activation of ROS-producing plasma membrane NADPH oxidase and by down-regulation of metallothionein, a reactive oxygen scavenger in rice (Kawasaki *et al.*, 1999; Ono *et al.*, 2001; Wong *et al.*, 2004, 2007). Trujillo *et al.*, (2006) provided evidence that a barley NADPH oxidase (HvRBOHA) contributes to susceptibility to penetration by *Bgh*. Hence, one can speculate that HvRAC1 co-operates with an NADPH oxidase in susceptibility to *Bgh* or in HR or both. In contrast to what was observed in interaction with *Bgh*, CA HvRAC1-G23V expression resulted in resistance to hemibiotrophic *M. oryzae* in barley, which is associated with local formation of CWAs and no fungus induced cell death was observed (Pathuri *et al.*, 2008). In rice, HvRAC1 homologous CA OsRAC1 expression induced HR-like cell death associated with resistance to *M. oryzae* (Fig. 4.1; Kawasaki *et al.*, 1999; Ono *et al.*, 2001). Thus, HvRAC1 is involved in basal resistance to *M. oryzae* in barley, whereas OsRAC1 is involved in race-specific resistance to *M. oryzae* in rice. It is intriguing that CA HvRAC1-G23V modulates penetration resistance to two directly penetrating fungi (*Bgh* and *M. oryzae*) with opposite outcomes. This supports speculations that ROPs, similar as the receptor-like MLO, have distinct functions in interaction with pathogens of different life style and further supports that MLO and ROPs could be functionally linked (Jarosch *et al.*, 1999, Schultheiss *et al.*, 2002; 2003b; Consonni *et al.*, 2006; Miklis *et al.*, 2007). However, preliminary

data of gene-expression profiling of CA HvRACB-G15V and CA HvRAC1-G23V barley showed no variation in the expression levels of MLO (Supplemental fig. 1) Nevertheless, the fact that HvRAC1 can influence both HR and fungal penetration concurrently during barley-*Bgh* interaction suggests that ROPs can modulate both susceptibility and cellular defense responses. This signifies RAC/ROPs as potential interfaces of different pathogen signals.

4.5 HvRAC1 disturbs cell vitality in barley epidermal cells when transiently over-expressed

Microprojectile-mediated transient transformation of single cells of cereals with subsequent challenge by *Blumeria graminis* has been widely used to assess gene function in defense and susceptibility (Panstruga 2004). It has been reported that stable expression of CA HvRACB-G15V induced a similar degree of enhanced susceptibility to penetration by *Bgh* as compared to transient single-cell over-expression of CA HvRACB-G15V (Schultheiss *et al.*, 2003b; 2005). In the transient single-cell assays, CA HvRAC1-G23V expression did not induce susceptibility or resistance to *Bgh* (Schultheiss *et al.*, 2003b). However, CA HvRAC1-G23V promoted susceptibility to *Bgh* in stable transgenic plants (Fig 3.11). This discrepancy might rely on non-cell autonomous functions of HvRAC1 or, alternatively, on the effect of HvRAC1 on cell vitality, which might superimpose susceptibility effects when CA HvRAC1-G23V is strongly over-expressed in single cells. CA HvRAC1-G23V induced H₂O₂-mediated hypersensitive cell death in host epidermal cells during barley-*Bgh* interaction in stable transgenic barley plants (Fig. 3.11). To test whether it might be altering cell vitality, CA HvRAC1-G23V was transiently over-expressed in single epidermal cells of barley independent of fungal attack. Indeed, CA HvRAC1-G23V reduced vital movement of the cytoplasm, whereas CA HvRACB-G15V did not (Fig. 3.15b). Hence, over-expression of CA HvRAC1-G23V in single cells seems to limit cell survival, which might have superimposed CA HvRAC1-G23V induced susceptibility to biotrophic *Bgh*. The cessation of cytoplasmic streaming in CA HvRAC1-G23V-expressing barley cells is probably a consequence of oxidative stress due to the excessive production of ROS. This view is supported because the expression of H₂O₂

detoxifying enzyme, barley ASCORBATE PEROXIDASE (APX) reduced CA HvRAC1-G23V-induced cessation of cytoplasmic streaming (Fig. 3.15c). Over-expression of APX can suppress cell death induced by H₂O₂ in *Arabidopsis* (Murgia *et al.*, 2004). There is increasing evidence that ROS serve as direct and indirect mediators of PCD in plant cells in response to biotic and abiotic stresses and developmental cues (Apel and Hirt 2004; Beers and McDowell 2001; Laloi *et al.*, 2004; Love *et al.*, 2008). Previous studies suggest that ROS and mitochondrial permeability transition are the critical regulators of PCD in plants (Beers and McDowell 2001; Jabs 1999; Lam *et al.*, 2001; Reape and McCabe 2008; Scott and Logan 2008; Williams and Dickman 2008; Yao *et al.*, 2004). OsRAC1, a homologue of HvRAC1, promotes pathogen-independently ROS-mediated cell death in rice, which is marked by the occurrence of cell shrinkage, condensation of the nucleus, condensed and fragmented chromatin and blebbing of the plasma membrane (Kawasaki *et al.*, 1999). In contrast to that, CA HvRAC1-G23V over-expressing cells showed only the cessation of cytoplasmic streaming and spontaneous cell death was never observed in stable transgenic CA HvRAC1-G23V barley plants. CA OsRAC1 expression induced ROS-mediated cell death that resulted in race-specific immunity, when challenged by the rice blast fungus *M. grisea* (Ono *et al.*, 2001). In contrast, single cell CA HvRAC1-G23V-induced cytoplasmic cessation did not confer resistance to *Bgh* in barley (Schultheiss *et al.*, 2003b). Even though, both HvRAC1 and OsRAC1 play roles in the regulation of ROS production, there seems to be qualitative differences in the final outcome of their signaling in respective plant species and plant-pathogen combinations. This is again supported because *M. oryzae* resistance in CA HvRAC1-G23V barley is associated with local formation of CWAs (Pathuri *et al.*, 2008) whereas CA OsRAC1 induced resistance to *M. oryzae* is associated with HR-like cell death (Kawasaki *et al.*, 1999; Ono *et al.*, 2001).

The mammalian cell death inducer BAX promoted cell death when over-expressed in barley epidermal cells. BAX-induced cell death in barley shows signs of apoptosis such as fragmentation of the cytoplasm (Eichmann *et al.*, 2006). CA HvRAC1-G23V expression in barley epidermal cells led to the cessation of cytoplasmic streaming but the cell phenotype was not similar to that of BAX-induced cell death, in that no fragmentation of the cytoplasm was obvious.

