Generation and molecular analyses of transgenic barley (*Hordeum vulgare* L.) in response to relevant pathogens

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1. INTRODUCTION

1.1 Barley

Barley (*Hordeum vulgare* L.) is an annual cereal**,** which is cultivated in all temperate climate zones, worldwide. It serves as a major animal feed crop, with lower amounts of use for malting and human food. Barley was one of the first domesticated cereals, most likely, originating in the Fertile Crescent in Middle East. Archaeological evidence found date back to 8000 BC for barley cultivation in Iran. Cultivated barley is one of 31 *Hordeum* species, belonging to the tribe Triticeae, family Poaceae. It is a diploid species with 14 chromosomes (2n=14). The genetic system is, relatively, simple; however the species is, genetically, diverse that renders it an ideal organism as a research model in cereals. Molecular evidence has revealed significant homology among barley, wheat and rye (Feuillet *et al.* 2009[1]). Different ploidy levels, i.e., diploid, tetraploid and hexaploid are existed amongst the wild *Hordeum*. Barley is ranked fourth in terms of production and area under cultivation (560,000 Km^2) in cereal crops. The rate of the world barley production during 2005-2007 was 139.2, 138.3 and 136.4 million tons, respectively[2]. Barley exists in two growing season types: Winter barley, which is usually sown in the fall. It needs vernalization, i.e., exposure to a period of cold temperature, which later ensures the normal development of heads and grains. It completes its development during the following spring and summer. Due to climatic needs, the growing region for winter barley is, predominantly, restricted to Europe and, mainly, used as livestock feed, because the kernels are rich in carbohydrates with moderate amounts of protein, calcium and phosphorus. In contrast, spring barley requires only short exposure to low temperature and can, thus, be sown in spring. Globally, the spring form is suitable for utilization in malting and alcohol production processes. A small amount of the produced barley is used for human food in form of pearl barley or flour. Barley is quite undemanding in terms of climate condition and soil quality. It needs a shorter growing season compared with wheat. Barley is more

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[1] http://barleyworld.org/whatisbarley/BarleyOriginTaxonomy.php
[2] http://www.fao.org

resistant to frost than wheat. It produces better in poor environments than wheat as if it is, often, found in acidic, drought-prone and thin soils at higher altitudes. Barley is, typically, much less stiff than wheat, so it tends to go flat, if it is over-fertilized and does not yield as much as wheat. Like other plants, several pathogens and insects can attack barley. The most common diseases that, particularly, affect spring barley in Europe are net blotch (caused by *Drechslera teres*), scald (caused by *Rhynchosporium secalis*), leaf rust (caused by *Puccinia hordei*) and powdery mildew (caused by *Blumeria graminis* f.sp. *hordei*).

1.2 The barley-powdery mildew interaction

Powdery mildew is a widespread fungal disease of many mono- and dicotyledonous plant species. In moderate temperate and humid climate, powdery mildew fungi cause severe yield loss in a wide range of crops. The fungus produces white to gray powdery-surfaced colonies that can appear on all aerial parts of plant. Barley is, usually, very susceptible to powdery mildew, and it has been reported to cause, approximately, 10% yield reduction in cold climate in no–fungicide farming (Jørgensen *et al.* 1988). During strong epidemics, the disease causes yield loss up to 25%. Early infection, negatively, affects crop density and number of seeds per ear, whereas the late infection, rather, reduces the seed weight. Intense electron microscopic and molecular inspections led to certain changes in the taxonomic classification of powdery mildew fungi. They are, currently, grouped in the order of Erysiphales with the family of Erysiphaceae, which splits into five tribes (Erysipheae, Golovinomycetinae, Cystotheceae, Phyllactinieae and Blumerieae) and several sub-tribes with more than 10 genera (Braun *et al.* 2002). The taxonomic classification of cereal powdery mildew fungi is:

Kingdom: Fungi / Phylum: Ascomycota / Class: Plectomycetes / Order: Erysiphales / Family: Erysiphaceae / Genus: *Blumeria* / species: *graminis*.

Powdery mildew fungi of the genus *Blumeria* infect plants of *Poaceae*, thereby, showing high host-species specificity.

Each forma specialis (f. sp.) of *B. graminis* is specialized to only one cereal species. In the case of barley, powdery mildew agent is *Blumeria graminis* (DC) Speer f. sp. *hordei* Em. Marchal (*Bgh*) (synonymous with *Erysiphe graminis* (DC.) ex. Merat f. sp. *hordei*). The fungus can complete its life cycle on barley plants, but it does not grow on wheat. Barley powdery mildew fungus is ecto-parasitic on the epidermal cells of barley leaves. When a *Bgh* conidium lands on a leaf surface of susceptible host, it starts to germinate in 2-30°C with an optimum range of 15-20°C and produces a primary germ tube (PGT), which is, fully, developed within 1-2 hours after inoculation (hai). The PGT produces a short penetration peg, which only, partially, breaches on epidermal cell wall but they can't produce haustorium (Zeyen *et al.* 2002). The PGT function is attaching of germinated conidium of fungus to host surface for absorbing the water and accompanying solutes from the host and recognizing the characteristics of the contact surface (Yamaoka and Takeuchi 1999; Carver and Bushnell 1983; Carver and Ingerson 1987; Zeyen *et al.* 2002). Afterward, appressorial germ tube (AGT) emerges from 8 hai, which is essential to form appresorium from appresorial lobes at the germ tube apex. The fungus, then, attempts to penetrate the cell by driving a penetration peg (PP) through the cell wall during 10–12 hai (Thordal-Christensen *et al.* 2000). Up to three PP can be observed from the same appressorial lobe after failure of the first. The fungus penetrates into host cell wall using a combination of mechanical (appressorial turgor pressure) and chemical (cutinase and cellulose) forces (Fric and Wolf 1994; Suzuki *et al*. 1998). After penetration of PP through the host cell wall and papilla, the tip of hyphal PP enters the epidermal cell and grows to form a specialized absorption structure, termed haustorium. The haustorium surrounded by host plasma membrane is, fully, mature around 30 hai with finger-like hyphal structures (Supplementary Fig. 1. C). This shape provides an extended surface area and facilitates the absorption of nutrients (Braun *et al.* 2002). Later, the primary appresorium starts to develop elongating secondary hyphae (ESH) during 36 to 48 hai, which can attack adjacent epidermal cells by forming new appresoria and secondary haustoria. The fungus starts to sporulate from conidiophores on the hyphae 3-4 dai, which has a club shaped basal-

cell with about eight conidia attached to each other forming a chain. The mature conidia that are separated from the conidiophore will spread by water or wind and start their way to a new infection cycle by completing asexual reproduction cycle (Ellingboe 1972). The asexual conidia are the main source of the disease (Aist and Bushnell 1991). However, the sexual reproduction takes place when condition is unfavorable for conidia formation. The heterothalic fungus develops, sexually, by fusion of compatible cells on the surface of plant tissue to produce sexual structure ascocarp (cleistothecium). The mature ascocarp contains upto 25 asci, each consisting of 8 ascospores. These are round-shaped and vary in color from brown to black. Under favorable condition, the ascospores are released and germinate (Ellingboe 1972; Agrios 2005). In suitable condition, epidemics can occur as powdery mildew can complete its life cycle in just three to four days at 20°C. In less favorable condition, this latent period, the time between infection and the development of visible symptoms, might take longer, e.g., 12 days at 10°C and 30 days at -2°C (Schulze-Lefert and Vogel 2000).

1.3 Plant defense systems

Plants challenged by diverse pathogens and pests, can build defense barrieres to infections, structurally and genetically. Sometimes, due to some sophisticated mechanisms, pathogens can suppress the host defense system and under favorable condition, cause severe infections that their effective management is hinged, solely, upon agrochemicals application. On the other hand, in most cases plants are very successful in resisting against many potential pathogens. Hence, plants have evolved defense systems to counteract pathogens, which use various infection strategies.