However, the cessation of cytoplasmic streaming has been shown as an early event of cell death in plants (Eichmann *et al.*, 2006; Yoshinaga *et al.*, 2005). BAX-induced cell death can be partially rescued by the barley cell death inhibitor, BAX INHIBITOR-1 (HvBI-1) (Eichmann *et al.*, 2006). Interestingly, CA HvRAC1-G23V influence on cell vitality was not rescued by HvBI-1 expression in barley single epidermal cells (Fig. 3.15b). Preliminary data of gene-expression profiling of CA HvRAC1-G23V stable transgenic barley plants show no (pathogen-dependent) variation in the expression level of *HvBI-1* when compared to control plants (supplemental fig. 1) indicating that the increase in HR frequency in HvRAC1-G23V plants does not affect abundance of the cell death inhibitor.

It is intriguing that mammalian cell death antagonist BCL-X_L can rescue barley cells from both BAX- and CA HvRAC1-G23V-induced lethality (Fig. 3.15c; Eichmann *et al.*, 2006). BCL-X_L is an anti-apoptotic Bcl-2 family member that regulates mitochondrial membrane permeabilization and a proteolytic caspase cascade in mammals (Green and Kroemer 2004). It is also reported that BCL-X_L inhibits plant cell death and is considered to function mainly at mitochondria (Qiao *et al.*, 2002). Hence, CA HvRAC1-G23V-mediated lethality may possibly involve the role of mitochondria. Exposure of *Arabidopsis* cell cultures to H₂O₂ increased subsequent H₂O₂ production in mitochondria and this led to ATP depletion, opening of the mitochondrial permeability transition pore, release of cytochrome c and PCD (Tiwari *et al.*, 2002). Likewise, CA HvRAC1-G23V-induced ROS production might promote mitochondria-mediated cell death-like responses in barley single epidermal cells. Mitogen-activated protein kinases (MAPK) were also known to mediate ROS-associated cell death in plants (Colcombet and Hirt 2008; Gechev and Hille 2005; Huang and Huang 2008; Ren *et al.*, 2002; Takabatake *et al.*, 2007; Takahashi *et al.*, 2007; Zhang *et al.*, 2006). Biochemical evidence indicates that a MAPK cascade can be responsible for transmitting an H₂O₂ signal during oxidative stress in plants (Kovtun *et al.*, 2000). The effects of ROS on components of the MAPK cascade result in the indirect activation of transcription factors. In addition to the MAPK cascade network, the H₂O₂ signal may be transmitted through alterations in calcium ion fluxes and cellular redox state (Gechev and Hille 2005; Laloi *et al.*, 2004). In rice, CA OsRAC1 is known to regulate the activation of an elicitor induced MAPK at the protein level and

proteomic studies of CA OsRAC1-dependent signaling also revealed the induction of MAPK expression by CA OsRAC1 (Lieberherr *et al.*, 2005; Fujiwara *et al.*, 2006). MAP kinases were also known to regulate NADPH-oxidase-mediated oxidative burst (Asai *et al.*, 2008). Hence, it might be possible that CA HvRAC1-G23V-induced NADPH oxidase-mediated ROS production stimulates mitochondrial permeability, perhaps via MAPK activation, which finally ends up in cell death. However, a functional role of MAP kinases in CA HvRAC1-G23V-induced HR and/or suppression of susceptibility needs to be examined.

In conclusion, transient expression studies elucidate the interference of CA HvRAC1-G23V-induced susceptibility to *Bgh* by CA HvRAC1-G23V-induced cytoplasmic cessation in barley single epidermal cells. Since *Bgh* is a biotrophic fungus, it seems to be consistent that CA HvRAC1-G23V-mediated disruption of cell vitality resulted in the impediment of *Bgh* penetrations in barley epidermal cells.

4.6 Phylogenetic analysis of RBOH family members from various plant species

In plant species such as *Arabidopsis*, rice and barley, several NADPH oxidases, also referred to as RESPIRATORY BURST OXIDASE HOMOLOGUES (RBOHs), were identified and partially characterized (Lightfoot *et al.*, 2008; Torres and Dangl 2005; Torres *et al.*, 1998; Wong *et al.*, 2007). Plant RBOHs have been found to play prominent roles in ROS production during several plant processes. The barley RBOH gene family consists of six members: HvRBOHB1, B2, E, F1, F2 (A), and J (Lightfoot *et al.*, 2008). A phylogenetic analysis was carried out by comparing the deduced amino acid sequences of barley RBOH proteins to various RBOHs from *Arabidopsis* and rice to infer the evolutionary relationships among divergent plant RBOHs. For this purpose, nine members of rice RBOH protein family OsRBOH A-I (Wong *et al.*, 2007) and ten RBOH proteins from *Arabidopsis thaliana* AtRBOH A-J (Torres and Dangl 2005; Torres *et al.*, 1998) were used. A phylogenetic tree was constructed which analyses the phylogenetic relationship of homologous sequence of different RBOH proteins from *Arabidopsis*, barley and rice (Fig. 4.2). Barley HvRBOHB1 and HvRBOHB2 are very closely related with each other and

their closest relative being OsRBOHB of rice. Barley HvRBOHF1 is phylogenetically close to OsRBOHA, where as HvRBOHF2 (A) is similar to OsRBOHC of rice. On the whole, barley RBOH proteins share high sequence homologies with the rice RBOHs rather than the *Arabidopsis* RBOHs (Fig. 4.2).