Some causal agents of plant diseases, e.g., fungi, viruses and bacteria require, at least in certain stages of their life cycle, living host cells for growth or reproduction (obligate biotrophs and hemibiotrophs), whereas some bacteria and fungi (necrotrophs) use toxins or enzymes to kill host and live on dead host cells. Plants employ diverse defence layers that are based on preformed barriers and induced responses (Bryngelsson and Collinge 1992). The first line of defense includes the waxy cuticle of the epidermal cell wall that

provides an effective barrier to inhibit the majority of potentially pathogenic microbes from entering plant tissues. When specific pathogens succeed to break this defense layer, either through wounds or stomata or by producing cutinase or cell wall degrading enzymes or by mechanical force, plants employ the second line of defense: large amounts of so-called preformed antimicrobial compounds aimed at inhibition of pathogen growth.

Additionally, plants have developed some inducible defense mechanisms, which are frequently mediated by plant signaling molecules, salicylic acid, jasmonic acid and ethylene. Within the induced responses, Resistance (R) gene-mediated defenses are most broadly characterized (Dangl and Jones 2001; Feys and Parker 2000; McDowell and Dangl 2000). In this case, a plant *R*-gene product recognizes (directly or indirectly) a matching pathogen *Avirulence* (*Avr*) gene product. This detection is often, but not always, associated with a rapid hypersensitive response (HR), a kind of programmed cell death (PCD) in plant cells (Dangl *et al.* 1996; Dangl *et al.* 2000; Heath 2000; Shirasu and Schulze-Lefert 2000). HR in plants displays many similarities with apoptosis, a programmed cell death phenomenon observed in animal cells. At the site of HR, and in surrounding cells, one of the earliest events observed is an oxidative burst whereby reactive oxygen species (ROS) including superoxide ($O_2^{\bullet -}$) and its dismutation product, hydrogen peroxide (H_2O_2), are produced (Doke 1983; Lamb and Dixon 1997; Ren etal. 2002; Yoda *et al.* 2003). Nitric oxide (NO), a redox-active molecule that is involved in mammalian defense responses (Schmidt and Walter 1994) is, also, generated and has been shown to serve as a signaling molecule in plant resistance (Delledonne *et al.* 1998; Durner *et al.* 1998). Barley resistance genes to powdery mildew agent can be divided into two broad categories including mutant alleles of the *MLO* gene, which confers broad spectrum nonspecific resistance against all *Bgh* isolates, and race specific resistance against specific isolate of *Bgh* which are under control of more then 40 genes like *MLa* and *MLg* (Wiberg 1974; Jørgensen 1994). These non race-specific and race-specific resistance mechanisms act through independent effector signalling components including *Ror 1* and *Ror 2* genes (*Ror*

=Required for *mlo* resistance) and the *Rar* 1 (*Rar*= Required for *Mla* resistance) and *Sgt1* genes respectively (Jørgensen 1988; Freialdenhoven *et al.* 1994, 1996; Hückelhoven *et al.* 2000; Peterhänsel *et al*. 1997; Azevedo *et al*. 2002). In this chapter, an overview is presented about some of the different plant defense mechanisms.

1.3.1 Cell wall apposition or papilla formation

Phytopathogenic fungi secrete cutinases to break and breach the plant surface polyester cutin layer. Afterwards, cutin derivatives can activate plant defense responses and prime plants for faster and stronger defense reaction to pathogen-derived elicitors (Fauth *et al*. 1998). However, it is not understood how plants assess intactness of their cuticles in pathogen interactions. After crossing the cuticle layer, pathogen breaches the host cell wall by mechanical and enzymatic pressures. Based upon genomic analyses, fungus strategy determines whether or not a fungus secretes a diversity of cell wall degrading enzymes (Kämper *et al.* 2006). Plant epidermal cells can resist actively against the penetration attempts by local cell wall reinforcement underneath appressorium and penetration peg (Aist 1976). Formation of cell wall apposition, which is also known as papilla formation (Supplementary Fig. 1. D), refers to the active deposition of the polysaccharide callose (1,3- glucans) and phenolic compounds as well as protein cross-linking (Kita *et al*. 1981; Ebrahim-Nesbat *et al.* 1993; Von Röpenack *et al.* 1998; Zeyen *et al.* 2002a; Jacobs *et al.* 2003). The main function of papillae seems to be repair of cellular damage, particularly, if papillae form before inoculation. They also seem to stop the pathogen penetration (Agrios 2005). Also, it is speculated that by this phenomenon epidermal cells protect themselves from powdery mildew fungi injury. In host plant upon contact with a fungus spore, reorganization of the cytoskeleton, especially the actin scaffold, towards the site of attempted penetration can be observed within the host cell (Kobayashi *et al.* 1993, 1997; Takemoto and Hardham 2004). This process is, probably, related to effective defense as in the compatible interaction polarized actin remodeling is noticeably reduced (Opalski *et al.* 2005). As well, streaming of Golgi and other small vesicle-like structures are known to be influenced by

actin microfilaments and may contribute to the accumulation of papilla material where required (Takemoto and Hardham 2004). It is documented in barley-*Bgh* interaction; vesicles formed during 10 to 15 hai and fused with the plasmamembrane, deposit papilla material onto the inner surface of the cell wall (McKeen and Rimmer 1973; Bushnell and Berquist 1975; Zeyen *et al.* 2002b). These papillae associated-vesicles can contain antimicrobial compounds including hydrolytic enzymes, phenolic conjugated polyamines and hydrogen peroxide (H_2O_2) that may be directly toxic to the pathogens (Hückelhoven *et al.* 1999, 2001; Trujillo *et al.* 2004; Collins *et al.* 2003; Thordal-Christensen *et al.* 1997). Additionally, visible responses in papillae zones are (i) a change in the epidermal cell wall staining affinity or 'halo' effect due to local change in cell wall pH, which can absorb acidic dyes and (ii) aggregation of dynamically moving epidermal cell cytoplasm directly in the appressorium contact site (Zeyen *et al.* 20002a,b). Due to incorporation of phenolic lignin-like compounds, papillae fluoresce intensely upon UV-light excitation (Kunoh *et al.* 1982; Koga *et al.* 1988).

The formation of cell wall appositions have been reported in both compatible and incompatible interactions of plants and fungi. Cytological examination has shown that local deposition of chemically modified cell wall material is critical for penetration resistance associated with papillae formation (Zeyen *et al*. 2002b). In compatible interaction, this type of response is ineffective and the fungus simply penetrates the papilla. Interestingly, it is documented that H_2O_2 accumulation cannot be observed in penetrated papillae (Hückelhoven *et al.* 1999). The basic genetic control of papillae responses in most of cereals and the others plants remains mostly unclear, but in the case of monocot barley and dicots *Arabidopsis* and tomato, it has been reported that mutation in *MLO* loci lead to broad-spectrum resistance against powdery mildew fungi agents (Büschges *et al.* 1997; Hückelhoven *et al.* 1999; Consonni *et al.* 2006; Bai *et al.* 2005).

Although papilla formation is one of the resistance mechanisms against fungi, it is documented that bacterial flagellin and (Type III secretion system) TTSS-deficient

Pseudomonas syringae strains induce papilla formation at inoculation sites (Gomez-Gomez *et al.* 1999).

1.3.2 Hypersensitive response (HR)

Resistance against pathogens in plants relies on multilayered mechanisms that ultimately lead to the inhibition of pathogen growth and development. Some plant defenses are constitutive, while others are induced by attempted-pathogen attack (Aviv *et al.* 2002). In case of successful penetration, the fungus must overcome the induced physical papilla-based barrier and start haustorium formation; then it may face another line of defense, i.e., the plant's hypersensitive response (HR). It is a mechanism used by plants to prevent the spread of infection by pathogens. This phenomenon is characterized by the rapid cell death in the local region surrounding an infection (Complementary Fig. 1.B). This post-penetration defense response causes disruption of nutrient uptake by the invader through a rapid and localized host cell death and is targeted mainly to restrict biotrophic pathogens (Koga *et al.* 1990; Heath 2000). Although, HR is not the only reason of resistance, but it is important for resistance to diverse diseases concerning obligate parasites (fungi, viruses, mollicutes and nematodes) as well as non-obligatory parasites (fungi and bacteria). HR is genetically controlled, and some of physiological features of HR seem to be same with programmed cell death (PCD) in animal (Freialdenhoven *et al*. 1994; Heath, M. 2000; Lam *et al.* 2001; Mittler *et al.* 1997). HR is the ultimate defense response initiated by the plant to specific pathogen produced signal molecules, known as elicitors. Recognition of pathogen elicitor by host plant activates a cascade of biochemical reactions in the attacked and neighboring cells, commencing new or altered cell functions and, thereby production of defense related compounds (Meindl *et al.* 2000; Czernic *et al.* 1999; Montesano *et al.* 2003). HR is accompanied by a high level production of antimicrobial compounds such as phytoalexines and PR proteins (Koga *et al.* 1990; Hammond-Kosack and Jones 1996).

In animals, release of cytochrome *c* from mitochondria, which have been affected by apoptotic agents like cell death regulators (e.g. BAX and BCL-XL), culminates in cell death (Green and Reed 1998). It has, also, been shown that these agents can induce cell death in plants through organelle destruction (Baek *et al.* 2004; Yoshinaga *et al.* 2005; Eichmann 2006). Mitochondria are the main source of ROS and alteration of the cellular redox state is known to be an alternative mechanism for triggering the caspases during apoptosis (Green and Reed 1998).

In spite of some differences, plants and animal share common characteristics regarding PCD. DNA laddering (fragmentation), nuclear condensation, shrinkage of cytoplasm, releasing of cytochrome *c* and alteration of membrane function are observed in both plants and animals (Hammond-Kosack and Jones 1996; Jones 1996; Dangl *et al.* 1996; Heath 2000; Grey 2002; Greenberg and Yao 2004).

HR is, obviously, distinguishable from necrosis as it is dependent on highly regulated signal transduction and *de novo* protein biosynthesis. By contrast, necrosis refers to coincidental irreversible cell death due to an injurious environmental factor and is not under genetic control. On the other hand, HR is rather considered to be a form of PCD similar to apoptosis in animals (Dangl *et al.* 1996).

The contribution of SA and *NPR1* in plant responses to PCD have been shown in several reports. Mutations in *NPR1* and SA responsive genes leads to suppression of cell death (Hunt *et al.* 1997; Rate *et al.* 1999; Shah *et al.* 1999; Weymann *et al*. 1995; Rate *et al*. 2001; Vanacker *et al*. 2001; Fitzgerald *et al*. 2004). It is well documented that *BAX INHIBITOR-1* (*BI-1*) is a factor, which makes plants hypersusceptible to biotrophic *Bgh* pathogen and confers resistance against necrotrophic pathogenes by suppressing PCD in plants (Hückelhoven *et al*. 2003; Hückelhoven 2004; Eichmann *et al*. 2006; Eichmann and Hückelhoven 2007; Babaeizad *et al*. 2009). Additionally, it is speculated that *MLO* gene playes a role in cell death regulation since mutations at different sites of the gene lead, generally, to spontaneous cell death in plants (Jarosch *et al*. 1999, Kumar *et al*. 2001, Jansen *et al*. 2005*,* Peterhänsel *et al.* 1997; *Consonni et al*. 2006). It has been reported that expression of *BI-1* and *MLO* genes in *mlo5*-mutant

barley plants facilitates the penetration of *Bgh* by upsetting the local accumulation of defense associated hydrogen peroxide (H_2O_2) due to suppression of penetration resistance. Interestingly, these effects in MLO expressor plants were significantly more than those in *BI-1* plants (Eichmann *et al*. 2006).

In barley-*Bgh* interaction, HR can, simply, be recognized by either UV-light excitation with whole cell autofluorescence due to cross-linking of phenolic compounds or by visualization of H_2O_2 accumulation inside the cell (supplementary Fig. 1.B), (Koga *et al.* 1990; Hückelhoven *et al.* 1999; Eichmann *et al*. 2006).

1.3.3 Pathogenesis–related (PR) proteins

Although plants possess some of physical barriers such as cell wall components (lignin and cellulose), cuticle and chemical compounds like tannins and phenolics to defend themselves against invaders, they must challenge against the agents that can pass through the mentioned defense layers. These later mechanisms are including inducible components, which are deployed, only, when needed. In this case, plants synthesize a variety of compounds when exposed to biological agents, i.e., fungi, bacteria, viruses, insects or herbivores and in response to wounding and certain abiotic stresses (Van Loon *et al.* 2006). Inducible defense compounds include reactive oxygen species, phytoalexins, cell wall components (callose, glycine or hydroxyproline rich proteins) and other groups of proteins called pathogenesis-related (PR) proteins. PR proteins have low molecular weight (10-40 KDa), which can tolerate extreme conditions due to their biochemical properties. They are soluble and very stable at low pH, where most other plant proteins are denaturized. These proteins are, also, resistant against proteolytic cleavage with extreme isoelectric points (pI) and, generally, localized in the vacuole, cell wall, intra- and intercellular spaces of different tissues (Stintzi *et al*. 1993). The PRs are, structurally, diverse within the panel of plant proteins that are toxic to invader pathogens. They are distributed, generally, in plants in low amounts, but this rate can elevate to much greater concentration in response to pathogen attack or stress. Different kinds of PR proteins have been isolated from several crop plants. Interestingly, different

plant organs, e.g., leaves, seeds, and roots may produce different types of PR proteins (Agrios 2005). Firstly, they were identified as new proteins accumulating during interaction of tobacco and Tobacco mosaic virus (TMV) (Van Loon and Van Kammen 1970; Van Loon 1985, 1999). They showed five distinct bands with different electrophoretic motilities, which were absent in mock plants and referred to as PR-1 to PR-5. Thereafter, several types of PR proteins have been identified and on the bases of molecular weight, serological or biochemical functions and other properties have been classified into 17 families as shown in supplementary table 3 (for review, see Van Loon 1999; Van strien, 1999; Broekart *et al*. 2000; Van Loon *et al*. 2006; Wladimir *et al*. 2007). The PR proteins are defined as "Proteins encoded by the host plant but induced, specifically, in pathological or related situation" (Van Loon 1999) or those proteins generally non-detectable in intact tissues. Most of PR proteins are induced through the action of the signaling molecules salicylic acid, jasmonic acid, or ethylene and have antimicrobial activities *in vitro* by hydrolytic activities on cell wall, contact toxicity, and perhaps involvement in defense signaling. Here, some of them that are associated with systemic acquired resistance (SAR) or the most prominent one of each family are explained, briefly.

1.3.3.1 PR-1 family

The PR-1 family contains the first identified PRs in tobacco infected with TMV (Van Loon and Van Kammen 1970; Van Loon 2006). Later, these proteins were isolated in rice, wheat, maize, *Arabidopsis*, barley and many other plants (Agarwal *et al.* 2000; Bryngelsson *et al.* 1994; Molina *et al.* 1999; Muradoy *et al.* 1993; Mendgen and Deising 1999). They are homologous to the super family of cysteine-rich proteins and divided in two acidic and basic group proteins with low molecular weight (15-17 KDa). In barley, expression of a gene of the PR-1 family, *PR-1b*, is frequently used as a reliable marker during challenge with *B. graminis* and other pathogens. Although its actual biological function is unknown, a certain antimicrobial impact of the protein on *Phytophthora infestans* and *Uromyces fabae* has been noted (Niderman *et al.* 1995;

Rauscher *et al.* 1999). Actually, involvement of *PR-1b* in penetration resistance of barley to the powdery mildew fungus (*Bgh*) has been demonstrated (Schultheiss *et al.* 2003). Different *PR-1* proteins can localize in diverse infected tissues like vacuoles, stomata guard cells, vascular bundles, cell walls, and also in xylem sap (Vera *et al*. 1989; Sessa *et al*. 1995; Hoegen *et al*. 2002; Lee *et al*. 2000; Grunwald *et al*. 2003).