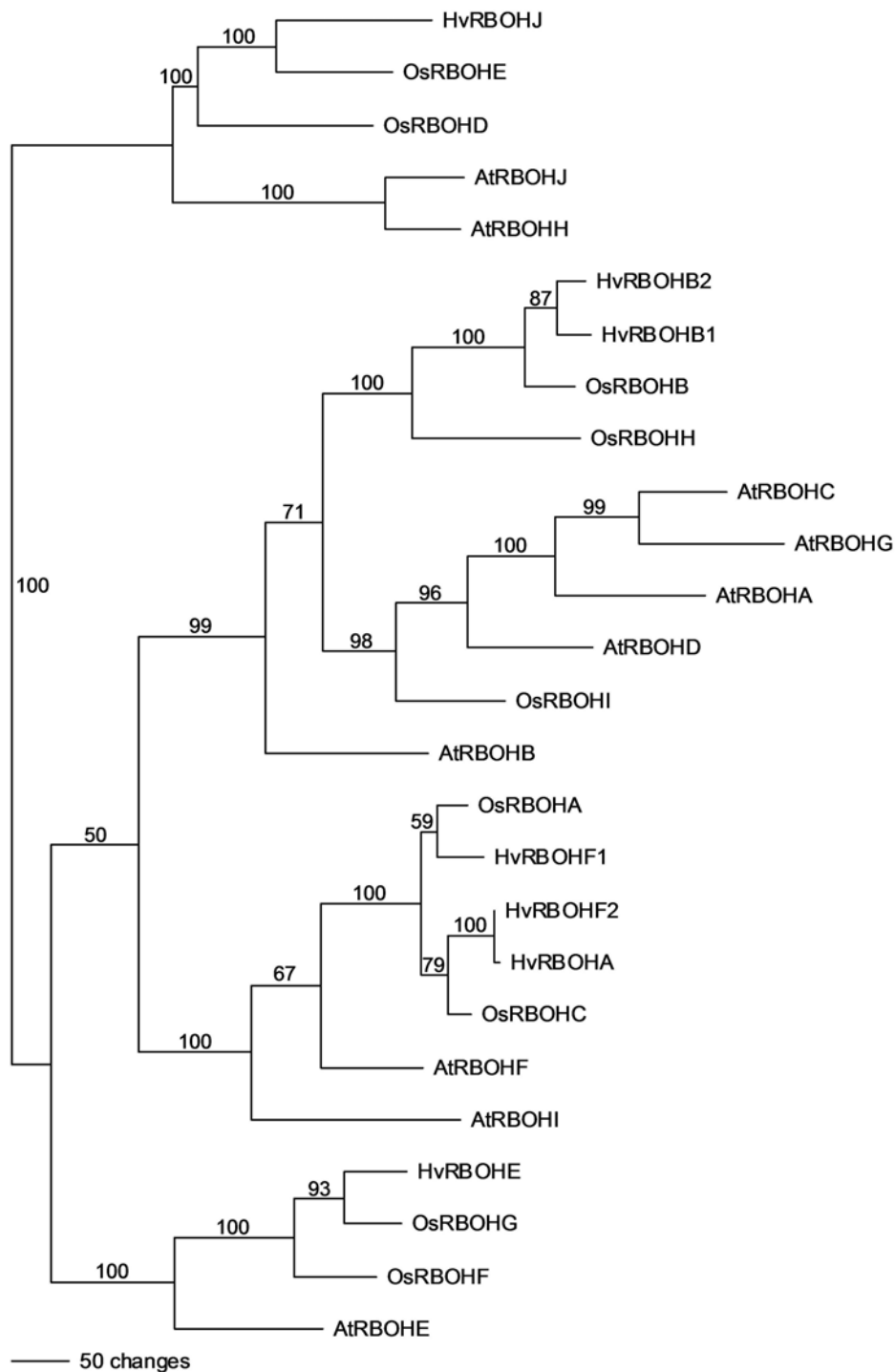


Figure 4.2: Tentative phylogram of the plant respiratory burst oxidase homologue (RBOH) protein family. The tree was constructed by alignment of deduced amino acid sequences using PAUP 4.0 (Phylogenetic Analysis Using Parsimony, <http://paup.csit.fsu.edu/about.html>). Phylogenetic analysis was carried out by A. Zuccaro (Institute of Phytopathology and Applied Zoology, University of Giessen, Germany). Sequences were obtained from GenBank. Abbreviations: *Hordeum vulgare* (Hv), *Oryza sativa* (Os), *Arabidopsis thaliana* (At). GenBank accession numbers: HvRBOHB1 (ACB56481), HvRBOHB2 (ACB56482), HvRBOHE (ACB56483), HvRBOHF1 (ACB56484), , HvRBOHF2 (ACB56485), , HvRBOHJ (ACB56486), HvRBOHF1a (ACB56487), HvRBOHF2a (ACB56488), HvRBOHA (125859103), OsRBOHA (NP_916447), OsRBOHB (AAT35117), OsRBOHC (AAU43962), OsRBOHD (ABA94089), OsRBOHE (BAD36069), OsRBOHH (ABA99453), AtRBOHA (At5g07390), AtRBOHB (At1g09090), AtRBOHC (At5g51060), AtRBOHD (At5g47910), AtRBOHE (At1g19230), AtRBOHF (At1g64060), AtRBOHG (At4g25090), AtRBOHH (At5g60010), AtRBOHI (At4g11230), AtRBOHJ (At3g45810).

4.7 Possible regulation of RBOH-produced ROS-mediated plant signaling by barley RAC/ROP proteins

Plant RBOH-mediated ROS production is known to play crucial roles in signal transduction processes during plant development and stress responses such as drought stress, wounding, suberization and stomatal closure (Desikan *et al.*, 2006; Kwak *et al.*, 2003; Macpherson *et al.*, 2008; Nepomuceno *et al.*, 2000; Razem and Bernards 2003; Sagi *et al.*, 2004; Torres and Dangl 2005). Plant NADPH oxidases are also emerging as key players in various plant-microbe interactions (Bolwell and Wojtaszek 1997; Kobayashi *et al.*, 2006; Simon-Plas *et al.*, 2002; Torres *et al.*, 2002, 2005; Trujillo *et al.*, 2006; Yoshie *et al.*, 2005; Yoshioka *et al.*, 2001; 2003). Several lines of evidence indicate that the activity of small GTPases is required for the appropriate execution of NADPH oxidase-mediated ROS production during various plant processes (Baxter-Burrell *et al.*, 2002; Carol *et al.*, 2005; Jones *et al.*, 2007; Morel *et al.*, 2004; Park *et al.*, 2004). Hence, it may be speculated that CA HvRAC1-G23V operates with barley RBOHs in inducing HR cell death when challenged by *Bgh*. Efforts to manipulate HR rates in *Bgh* infected CA HvRAC1-G23V barley leaves by chemical inhibition of the NADPH oxidase-mediated oxidative burst with diphenylene iodonium (DPI, Kawasaki *et al.*, 1999; Kobayashi *et al.*, 2006; Levine *et al.*, 1994; Sagi and Fluhr 2001; Yoshioka *et al.*, 2001) were not successful. Surprisingly, most of the *Bgh* spores on DPI-treated barley leaves

showed perturbed germination even at very low DPI concentrations (Supplemental fig. 3). This might suggest the contribution and requirement of fungal NADPH oxidase-mediated ROS in germination, growth and pathogenicity of *Bgh*, because NADPH oxidases were reported to play role in pathogenicity of various plant interacting fungi (Egan *et al.*, 2007; Giesbert *et al.*, 2008; Lara-Ortiz *et al.*, 2003).