1.3.3.2 PR-2 family

The PR-2 (β-1, 3-glucanase) family is, generally, distributed among plants including tobacco, *Arabidopsis*, pea, sorghum (Waniska 2000; Cote *et al.* 1991; Kim and Hwang 1997; Rezzonico *et al.* 1998). They have molecular weight of about 33 to 36 KDa. It is believed that antifungal activities of plant ß-1, 3-glucanases are due to hydrolysis of ß-1, 3-glucans present in the fugal cell walls resulting in a weak cell wall (Leubner-Metzger and Meins 1999). The PR-2 proteins are, mostly, accumulated in the hyphal tip of fungi. This weakened cell wall results in cell lysis, cell death, and release of oligosaccharides (Hernadez *et al.* 2005; Mauch and Staehelin 1989). These released fragments can be recognized as elicitors by plant and, finally, activate further defense responses. For instance, in soybean infected by *Phytophthora megasperma* f. sp. *glycinea* oligosaccharides released from cell walls of the pathogen due to digestion by β-1,3-glucanases act as elicitors, which lead the accumulation of a phytoalexin, glyceollin (Sharp *et al.* 1984). This family has two main groups, basic and acidic which are different in their enzymatic and antifungal functions (Kauffmann *et al*. 1987; Sela-Buurlage *et al*. 1993). Several experiments have shown the antifungal effects of basic class I of *β-1, 3-glucanases* on a wide range of fungi, either alone or in combination with PR-3 (Mauch *et al*. 1988; Ludwig & Boller 1990; Sela-Buurlage *et al*. 1993). It has been shown taht *PR-2* and *PR-3* have synergistic effect in double-transformed plants (Zhu *et al*. 1994). Studies on hyphal tips of *Trichoderma longibrachiatum* showed that *PR-2* and *PR-3* together affect the hyphal tip causing lysis in this point (Mauch *et al*. 1988; Arlorio *et al*. 1992). Both *PR-2* and *PR-3* are likely to play a dual role in plant defense directly by hydrolyzing structural components from fungal cell walls and

indirectly by releasing elicitors that may elevate the defense response in the plant (Stintzi *et al*. 1993). The *PR-2* proteins can localize in vacuoles in bean leaves (Mauch and Staehelin 1989; Mauch *et al*. 1992); *PR-2* in wheat leaves was recovered, mainly, in the domain of the host cell wall nearby to plasmalemma, cell wall appositions, intercellular space, guard cells and cell wall (Hu and Rijkenberg 1998). Tomato roots infected with *Fusarium oxysporum* showed *PR-2* localized, mainly, in the cell walls and vacuoles of the host, and in the cell wall and septa of the fungus.

1.3.3.3 PR-5 family

PR-5 proteins share significant amino acid homology to thaumatin (the sweet-tasting plant protein thaumatin from *Thaumatococcus danielli*), and are known as thaumatin like (TL) proteins (Linthorst 1991). The TL proteins have been isolated from *Arabidopsis*, corn, soybean, rice, wheat, barley, tobacco, tomato and many others (Hu *et al.* 1997; Huynh *et al.* 1992; Koiwa *et al.* 1997; Singh et al. 1987; Moralejo *et al.* 1999). Most of PR-5 proteins have molecular weight of about 22 KDa. Like other PRs, PR-5 proteins have acidic-neutral and basic isoforms. The PR-5 proteins have different functions such as antifungal activity, protection against osmotic stress and tolerance to freezing (Kononowicz *et al*. 1992; Hon *et al*. 1995). It is documented that PR-5 proteins exhibit significant inhibitory activity *in vitro* on hyphal growth, spore germination or development of germ tubes, possibly by permeabilizing of fungal plasmamembrane (Velazhahan *et al*. 1999). The PR-5b protein has inhibitory activity *in vitro* against germ tube development of *Bgh* and overexpression of *HvPR5b* fused with pathogen-inducible epidermis specific promoter in barley resulted in enhanced diseases resistance to *Bgh*, scaled (*Rhynchosporium secalis*) and net blotch (*pyrenophora teres*) (Poulsen, 2001). As well, two basic barley PR-5 proteins inhibited growth of *Trichoderma viride* and *Candida albicans* (Hejgaard *et al*. 1991). Furthermore, it has been shown that a basic PR-5 from tobacco can inhibit growth of *P. infestans, Neurospora crassa, Trichoderma reesei* and *C. albicans* (Woloshuk *et al*. 1991, Vigers *et al*. 1992). Additionally, this protein induces spore lysis, inhibits spore germination or reduces

spore viability in different species of *Bipolaris*, *Fusarium* and *Phytophthora*. Overexpression of PR-5 in potato postponed development of disease symptoms of *P. infestans* (Liu *et al*. 1994*)*, whereas transgenic potato plants expressing antisense *PR-5* did not exhibit any higher susceptibility (Zhu *et al*. 1996). Moreover, expression of a rice *PR-5* in tobacco caused enhanced resistance to *Alternaria alternata* (Velazhahan and Muthukrishnan 2003). In barley, PR-5 mRNA transcripts were increased in the mesophyll cells after infection with the necrotrophic fungus *Rhynchosporium secalis* (Steiner-Lange *et al*. 2003). It is, also, documented that PR-5 proteins are present in high amount in the xylem sap of intact *Brassica napus* plants (Kehr *et al*. 2005).

1.3.3.4 Other PR proteins in cereals

The PR-3 proteins (Chitinases), which are able to degrade fungal cell wall have been, frequently, used in genetic engineering for plant disease resistance (Schlumbaum *et al.* 1986; Datta and Datta 1999). Most PR-3 proteins have molecular masses of between 26 to 43 KDa. Chitinases have been isolated from fungi, bacteria, as well as plants, e.g., tobacco, cucumber, beans (Kang *et al.* 1999; Melchers 1994; Huynh 1992; Lee 1999; Chernin 1997). They exhibit antifungal activities against a wide range of human and plant pathogens except oomycetes, which lack of chitin in their cell wall compositions (Mauch *et al*. 1988). It is documented that basic isoforms of PR-2 and PR-3 have inhibitory affects on *Bgh* (Poulsen 2001). Additionally, several studies have shown the synergistic effect of PR-3 and PR-2 against many fungi (Mauch *et al.* 1988).

The PR-4 proteins are chitin-binding, with molecular weight of 13-14 KDa (Van Damme 1998). These proteins have potent antifungal activity against a variety of pathogens. In wheat, *PR-4* gene can induce by activators of SAR, *Fusarium culmorum* infection and wounding (Bertini *et al.* 2003). As well, wheat PR-4 proteins inhibited *in vitro* growth of some pathogenic fungi and showed ribonuclease activity (Caruso *et al.* 2001a, b; Caporal *et al.* 2004).

The PR-6 proteins are shown to be inhibitors of proteases (Green and Ryan 1972). Presumably, their targets are insect or microbial proteases. The PR-7 proteins are

endoproteases (Vera and Conejero 1988), and are the most common PR protein in tomato (Jord´a *et al*. 2000), functioning in microbial cell wall dissolution. PR-8 family members possess lysozymic activity and may be directed against bacteria. The PR-9 proteins are lignin-forming peroxidases (Reimers *et al.* 1992; Baga *et al.* 1995; Johansson and Nyman 1996) acting in cell wall fortification by catalyzing lignification (Passardi *et al.* 2004) and enhancing resistance against several pathogens. For instance, in the barley-powdery mildew interaction, peroxidases (PR-9) are enzymes with possible implications in the oxidative cross-linking of plant cell wall components to prevent penetration of pathogen (Thordal-Christensen *et al.* 1992).

The PR-10 proteins have been shown to have RNase activity (Somssich *et al.* 1986; Moiseyev *et al.* 1994) and some of them present weak ribonuclease activity (Bufe *et al*. 1996). The PR-10 proteins are unique in PR families owing to the direct specificity against viruses, and it has been assumed that the ribonuclease activity of PR-10 type proteins points out a role in defense against these pathogens (Park *et al*. 2004). PR-12 and PR-13 are known as defensins and thionins, respectively. They have been well studied and are known to permeate pathogen membranes (Edreva 2005). These proteins are inducibly expressed in leaves. Expression of oat thionin in rice increased resistance to seed-transmitted bacterial diseases (Iwai *et al.* 2002). As well, expression of barley *Thionin* gene in tobacco plants reduced lesion size when the plants were challenged with two strains of *Pseudomonas syringae* (Carmona *et al.* 1993; Florack *et al.* 1993). Recently, it is documented that silencing of *PR-13*/*thionin* in *Nicotiana attenuate* increases susceptibility to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC 3000 (Rayapuram *et al.* 2008). PR-15 and -16 are typical for monocots and comprise families of germin-like oxalate oxidases and oxalate oxidase-like proteins with superoxide dismutase activity, respectively (Bernier and Berna 2001). These proteins generate hydrogen peroxide that can be toxic to different types of attackers or could, directly or indirectly, stimulate plant-defense responses (Donaldson *et al.* 2001; Hu *et al.* 2003). PR-17 proteins have been found as an additional family of PRs in infected tobacco, wheat and barley and contain sequences similar to the active site of zinc-

metalloproteinases (Christensen *et al.* 2002). A putative novel family, PR-18, comprises fungus and SA-inducible carbohydrate oxidases, as exemplified by proteins with hydrogen peroxide generating and antimicrobial properties from sunflower (Custers *et al.* 2004). An overview on PR proteins, which have been already reported in barley, is given in supplementary table 4.

1.4 Systemic acquired resistance (SAR)

Plants, like animals, have evolved different sophisticated defense mechanisms like innate and acquired immunities for responding to microbial pathogens. Systemic acquired resistance (SAR) is one the most famous defense mechanism that is characterized by an activation of a broad spectrum of host defense responses, locally at the site of the initial pathogen attack and, systemically, in distal uninfected parts of the plant by the pathogen (Zhang and Klessig 1997). SAR was described by Ross (1961) in tobacco infected with tobacco mosaic virus (TMV). He demonstrated that infections of TMV were limited during the whole plant by a prior infection. This resistance was shown to be effective not only against TMV, but also to tobacco necrosis virus (TNV) and certain bacterial pathogens. He called the term "SAR" to refer to the inducible systemic resistance (Ross 1961).

SAR can provide resistance against diverse organisms such as fungi, bacteria and viruses. It is associated with induced defense reactions including biochemical and cytological changes and depends on the production of a signal that is translocated to other parts of the plant. Recognition of a pathogen frequently triggers a localized resistance reaction, known as the hypersensitive response (HR), which is characterized by rapid cell death at the site of infection (Hammond-Kosack and Jones 1996). The recognition of pathogen and subsequent HR lead to SAR induction in plants. A major feature of SAR is that resistance is expressed against pathogens that can be extensively different from the primary infecting agent. Although plants do not possess immunoglobulins, the general phenomenon of SAR is comparable to immune system in animals and human.

SAR is completely dependent on the signal molecule salicylic acid (Gaffney *et al*. 1993; Cao *et al.* 1994; Glazebrook *et al*. 1996; Shah *et al*. 1997). The evidence came from plants unable to accumulate SA due to the expression of a bacterial salicylate hydroxylase (NahG), which converts SA to the biologically inactive catechol. Transgenic tobacco and *Arabidopsis* expressing NahG accumulated very little SA after pathogen infection, failed to express *PR* genes, and were impaired in SAR (Delaney *et al*. 1994; Gaffney *et al*. 1993). Additionally, loss of function of phenylalanine, which is required for the SA synthesis, leads to reduction of SAR (Pallas *et al.* 1996). These findings confirmed the crucial role of SA as a signal transducer between pathogen elicitation and disease resistance in plants. Accumulation of SA in plant tissues, either as the result of a necrotic lesion or by pathogen challenge after exogenous application of the same inducer, results in the induction of distinct set of *PR* genes expression, in both local and systemic tissues. These proteins were first described in the 1970s by Van Loon, who observed accumulation of various novel proteins after infection of tobacco with TMV (Van Loon, Van Strien 1999; Van Loon and Van Kammen A. 1970; Ryals *et al*. 1996). They include glucanases, chitinases, and peroxidases. Some of these proteins may have their individual role against fungal or bacterial pathogens via hydrolytic action on their cell walls.

The SAR conferred resistance is long lasting, sometimes for the whole life of the plant, and it is effective against viral, bacterial and fungal pathogens. It can also be induced by the SA and its analogs like 2, 6- dichloroisonicotinic acid (DCINA) Métraux *et al*. 1991 and Benzolar-S-methyl [benzo (1,2,3) thiadiazole-7- carbothioic acid S-methyl ester (BTH)] that may have similar effects in disease resistance against various pathogens (Kogel *et al*. 1994; Görlach *et al*. 1996; Rairdan *et al.* 2001, 2002; Schweizer *et al*. 1997; Morris *et al*. 1998). These inducers activate defense genes expression via Nonexpresser of *Pathogenesis-Related* genes 1 (*NPR1*), as key mediator of SAR (Shah *et al*. 1997; Datta and Muthukrishnan 1999; Dong 2004).

1.5 *NPR1* and its role in plant disease resistance

Nonexpresser of *Pathogenesis-Related* genes 1 (*NPR1*); Cao *et al.* 1994) also known as *NIM1* (non-inducible immunity; Delaney *et al.* 1995), and *SAI1* (salicylic acid–insensitive; Shah *et al.* 1997) is essential for transduction of the SA signal to activate *PR* genes and induction of SAR, which confers long lasting broad spectrum disease resistance in plants (Shah *et al.* 1997; Cao *et al.* 1998; Dong 2001; Me´traux 2001). It affects the SAR pathway downstream of the SA signal. *NPR1* gene firstly identified in *Arabidopsis* through a genetic screen for SAR compromised mutants (Cao *et al.* 1994; Glazebrook *et al.* 1996; Shah *et al.* 1997; Delany *et al.* 1995). Afterward, it was reported from other plants like tobacco, wheat, rice, barley and apple (Chern *et al.* 2005, Kogel and Langen 2005; Malnoy *et al*. 2007*)*. Overexpression of *AtNPR1* in *Arabidopsis* and its expression in rice, tomato, wheat and apple enhanced pathogens resistance by elevation of *PR* genes expression (Cao *et al.* 1998; Lin *et al.* 2004; Fitzgerald *et al.* 2004; Chern *et al*. 2001, 2005; Fitzgerald *et al.* 2004; Friedrich *et al.* 2001; Lin *et al*. 2004; Makandar *et al*. 2006, 2000; Malnoy *et al*. 2007). As well, it is documented that induced resistance due to *NPR1* expression is correlated with rather fast and higher expression of *PR* genes (Cao *et al.* 1998; Friedrich *et al*. 2001, Makandar *et al.* 2006; Malnoy *et al*. 2007). Accordingly, it has been reported that mutated *npr1* in *Arabidopsis* failed to respond to various SAR inducing agents and, thus, exhibited enhanced susceptibility to pathogens (Cao *et al.* 1997; Chern *et al.* 2001). Beside, silencing of the *NPR1* gene in tomato enabled *Pseudomonas syringae* pv. *tomato* (*Pst*, DC3000), carrying the avirulence gene *avrPto*, to develop disease symptoms in the Rio Grande-PtoR (RG-PtoR) background (near isogenic line containing the *Pto* locus from *Lycopersicum pimpinellifolium*), which normally shows Pto mediated resistance to this bacterium (Ekengren 2003). In rice and tobacco, silencing of *NPR1* via RNA interference (RNAi) results in the higher susceptibility to pathogens and herbivores (Rayapuram and Baldwin 2007; Yuan *et al.* 2007). *NPR1* encodes a protein with a bipartite nuclear localization sequence and two potential protein–protein interaction domains: an ankyrin repeat domain and a BTB/POZ (Broad-

complex, Tramtrack and Bric-a-brac/Pox Virus and Zink finger motif; Cao *et al*. 1997). Activity of *NPR1* is dependent on the cellular oxidoreduction (redox) status. Some studies have shown that increasing of SA concentration after pathogen infection leads to change of redox state of the cell (Chen *et al.* 1993; Noctor *et al.* 2002; Vanacker *et al.* 2000). After SAR induction and subsequent oxidative burst, plant cells attain a more reducing environment owing to the accumulation of antioxidants like SA, and *NPR1* is converted from oligomeric form to monomeric form through reduction of intermolecular disulfide bonds. The monomeric *NPR1* then moves into the nucleus to activate SAR associated gene expression. Also, mutation at each ten conserved *AtNPR1* cysteines revealed that mutations at C82 and C216 result in constitutive expression of monomeric nuclear *NPR1* and *PR1* expression even in the absence of SAR inducer (Mou *et al.* 2003; Tada *et al.* 2008).