There are evidences that NADPH oxidase-derived ROS are required for tip growth and cell expansion during plant development and organ morphogenesis (Foreman *et al.*, 2003; Gapper and Dolan 2006; Monshausen *et al.*, 2007; Takeda *et al.*, 2008) and NADPH oxidase-mediated ROS production is under the spatial regulation of RAC/ROP GTPases (Carol *et al.*, 2005; Carol and Dolan 2006; Jones *et al.*, 2007; Kost 2008; Potocký *et al.*, 2007; Uhrig and Hülskamp 2006). Constitutive expression of all three HvROPs largely affected epidermal cell morphogenesis and root hair polar growth in barley (Fig. 3.3; 3.5; 3.6). In yeast two hybrid assays, CA HvRAC1-G23V specifically interacted with HvrBOHB1 and HvrBOHB2, whereas HvrRACB and HvrRAC3 showed weak or no interaction with the HvrBOHs tested (Fig. 3.17). In spite of functional redundancy, ROPs show a high degree of specificity towards their interacting partners. One such example is the interaction of a 171 amino acid, RAC-interacting CRIB-motif containing protein, HvRIC171, with CA HvrRACB-G15V and CA HvrRAC3-G17V but not with CA HvRAC1-G23V (Schultheiss *et al.*, 2008). The selective interaction between HvROPs and HvrBOHs offers a possibility for divergent regulation of signal transduction processes and indicates that RBOHs are not the only source of ROS during plant development and cellular growth. However, it can not be ruled out that HvrRACB and HvrRAC3 may interact with other RBOH proteins of barley that have not been tested yet.

Rice ROPs were shown to interact with various RBOHs at protein level in a yeast two hybrid analysis. OsRAC1 and HvRAC1 interact with OsRBOHB and HvrBOHBs of rice and barley respectively (Fig. 3.17; Wong *et al.*, 2007) and the specificity of these interactions seems to be phylogenetically conserved since both OsRAC1-HvrRAC1 and OsRBOHB-HvrBOHBs of rice and barley are phylogenetically similar (Fig. 4.1; Fig. 4.2). In contrast, both HvrRACB and HvrRAC3 showed no interaction with either HvrBOHA (F2) or HvrBOHJ (Fig. 3.17), where as corresponding rice

homologues OsRACB (OsRAC6) and OsRAC3 interact with OsRBOHC and/or OsRBOHD (Fig. 4.1; Fig. 4.2; Wong *et al.*, 2007). The tendency of ROP-RBOH interactions of rice and barley suggests cross-species conservation as well as diversity in regard to the specificity of interactions.

In all three CA HvROP-expressing plants, ROS accumulation was observed in CWAs in response to *Bgh* infection but, enhanced HR was found only in CA HvRAC1-G23V barley (Fig. 3.11). Therefore, HvRAC1-specific ROS production that resulted in HR-cell death might be a consequence of the activation of HvrBOHB1 and HvrBOHB2 by CA HvRAC1-G23V (Fig. 3.11; 3.17). This is reminiscent of the situation in rice, where oxidative burst-inducing OsRBOHB is regulated by the small G-protein OsRAC1 through binding to its N-terminal region (Wong *et al.*, 2007). However, a bimolecular fluorescence complementation assay did not confirm *in planta* interaction of RAC/ROPs and RBOHB proteins in transiently transformed barley epidermal cells (Supplemental fig. 4). This may indicate either a transitory interaction between the two proteins or suggest that BiFC is not sensitive enough to detect it. Another possibility could be that the cytosolic calcium levels in the transiently transformed cells might have disturbed the stability of the interaction. Ca²⁺ binding and phosphorylation were suggested to play significant roles in the activation and regulation of RBOH-mediated ROS production in plants (Kobayashi *et al.*, 2007; Ogasawara *et al.*, 2008; Sagi and Fluhr 2001, 2006; Takeda *et al.*, 2008; Yoshioka *et al.*, 2001). Wong *et al.*, (2007) found that the interaction of OsRAC1 with OsRBOHB is influenced by changes in Ca²⁺ concentrations and suggested that the ROP-RBOH interaction could be suppressed by cytosolic Ca²⁺ elevation. Hence, it would be interesting to investigate a Ca²⁺-dependency of the *in planta* interaction between HvRAC1 and HvrBOHBs.

Trujillo *et al.*, (2006) reported that transient RNA interference-mediated gene silencing of HvrBOHA led to an increase in basal penetration resistance to *Bgh* without altering H₂O₂ accumulation. It was suggested that superoxide and associated hydroxyl radicals produced by HvrBOHA in response to *Bgh* infection could induce cell-wall softening, therefore supporting haustoria establishment in compatible interactions (Trujillo *et al.*, 2006). Although both HvROPs and HvrBOHA seem to play potential role in cellular accessibility to *Bgh* in barley, no

interaction of HvrBOHA was observed with either of the three CA HvROPs tested (Fig. 3.17). This implies that HvROPs and HvrBOHA may not share the same pathway to support *Bgh* susceptibility.

It is well established that small GTPases of plants act as signaling molecules in plant development and pathogen response in dicot plants. The present model of RAC/ROP protein functions in barley proposes a multitude of processes that involve ROP signaling (Fig. 4.3). Barley ROPs collaborate with other cellular factors such as Ca^{2+} , ROS, actin cytoskeleton and membrane dynamics to perform various functions in plants. Barley ROPs may interact with the downstream effectors like ROP-interacting proteins or RBOHs to transmit the signals during ROP-mediated plant processes (Fig. 4.3). Constitutively activated HvrRAC1 induced H_2O_2 -associated hypersensitive response in barley epidermal cells in response to *Bgh* infection. Activated HvrRAC1 displayed cell death inducing property in transiently transformed barley cells. According to the scheme proposed in figure 4.4, HvrRAC1 acts as a signal switch in inducing ROS-mediated cell death in barley. Given that HvrRAC1 interact with barley RBOHs and the mitochondrial cell death inhibitor (BCL-X_L) and ASCORBATE PEROXIDASE rescue the HvrRAC1-mediated cessation of cytoplasmic streaming, it is possible that RAC1-induced cell death involves RBOH-mediated ROS production and mitochondrial dysfunction (Fig. 4.4). Similar to OsRAC1 (Wong *et al.*, 2007), HvrRAC1-HvrRBOH interaction may entail Ca^{2+} for the feedback regulation of RBOH-mediated ROS production because, the EF-hand motifs in the N-termini of RBOHs are more conserved and are predicted to bind with Ca^{2+} .

On the whole, over-expression studies of barley ROPs (HvrRACB, HvrRAC3 and HvrRAC1) suggested the involvement of ROPs in plant developmental processes such as cell morphogenesis, root hair polar growth, and in susceptibility to barley powdery mildew fungus. Barley ROPs from different phylogenetic clades (Fig. 4.1) induced similar effects supporting redundancy of protein functions or cross-activation of downstream effectors. Expression of barley ROPs in tobacco revealed that RAC/ROPs carry out conserved as well as specific functions across the monocot and dicot plant species.

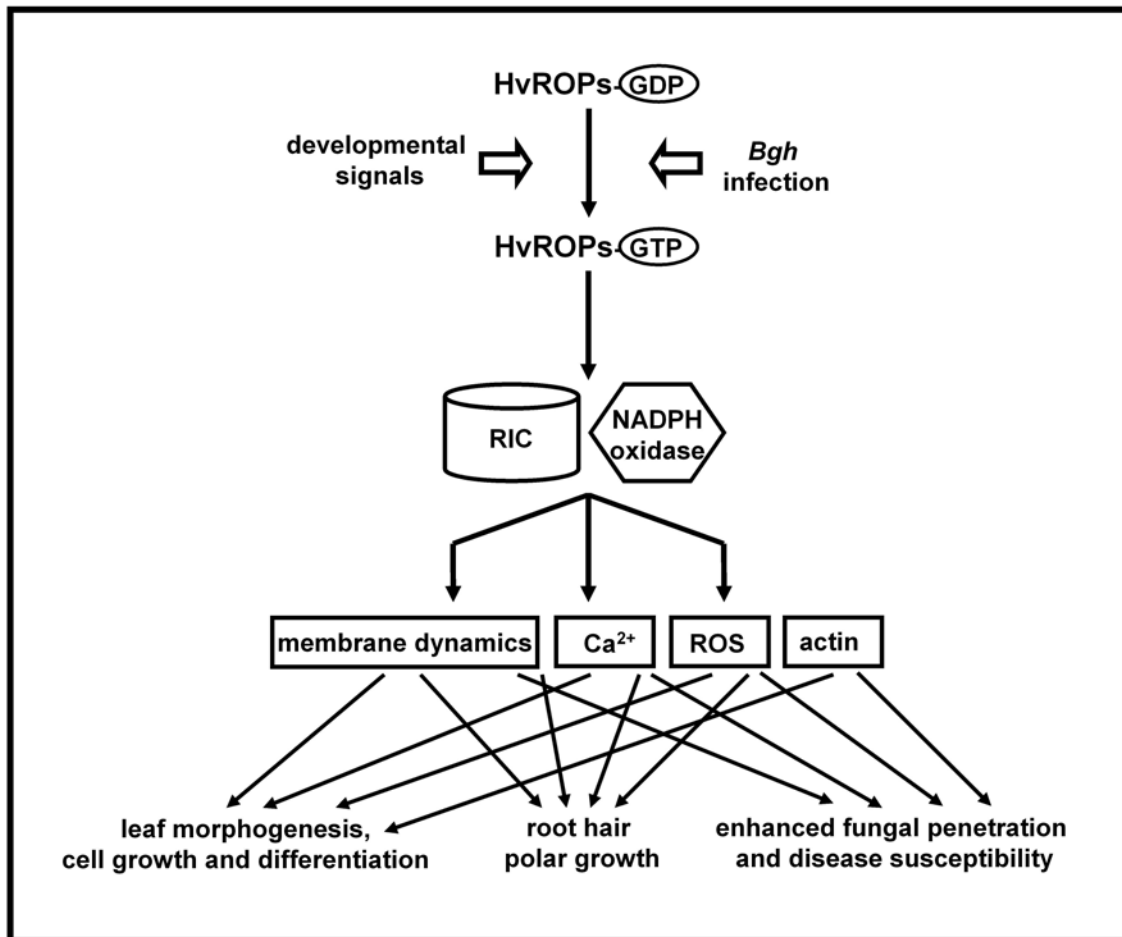


Figure 4.3: Schematic representation of suggested signaling pathways for HvROP-regulated functions in barley. Developmental signals or powdery mildew infection activate the exchange of HvROP-bound GDP with GTP. GTP-bound, active HvROPs (HvRACB, HvRAC3 and HvRAC1) interact with a number of proteins like RICs and NADPH oxidases to regulate cellular processes such as membrane dynamics, Ca²⁺ ion gradient, ROS production and actin polarization. A synchronized execution of these signal transduction pathways is essential for polar growth and development in barley plants. In case of *Bgh* infection, HvROPs are likely employed by the powdery mildew fungus to manipulate actin cytoskeleton to accommodate the fungal haustorium, thereby supporting susceptibility.

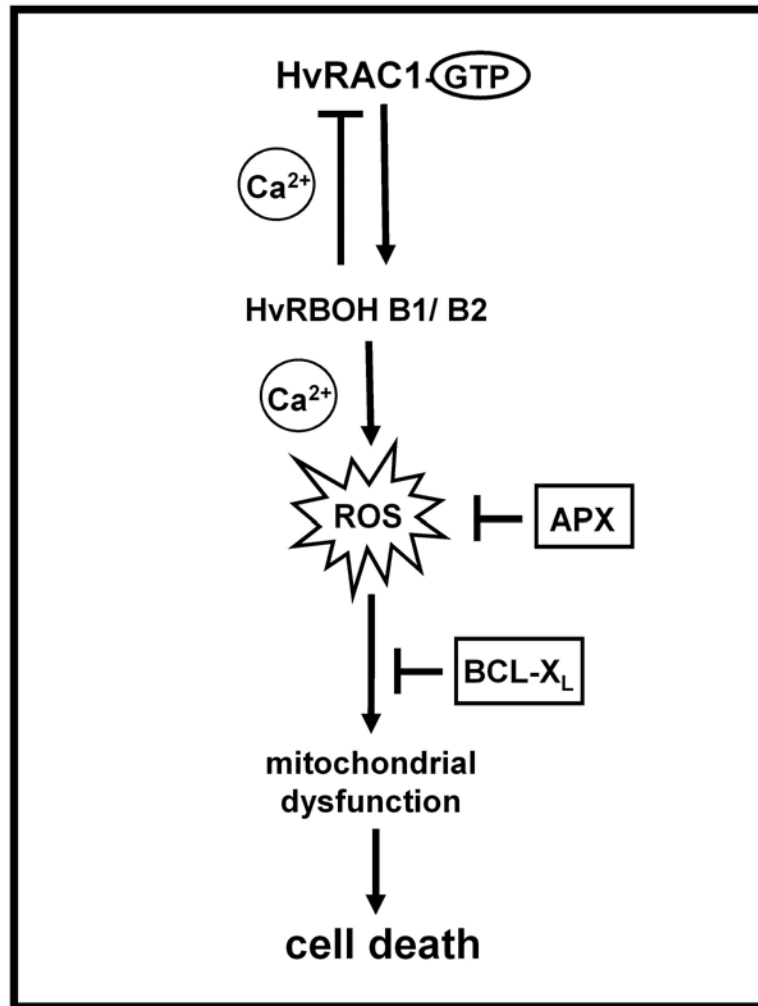


Figure 4.4: Schematic representation of a suggested signaling pathway for HvRAC1-mediated cell death in barley. HvRAC1 gets activated by the exchange of GDP with GTP in response to abiotic and biotic stress signals. GTP-bound active HvRAC1 may interact with HvRBOHB1 and/or HvRBOHB2 proteins to produce ROS. The interaction between HvRAC1 and HvRBOHBs is possibly negatively regulated by a Ca²⁺ ion gradient as was suggested for the OsRAC1-OsRBOHB interaction (Wong *et al.*, 2007). Excessive amounts of ROS lead to mitochondrial dysfunction and eventually cell death. ASCORBATE PEROXIDASE (APX) can rescue cells from HvRAC1-mediated cell death as it may scavenge the excess ROS produced (Fig. 3.15c). The mammalian apoptotic inhibitor BCL-X_L can also rescue the cells as it is a regulator of mitochondrial membrane permeability (Fig. 3.15c).