It is well known that *NPR1* interacts with several members of the TGA subclass of basic domain/leucine zipper transcription factors (Zhang *et al.* 1999; Després *et al.* 2000; Zhou *et al.* 2000). The presence of two protein-protein interaction domains in *NPR1* suggests that it might regulate SAR related gene expression through interaction with TGA factors (Mou *et al.* 2003). These TGA factors can bind to the SA-responsive elements present in *PR* genes' promoters and, subsequently, SAR will be activated (Lebel *et al.* 1998). In *Arabidopsis, NPR1* interacts with three TGA transcription factors (TGA2, TGA5 and TGA6) and it has been shown that a triple-mutation in all of them (tag2, tga5, tga6) is essential to suppress *NPR1* function by *PR* gene expression (Zhang *et al.* 2003). *In vitro* gel mobility shift assay showed that the DNA binding activity of TGA2 is enhanced by *NPR1* (Després *et al.* 2000). It is documented that TGA protein serves as a bridge between *NPR1* and *PR* genes induction (Chern *et al*. 2005). Besides TGAs, WRKY transcription factors, which have been shown to be effective in *PR* gene expression, can regulate the *PR* genes expression. For instance, overexpression of WRKY70 leads to constitutive *PR* gene expression, indicating WRKY70 is a positive regulator of *PR* genes (Li *et al.* 2006).

Activation of SAR by *NPR1* leads to a high level of PR proteins in vacuoles and apoplast as if the basal activity of the protein secretory pathway may not be sufficient to accommodate increased PR protein synthesis. Therefore, it is thought that a matched up-regulation in the protein secretory machinery is essential for proper folding, modification, and transport of PR proteins. Beside PR proteins induction, it has been shown that *NPR1* encodes members of the protein secretory pathway genes with more than 2-fold induction of endoplasmic reticulum (ER) localized proteins (Vitale and Denecke 1999*;* Trombetta and Parodi 2003). These secretion related-genes include those encoding Sec61 translocon complex, which provides a channel for proteins to cross the ER membrane and a signal recognition particle (SRP) receptor, which directs proteins with a signal peptide to the translocon complex. *NPR1* also regulates many genes encoding ER-resident chaperones, such as BiP2 and glucose regulated protein 94 (GRP94), as well as co-chaperones including defender against apoptotic death 1 (DAD1) (Fu and Kreibich 2000), calnexins (CNXs), calreticulins (CRTs) and protein disulfide isomerases (PDIs). These proteins function in the cotranslational folding and modification (e.g. disulfide bond formation and glycosylation) of nascent polypeptides destined for the apoplast or various organelles. Other genes in this group encode a Golgi-associated membrane trafficking protein; a clathrin, which is involved in packaging secretory proteins into small vesicles and a vacuolar sorting receptor (Wang *et al.* 2005).

1.6 MLO protein and its role in susceptibility to powdery mildew

Similar to *lsd1* (Lesion simulating disease) in *Arabidopsis*, *mlo* (mildew resistance locus o) mutants in barley, *Arabidopsis* and tomato exhibit resistance at the pre-lesion stage and confer non-race-specific (broad spectrum) resistance to the powdery mildew fungus (Buschges *et al*. 1997; Consonni *et al.* 2006; Bai *et al.* 2008). The *mlo*-mediated resistance requires, at least, two additional genes, designated *Ror1* and *Ror2*; mutations in either *Ror* genes leading to susceptibility in the *mlo* genetic background (Freialdenhoven *et al*. 1996).

In barley and *Arabidopsis*, the *mlo* mutation causes a lesion mimic phenotype in developed elder leaves. Homozygous barley mutant *mlo* plants are resistant to the normally virulent obligate biotrophic powdery mildew fungus *Bgh*. It is documented that in barley a single mlo locus is sufficient to render full, recessively inherited, resistance to powdery mildew, whereas in *Arabidopsis* partial redundancy of *MLO* family genes causes a more quantitative nature of single mlo-mutations (Jørgensen *et al.* 1992, Büschges *et al.* 1996; Consonni *et al.* 2006). As well, it has been shown that in *mlo* plants, papillae forms at the site of attempted *Bgh* penetration and the rate of papillae is significantly greater than that in WT *MLO* plants (Freialdenhoven *et al.* 1996; Hückelhoven *et al.* 1999). Additionally, barley genotypes lacking functional MLO, either due to natural genetic variation (Piffanelli 2004) or because of induced deletions in the *MLO* gene, (Büschges *et al.* 1997; Piffanelli *et al.* 2002) are resistant against all known isolates of the *Bgh* owing to intimate connection between cell death and disease resistance (Shirasu and Schulzel-Lefert 2000). On the other hand, barley compromised in *MLO* is susceptible to necrotrophic fungal pathogens like *Magnaporthe grisea* and *Bipolaris sorokiniana* (Jarosch *et al.* 1999; Kumar *et al.* 2001). Furthermore, inoculation in the immature caryopses with necrotrophic *Fusarium graminearum* progresses deeper into the tissue of *mlo*-barley compared with *MLO*-barley (Jansen *et al.* 2005). MLO may normally function as a negative regulator of cell death during HR (Lam *et al*. 2001). It is documented that *mlo* is functioning in penetration resistance, which is characterized by formation of cell wall appositions (papillae) and accumulation of phytoalexins, *PR* genes transcripts, and hydrogen peroxide (Stolzenburg *et al.* 1984; Zeyen *et al.* 1993; Peterhänsel *et al.* 1997; von Röpenack *et al.* 1998; Hückelhoven *et al.* 1999, 2000). All of these characteristics are also found, in a lower amount, in susceptible barley meaning that the *mlo* alleles confer a primed responsiveness for these defense reactions or the functional *MLO* is a controlling element of these basic resistance mechanisms (Büschges *et al.* 1997; Peterhänsel *et al.* 1997).

Accordingly, *Atmlo2*/*6* double and *Atmlo2*/*6*/*12* triple mutants are supersusceptible to necrotrophic *Alternaria* species, and show, as well, an enhanced cell death to

hemibiotrophic *Phytophthora infestans* when compared with WT (Consonni *et al.* 2006). The spontaneous cell death in *mlo* mutants suggests that *MLO* functions as a negative regulator of leaf cell death. This may indicate the relationship between developmental cell death control and pathogen resistance. Indeed, *mlo*-dependent spontaneous cell death is compromised in *mlo ror1* and *mlo ror2* double mutants, indicating at least overlapping genetic pathways leading to cell death and resistance (Peterhänsel *et al*. 1997). It has been shown that both *BI-1* and *MLO* can suppress barley BC Ingrid-*mlo5* resistance to powdery mildew, and the rate of penetration in *MLO* expressor plants increases, significantly, in relation to that in *BI-1* expressing plants (Eichmann *et al.* 2006).

Presence of the MLO protein is an absolute requirement for successful penetration of the host cell wall by compatible powdery mildew species, *Bgh* (Panstruga 2005). This gene could have a broad involvement in cell death protection in responses to biotic and abiotic stresses (Piffanelli *et al*. 2002; Lam *et al*. 2001). It is documented that like *BI-1*, *MLO* negatively controls *Bgh* induced apoplastic H_2O_2 burst at sites of fungal attack (Hückelhoven *et al.* 1999; Eichmann *et al.* 2006). Additionally, Opalski *et al.* (2005) showed, like RAC/ROP G-protein, MLO is involved in the modulation of actin reorganization and cell polarity of barley-*Bgh* interaction. As well, their findings showed that overexpression of RAC/ROP or MLO delays reorganization of Actin filaments (AFs) and their accumulation in response to *Bgh* in *mlo5* plants was much higher than in *MLO*-barley (Opalski *et al.* 2005). Actin filaments are important paths for intercellular organelle and vesicle transport. Local accumulation of defense-related compounds, such as callose and autofluorescent materials, occurs simultaneously with the radial arrangement of AFs at sites of fungal attack in barley, cowpea and potato (Kobayashi *et al.* 1997a; Schmelzer 2002; Skalamera *et al.* 1997). Existence of over 35 MLO family members in *Arabidopsis* genome (Shirasu and Schulze-Lefert 2000) indicates that they are conserved proteins with probably diverse functional roles within plant cells. It is thought that the fungal pathogen manipulates, directly or indirectly, MLO to suppress a vesicle–associated and soluble N-ethylmaleimide–sensitive factor

attachment protein receptor (SNARE) protein- dependent resistance response at the cell periphery (Panstruga and Schulze-Lefert 2003; Collins *et al.* 2003; Schulze-Lefert 2004). It has been shown that barley *Ror1* and *Ror2* genes are required for full expression of *mlo* resistance and thus assumed to be components of penetration resistance. Barley *Ror2* and its *Arabidopsis* ortholog, *PEN1*, encode PM-resident syntaxins containing a SNARE domain (Collins *et al.* 2003). The barley synaptosome associated protein of 25 KDa (SAP25) homolog, *HvSNAP34*, is also required for penetration resistance to *Bgh*, and has been shown to form a binary SNARE complex with *Ror2*. These finding have been interpreted as evidence for the existence of SNARE complex dependent resistance mechanism acting at the cell periphery against powdery mildew penetration (Collins *et al.* 2003; Schulze-Lefert 2004).

1.7 RNA interference (RNAi)

RNA interference refers to a mechanism that inhibits gene expression by causing the degradation of cellular mRNA molecules or preventing the transcription of specific genes. This mechanism begins when a gene that is homologous to an endogenous target gene is introduced into host cell, which can occur after virus infection or following gene transfer during transformation. Transcription of the introduced gene results in the formation of double- stranded RNA (dsRNA) which is cut into a smaller dsRNA species termed small interfering RNAs, siRNAs, by the RNAse III-like enzyme called "Dicer". The formation of siRNAs with about 20-25 bp in length is shared between the plants and animals during RNA silencing process. The SiRNAs associate with a protein complex termed "RNA–induced silencing complex" (RISC), which mediates the binding of one strand of siRNAs to messenger RNA (mRNA) transcribed from the native target gene. Homology-based recognition of unknown RNA initiates a series of events that results in disruption of the target gene. The RNA silencing story got started in plants following attempts to overexpressing the gene constructs encoding key enzymes in the anthocyanin biosynthesis pathway in transgenic petunia (Napoli *et al.* 1990; van der Krol *et al.* 1990). Contrary to expectation, the anthocyanin pigmentation

in the flowers of transformed plants was not enhanced. Interestingly, the flowers of transformed plants were de-pigmented, and significantly, endogenous gene mRNA transcript levels were significantly reduced because of suppressing of the transgene as well as the endogenous gene (Ruiz *et al.* 1998; Vaucheret *et al.* 1998; Jensen *et al.* 1999). Later, it was demonstrated that plants could target specific virus or mRNA sequences for degradation and this activity was the mechanism behind some examples of virus resistance in transgenic plants (Lindbo and Dougherty 1992). This process initially called RNA mediated virus resistance, or post-transcriptional gene silencing, is termed, now, RNAi. Additionally, it has been shown that there is a negative correlation between the infectivity of *Tobacco etches virus* (TEV) and the expression of the untranslatable coat protein of virus in transgenic plant. The rate of TEV coat protein expression decreased and transgenic plants were more resistant to this virus (Dougherty *et al.* 1994). This phenomenon usually occurs when virus genome possesses some similarities in RNA sequence with host plants genome. In cross-protection assays, it is proved that inoculation of plants with weak strains of virus protects the plant against highly virulenct viruses, which share a degree of sequence homology. Later, it was defined that this phenomenon is related to RNA silencing (Ratcliff *et al.* 1999; Voinnet 2001). This type of resistance in virus-infected plants operates at the RNA level, post-transcriptionally, and the silencing effect has been termed post-transcriptional gene silencing (PTGS).

To achieve RNA silencing of a gene full homologous interaction between the introduced gene and the target gene is not an absolute requirement. For instance, Voinnet *et al.* (1998) showed that introduction of a limited portion of the 5' or 3' end of the GFP coding region into stably transformed GFP expressing plants could successfully silence *GFP* gene. Remarkably, the homology required to initiate RNA silencing can be as short as 23 nucleotides (about 10% of the size of the target gene) (Thomas *et al.* 2001). Since discovery of RNA interference mechanism, scientists could silence several target genes via diverse methods in different organism such as nemathods, insects, bacteria, fungi and plants (Fire *et al.* 1998; Tavernarakis *et al.* 2000;

Bernstein *et al.* 2001; Wang *et al.* 2000; Tchurikov *et al.* 2000; Cogoni and Macino, 2000; Akashi *et al.* 2001). For instance, Silencing of the *NPR1* and *TGA* genes via virus-induced gene silencing (VIGS) technique in tomato enabled *Pseudomonas syringae* pv. *tomato* (*Pst*, DC3000), carrying the avirulence gene avrPto, to develop disease symptoms in the Rio Grande-PtoR (RG-PtoR) background, which shows, normally, Pto mediated resistance to this pathogene (Ekengren SK. 2003). In rice, silencing of Phytochelatin synthase gene *OsPCS1*, which is involved in cadmium accumulation, reduced cadmium content in rice seeds (Li *et al.* 2007). Rice and *Nicotiana attenuata* silencing of *NPR1* using RNAi led to a significant (50%) suppression of NPR1 transcript and, subsequently, increased susceptibility to pathogens and herbivore (Rayapuram and Baldwin 2007; Yuan *et al.* 2007). Additionally, it was documented that silencing of PR-13/thionin in *Nicotiana attenuate* increases susceptibility to *Pst*, DC 3000 (Rayapuram *et al.* 2008). An illustrative scheme of the molecular events in cell during RNA interference phenomenon is given in supplementary Fig. 2.

1.8 Objectives

The role of *NPR1* in disease resistance is well defined during past two decades, especially, in dicots such as *Arabidopsis* and tobacco. Even though, the function of *NPR1* has been considered in a few monocots like rice and wheat; and there is little information on the function of *NPR1* homologue in barley, *HvNH1*. On the other hand, many results showed that cell death suppressor *Bax inhibitor-1* in plants like barley is involved in susceptibility to biotrophic powdery mildew agent.

This study focuses on the functional analyses of these two distict genes, *HvBI-1* and *HvNH1* in barley-pathogen interactions. For that, transgenic barley plants for either overexpression of *HvBI-1* or silencing of *HvNH1* using RNAi strategy by *Agrobacterium*-mediated transformation were generated and different experiments, i.e., quantification of expression rate of *NH1* and *HvBI-1* in corresponding transgenic plants and different pathogens assays via macroscopic and microscopic techniques were

performed. Moreover, regulatory effects of *HvNH1* on cell death suppressor genes, *MLO* and *BI-1*, were inspected in *NH1* silenced plants. The effect of SAR inducer (BTH) on *NH1* silenced plants was examined, as well.

For a concise presentation of the information on these two different genes, the procedures and results of experiments regarding *HvNH1*-silenced barley will be elaborated in the coming pages, and the data on *HvBI-1* would be found in the published paper (Babaeizad *et al.* 2009).

2 MATERIALS AND METHODS

2.1 Plant and fungal materials

Transformation was carried out on barley (*Hordeum vulgare* L.) cultivar golden Promise (GP) (obtained from Prof. Steinbiss, MPI Cologne, Germany) grown in a climate chamber at 20°C/18°C (day/night) with 60% relative humidity and a photoperiod of 16 h with 240 µmol m^2 s^{-1} photon flux density.

Blumeria graminis f. sp. *hordei* (*Bgh*) race A6 was propagated in the same condition on GP plants. Wheat powdery mildew fungus (*Blumeria graminis* f. sp. *tritici*, *Bgt*) field isolate A95, which was gained near Aachen by Ulrich Beckhove was propagated in the same condition for *Bgh*. Induction of *F. graminearum* (strain WT 1003) conidiation was on synthetic nutrient agar (SNA) medium (Nirenberg, 1981) incubated at 18°C under near-UV and white light (TLD 36 W-08; TL 40 W-33 RS; Philips, Hamburg, Germany) with 12 hours photoperiod for 10–14 days. *Bipolaris sorokiniana* was propagated on SNA (containing Nitrocellulose filter paper) for 6-7 weeks at 25°C.

Bobwhite (Bob) wheat expressing *Arabidopsis thaliana NPR1* (*AtNPR1*) was kindly provided by Jyoti Shah (Division of Biology, Department of Plant Pathology, Kansas State University, USA).