5. Summary / Zusammenfassung

Small monomeric RAC/ROP GTPases act as molecular switches in plant signal transduction processes, which are involved in plant development and a variety of biotic and abiotic stress responses. In the present work, constitutively activated (CA) mutants of different barley (*Hordeum vulgare*) ROPs (HvRACB, HvRAC3, HvRAC1) were stably expressed in barley and tobacco (*Nicotiana tabacum*) to get a deeper insight into functions of ROPs in plant development and pathogen responses. CA HvROPs induced epidermal cell expansion and abolished polarity in tip growing root hairs in both barley and tobacco. CA HvROPs induced susceptibility to the biotrophic powdery mildew fungi in barley and tobacco. CA HvRAC3-G17V induced super-susceptibility to the bacterial wild fire pathogen *Pseudomonas syringae* pv. *tabaci*, whereas CA HvRACB-G15V did not alter wild fire susceptibility. This illustrates the involvement of ROPs in response to pathogens of different life style. Data presented in this work support partial functional overlap among different barley RAC/ROPs and conservation of RAC/ROP-mediated processes across the monocot and dicot plant species.

All three CA HvROPs supported susceptibility of barley to penetration by *Blumeria graminis* f.sp. *hordei* (*Bgh*), whereas only CA HvRAC1-G23V supported whole cell H₂O₂ production in non-penetrated cells. Despite increasing penetration by *Bgh*, CA HvRAC1-G23V supported callose deposition at sites of fungal attack. Transient over-expression of CA HvRAC1-G23V induced the cessation cytoplasmic streaming which is a hallmark of cell death, and the CA HvRAC1-G23V effect was rescued by the anti-oxidant ASCORBATE PEROXIDASE and mammalian mitochondrial cell death regulator BCL-X_L. Yeast two-hybrid assays suggest protein-protein interactions between CA HvRAC1-G23V but not dominant negative HvRAC1-T28N and barley respiratory burst homologues (RBOH) B1 and B2. Together, data supports the idea that CA HvRAC1-G23V interacts with barley RBOHs to trigger reactive oxygen species-mediated mitochondrial dysfunction.

Kleine monomere RAC/ROP GTPasen sind molekulare Schalter in der Signalübertragung von Pflanzen und involviert in Entwicklungsprozesse und in Antworten auf biotischen und abiotischen Stress. In der vorliegenden Arbeit wurden stabil transgene Gersten- (*Hordeum vulgare*) und Tabakpflanzen (*Nicotiana tabacum*) untersucht, die konstitutiv aktivierte (CA) ROPs (HvRACB, HvRAC3, HvRAC1) exprimieren, um ein tiefer gehendes Verständnis der Funktion dieser Proteine in Prozessen der Entwicklung und Pathogeninteraktion zu erhalten. Die CA HvROPs induzierten epidermale Zellexpansion und verhinderten das Spitzenwachstum in Wurzelhaaren in Gerste und Tabak. Außerdem waren die Tabak und Gerstenpflanzen anfälliger gegen biotrophe Echte MehltauPilze. CA HvRAC3-G17V aber nicht CA HvRACB-G15V induzierte eine erhöhte Anfälligkeit gegen das bakterielle Blattpathogen *Pseudomonas syringae* pv. *tabaci*. Das zeigt, dass ROPs die Anfälligkeit von Pflanzen gegenüber Pathogenen von unterschiedlicher Lebensweise regulieren. Die Daten unterstützen die Ansicht, dass individuelle ROPs partiell funktional überlappenden Funktionen haben und über die Grenze von Monokotyledonen und Dikotyledonen hinweg konservierte Funktionen aufweisen.

Alle getesteten CA HvROPs förderten die Anfälligkeit von Gerste gegen Penetration durch *Blumeria graminis* f.sp. *hordei* (*Bgh*), wohingegen nur CA HvRAC1-G23V die eine H₂O₂ Akkumulation in der ganzen Epidermiszelle unterstützte, wenn diese nicht penetriert war. Neben der Förderung der Anfälligkeit, förderte CA HvRAC1-G23V auch die Bildung von Callose am Ort der pilzlichen Attacke. Wenn CA HvRAC1-G23V in einzelnen Zellen transient überexprimiert wurde, stoppte in diesen Zellen die Zytoplasmaströmung, was als frühes Zeichen für Zelltod gilt. Die Ko-expression von Ascorbate Peroxidase oder des mitochondrialen Zelltodregulators BCL-X_L aus Säugern rettete einen Teil der Zellen vor dem CA HvRAC1-G23V Effekt. Ein Yeast Two Hybrid Assay zeigte die Interaktion von CA HvRAC1-G23V nicht aber von dominante negativem HvRAC1-T28N mit den Gersten NADPH Oxdasen RBOHB1 und RBOHB2 an. Die Daten schlagen vor, dass CA HvRAC1-G23V mit RBOHBs interagiert, was zu einer Bildung von reaktiven Sauerstoffspezies führt, welche mitochondriale Fehlfunktionen verursachen.

6. References

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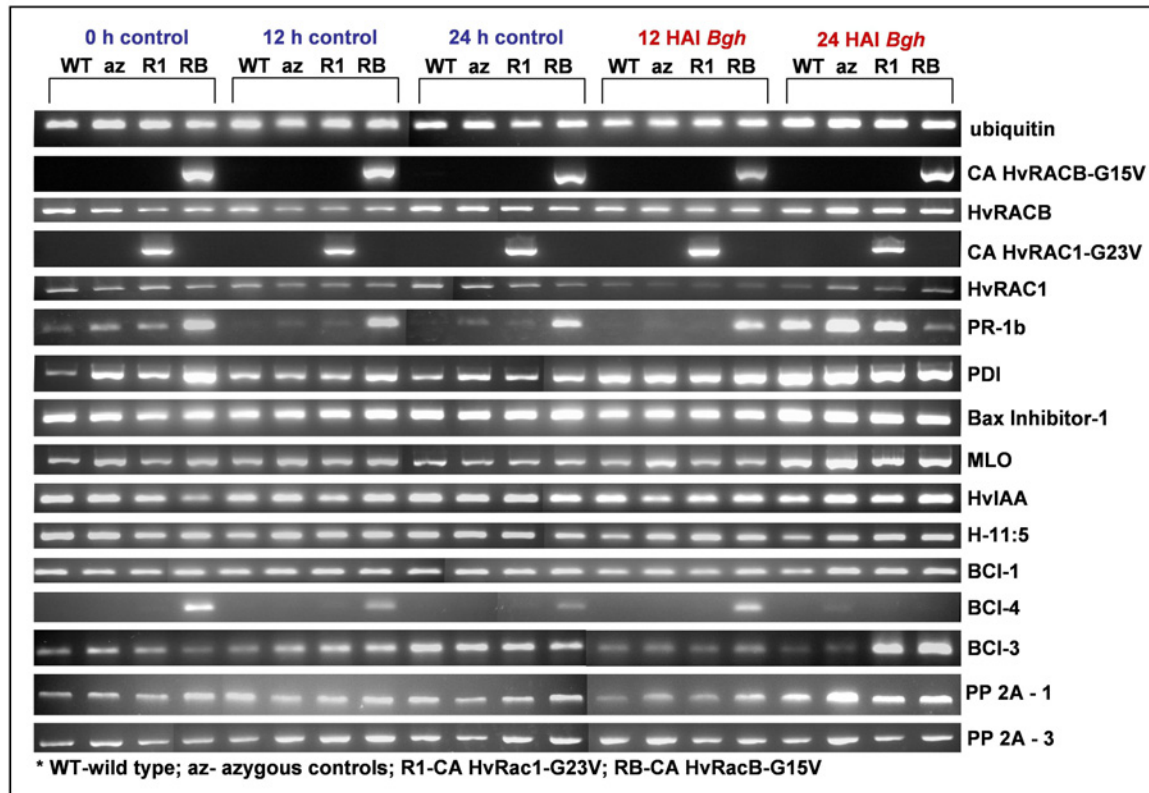
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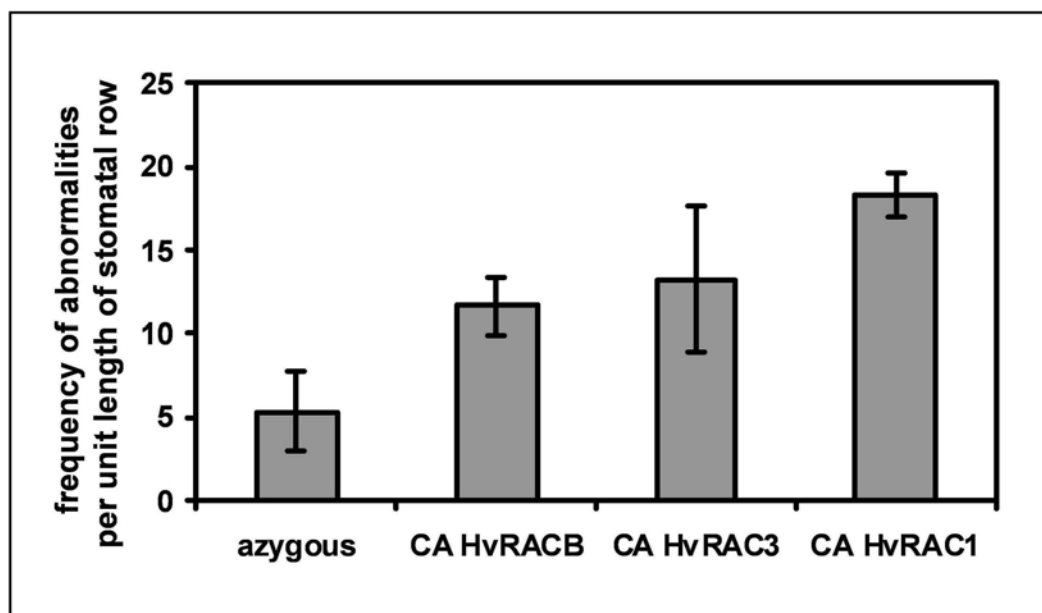
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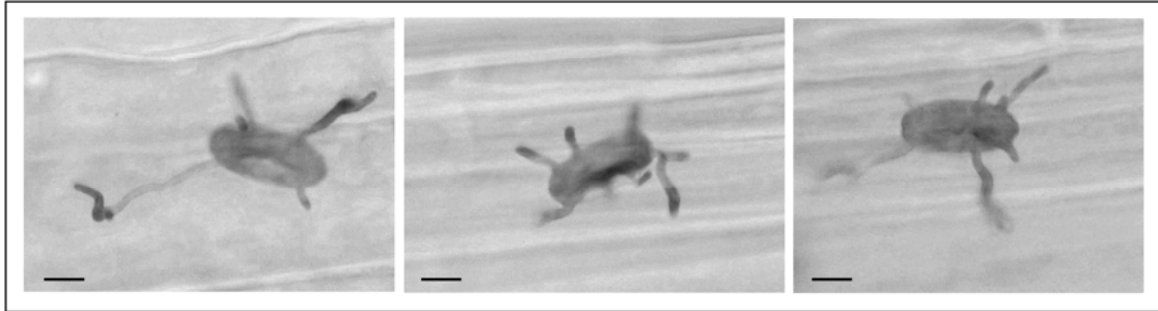
7. Supplement



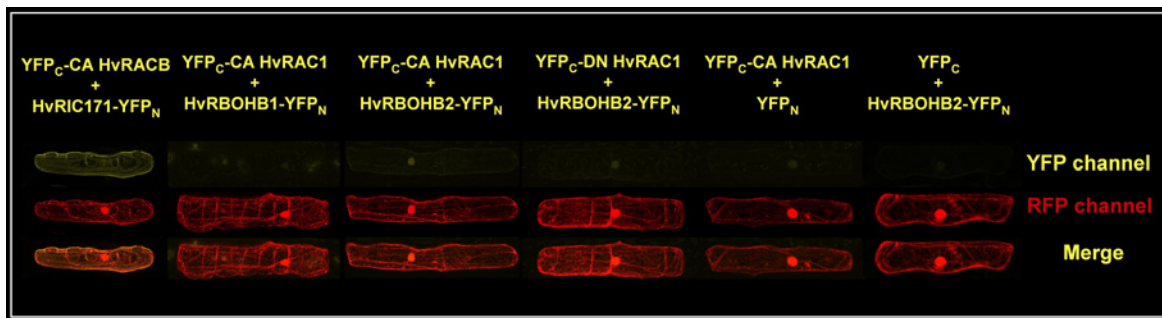
Supplementary figure 1: Semi-quantitative RT-PCR to detect the differential gene expression of CA HvROP-expressing barley in response to *Bgh* infection. At the time points indicated, RNA was extracted from the control and *Bgh* inoculated leaves. For the experimental procedure and PCR information, see chapter 2.3. The figure presents the gel photographs of ethidium bromide stained PCR products. Abbreviations: HAI-hours after inoculation, BCI-barley chemically induced. Accession numbers of the genes tested: *ubiquitin* (GenBank M60175.1), *HvRACB* (GenBank AJ344223.2), *HvRAC1* (GenBank AJ518933.1), pathogenesis-related-1b (*PR-1b*) (GenBank X74940.1), protein disulfide isomerase (*PDI*) (GenBank L33250.1), barley *Bax-inhibitor-1* (GenBank AJ290421.1), barley *MILDEW LOCUS 'O'* (*MLO*) (GenBank Z83834.1), auxin responsive *HvIAA* (TIGR accession TA32246_4513), auxin responsive *H-11:5* (TIGR accession TA58148_4513), methyl jasmonate-inducible lipoxygenase (BCI-1) (GenBank U56406.1), Ca²⁺-binding EF-hand protein (BCI-4) (GenBank AJ250283), acid phosphatase (BCI-3) (GenBank AJ250282), catalytic subunit of protein phosphatase 2A-1 (PP2A-1) (TIGR accession TA36140_4513), catalytic subunit of protein phosphatase 2A-3 (PP2A-3) (TIGR accession TA40824_4513).



Supplementary figure 2: Frequency of stomatal abnormalities observed in CA HvROP-expressing transgenic barley leaves. Several kinds of stomatal abnormalities were observed in CA HvROP-expressing barley leaves (Fig. 3.6). Average number of developmental abnormalities found in 5 cm of a stomatal row in transgenic plants expressing distinct CA HvROPs was quantified. For all samples, stomatal rows present on either side of the mid rib were counted in the leaf upper epidermis. Fully expanded leaves of 3 weeks old barley plants were used for the evaluation. Error bars show 95% confidence intervals. (Lines used: CA HvRACB-G15V 18-1-10, 17-1-11, 18-1-6; CA HvRAC1-G23V 27-3-17, 27-2-2; and CA HvRAC3-G17V 14-1-8, 17-3-16).



Supplementary figure 3: Perturbed germination of *Bgh* spores on the barley leaves upon treatment with DPI. Second leaves from wild type parent ‘Golden promise’ and CA HvrRAC1-G23V barley plants were used. The lower epidermis of the leaves were peeled off and then placed on the 0.2% water agar containing 30µM diphenyliodonium (DPI), an inhibitor of NADPH-oxidase, and inoculated with the *Bgh*. At 24 h after inoculation, leaf samples were fixed and microscopically analyzed. *Bgh* spores were stained by ink. Figure show the perturbed germination of the *Bgh* spores on the DPI-treated barley leaves. Bar=10µm.



Supplementary figure 4: Bimolecular fluorescence complementation assays by split YFP proteins fused to RAC1, RBOHB1 and B2 of barley. *In planta* interaction of CA HvrRACB-G15V with HvrRIC171 resulted in strong YFP fluorescence complementation at the cell periphery (column one) used as a positive control (Schultheiss *et al.*, 2008). Faint or no yellow fluorescence observed when CA HvrRAC1-G23V was co-expressed with either HvrBOHB1 or HvrBOHB2 (column two and three). Faint or no yellow fluorescence observed when DN HvrRAC1-T28N was co-expressed with HvrBOHB2 (column four) and when one of the split YFP proteins is expressed alone instead of being fused to either HvrRAC1 or HvrBOHB (column five and six). Red fluorescing protein (RFP) was a transformation marker localized in the cytoplasm and nucleus. All pictures represent maximum projections of 20–30 optical sections through the entire cell at 2 µm increments. All signals were recorded using the same laser beam intensity and detector voltage.

Acknowledgements

I wish to take this opportunity to express my sincere gratitude to Prof. P.B. Kirti and Prof. Karl-Heinz Kogel for giving me the opportunity to make my doctoral thesis in Germany.

A few lines are too short to make a complete account of my deep appreciation for my PhD supervisor Prof. Ralph Hückelhoven. He is clearly a master of his craft and I have benefited tremendously from observing him and conducting research with him. I am thankful to him for the inspiring scientific discussions and the motivation he offered. I wish to thank him for his steady support and constant encouragement through all these years.

My special gratitude goes to Dr. Ruth Eichmann, for providing numerous ideas and useful discussions during my PhD work. My sincere appreciation is extended to Ruth for spending time in reading and correcting my thesis. Her valuable suggestions and constant encouragement, especially during the writing period is highly treasured.

My heart-felt thanks to Jutta Preuss, for the scientific discussions and hilarious chitchats we had. Whenever I was in need, she was there to help me, especially to deal with German bureaucracy.

I wish to express my thanks to Dr. Christina Opalski, Dr. Marco Trujillo for helping me during my initial days of the course. Special thanks goes to Dr. Holger Schultheiss, Dr. caroline Höfle, Dr. Reinhard Pröls, Dr. Harald Schempp, Dr. Jürgen Leiminger, Dr. Jafargholi Imani and Mr. Valiollah Babaeizad for their help and support during the course of my work. I wish to thank Dr. Alga Zuccaro for providing the phylograms.

I would like to express my heartiest thanks to all my colleagues in Freising and Giessen for their kindness and affection, and for never letting me feel that I am away from my country. All their affection and friendliness gave me a very pleasurable stay in Germany.

ACKNOWLEDGEMENTS

I would like to thank Frau Helga Fritze, Frau Hildegund Mergans and Frau Gertraud Schröttle for their help to file the paper work for my stay in Germany and for thesis.

I am indebted to the entire faculty of Department of Plant Sciences at University of Hyderabad for motivating me into the pursuit of research. I express my deep respectful thanks to them.

The financial support of the STIBET programme of the German Academic Exchange Service (DAAD) and the BASF, and the German Research Foundation (DFG) are gratefully acknowledged.

I wish to extend my thanks to my friends in India and Germany for their friendship and support. I would like to thank my brothers Vijay and Bharath, and my uncle Venkatram Reddy who helped me to concentrate on completing this dissertation and supported mentally during the course of this work.

I take this opportunity to express my heart-felt gratefulness to Prasad for his endless help, unconditional support, and long-lasting patience through all these years without whom, making my PhD would have remained as a dream. I am so fortunate and blessed to have a wonderful person like you who made my dreams come true.

Finally, I would like to express special thanks to my parents and my grand mother for their prayers and constant encouragement throughout my life. It is their aspiration too to see me as a doctorate graduate. I dedicate this work to them, to honor their love, patience and support during these years.

Thanks almighty..!

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