2.2 Generation of transgenic barley plants

2.2.1 Construction of *GFP-BI-1* vector

BI-1 ORF (Hückelhoven *et al.* 2003) was amplified by PCR using the primers 5'-ggatcccaacgcgagcgcaggacaagc-3' (containing a *Bam*HI site) and 5'-gtcgacgcggtgacggtatctacatg-3' (containing a *Sal*1 site), and subsequently cloned into the *pGEM*-T vector (Promega, Mannheim, Germany). After sequence confirmation, the *Bam*HI-*Sal*I fragment was cloned into the expression vector *pGY*I (Schweizer *et al.* 1999). The GFP coding fragment was amplified using the oligonucleotides 5'-ggatccatggtgagcaagggcgag-3' (containing a *Bam*HI site) and 5'-ggatccttgtacagctcgtccat-

3’ (containing a *Bam*H1 site), which eliminates the stop codon, and was inserted in frame into *pGY*1-*BI-1* using the internal *Bam*HI site of the *BI-1* forward primer (i.e. at the N-terminal end of *BI-1*). For constitutive overexpression and for tagging expression, a cDNA fusion of *GFP* and *HvBI-1* was cloned by digestion of *pGY*1-*CaMV35S::GFP–HvBI-1* (Hüeckelhoven *et al*. 2003; Eichmann *et al*. 2004) into appropriate sites of the binary vector pLH6000 (DNA Cloning Service, Hamburg Germany; Fig. 3), which was, then, introduced into *Agrobacterium tumefaciens* strain *AGL1* (Lazo *et al*. 1991; Deshmukh et al. 2006). In this study transformation of barley (*Hordeum vulgare* cv. Golden Promise) was performed following Tingay *et al.* (1997) and Matthews *et al*. (2001). PCR analysis was performed to confirm integration of the transfer DNA using *PGY1*-frw2 and *GFP3'Bam*HI (as) (Fig. 3)**.** The GFP reporter protein was visualized with either a standard fluorescence microscope or a confocal laser scanning microscope.

2.2.2 Construction of *NH1*- RNA interference vector

The RNAi expression vector pJP26[1] was made by cloning the wheat *RGA2* intron in *pGY*1 between *Xba*I and *Sal*I sites of the multiple cloning sites. Then the NH1 fragment was cloned into the pJP26. Sense and antisense fragments were flanked by *Sma*I and *Bam*HI as well as *Spe*I and *Sph*I restriction sites, respectively. The subsequent cloning was carried out after sequence confirmation. The sense and antisense fragments sequences are shown in supplementary table 5.

To knock down the barley *NH1,* RNAi construct under the control of constitutive *CaMV35S* promoter was cloned into the plant transformation binary vector (pLH6000-*35S::NH1*-RNAi, Fig.1), which was introduced into *Agrobacterium tumefaciens* strain *AGL-1* (Lazo *et al*. 1991). In this study transformation of barley (*Hordeum vulgare* cv. Golden Promise) was performed following Tingay *et al.* (1997) and Matthews *et al*. (2001). The transgenic plants were selected on hygromycin-containing (50 mg/L) medium. PCR analysis was performed to confirm integration of the transfer DNA using *PGY1*-frw2 and *BamH1npr1*primers (Fig. 2).

[1] http://apsjournals.apsnet.org/doi/pdf/10.1094/MPMI.2004.17.1.109?cookieSet=1

2.2.3 *Agrobacterium*-mediated transformation

Constructs were introduced into the *Agrobacterium* AGL-1 (Lazo *et al.* 1991) and LBA4404 strains through electroporation (*E. coli* Pulser, Biorad, USA). *Agrobacterium tumefaciens*-mediated transformation, selection and regeneration of barley were performed as previously described by Tingay *et al.* (1997). Barley immature embryos were used for transformation. After 2 days co-culture of embryos and transformed *Agrobacterium* on callus induction medium in darkness, putative transformed embryos were transferred onto callus selection medium containing 150 mgL^{-1} Ticarcillin and 50 mgL^{-1} hygromycin B (Roche, Germany). Having done twice sub-culture with 2-week interval, the survived calluses were transferred onto regeneration medium containing 150 mgL^{-1} Ticarcillin and 25 mgL^{-1} hygromycin B. Regenerated plantlets were transferred into root induction medium supplemented with 75 mgL^{-1} Ticarcillin and 12.5 mgL^{-1} hygromycin B. Finally, rooted plants (T_0 plants) were transferred into sand: ceramics (1:2 v/v) under cover for 48 hours in acclimatization room. Three weeks later transgenic plants were transferred to greenhouse with the temperature ranged from 20 to 28°C and uncontrolled humidity. Transformants were selected by PCR check using gene specific primers (Fig 2 and 3). The results of *GFPHvBI-1*, *HvNPR1*-RNAi genes and *GFP* under control of CaMV*35S* promoter transformation are presented in supplementary tables 1and 2.

Barley callus induction medium (1L)

MS-stock (Duchefa M0221)	4.3 g
$CuSO_4.5H_2O$	1.2 mg (5 μM)
Maltose	30 g
Thiamine HCl	1 mg
Myo-inositol	250mg
Casein hydrosylate	1 g
L-Proline	690 mg
2,4-Dichlorophenoxyacetic acid (2,4-D)	2.5 mg
Phytoagar	6 g

pH: 5.9, Filter sterilization

Barley regeneration medium (1 L)

MS-stock (Duchefa M0238, NH_4NO_3 free)	2.7 g
$CuSO_4.5H_2O$	1.2 mg (5 μM)
NH_4NO_3	165 mg
Maltose	62 g
Thiamine HCl	0.4 mg
Myo-inositol	100 mg
Glutamine	150 mg
6-Benzylamino purine (BAP)	1 mg
Phytoagar	6 g

pH: 5.6, Filter sterilization

Barley root induction medium (1L)

MS-stock (Duchefa M0221)	2.15 g
$CuSO_4.5H_2O$	0.6 mg (2.5 µM)
Maltose	15 g
Thiamine HCl	0.5 mg
Myo-inositol	125 mg
Casein hydrosylate	0.5 g
L-Proline	345 mg
Phytoagar	6 g

pH: 5.9, Filter sterilization

2.3 Plant susceptibility bioassay

2.3.1 Powdery mildew (*Blumeria graminis* f. sp. *hordei*)

For powdery mildew (*Bgh*) assay to consider the rate of susceptibility and finding the most promising lines, surface-sterilized transgenic seeds by sodium hypochlorite solution containing 6% active chlorine (2 hours) were first germinated for 3 days on filter paper and then transplanted in soil (Fruhsorfer Erde, Hawita Gruppe, Vechta, Germany) and grown in a climate chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (240 µmol m^2 s^{-1} photon flux density).

After 7 days, first leaf segments were placed on 0.5% (w/v) water agar containing 20mg/L benzimidazole (Merk schunchardt, Munich, Germany) and, next, inoculated with *Bgh* spores (5 conidia per mm^2 density) by air current dispersion in an inoculation tower and saved in the same climate chamber for 6 days. Subsequently, the number of colonies was counted under binocular filed on the same size (2.5 cm^2) of segmented leaf. The means of colony number in 20 plants of each wild type, negative and positive segregants are presented in Fig. 4.A.

In the case of *Ubi1:AtNPR1*-expressing wheat, WT and transgenic plants were inoculated with *Blumeria graminis* f. sp. *tritici* (*Bgt*), accordingly**.** The means of colony number in 20 plants of each WT and transgenic lines are presented in Fig. 4.B.

2.3.2 *Fusarium graminearum* root rot

To test the resistance of transgenic barley to *F. graminearum* root rot, WT, *NH1* silenced line and one negative segregant line were surface-sterilized in 6% sodium hypochlorite for 2 h and rinsed husk free seeds were laid on sterile water-soaked filter paper to germination. Three-day-old seedlings were used for inoculation by *F. graminearum* spore solution (2.5×10^4 spores ml^{-1}), (Babaeizad *et al.*, 2009) and in parallel in 1:50000 Tween 20:water (v/v) and 0.5% Gelatin (w/v) solution as mock treatment for two hours. Inoculated seedlings were transplanted into a 2:1 mixture of expanded clay (Seramis®, Master foods) and Oil Dri® (Damolin) in a growth chamber at 22/18ºC day/night cycle with 60% relative humidity and a photoperiod of 16 h (photon flux density of 240 µmol m^{-2} s^{-1}), and fertilized weekly with 20 ml of 0.1% Wuxal top N solution (Schering, N/P/K: 12/4/6).

Afterward, shoot length and weight were measured, and root samples for quantitative measurement of fungal DNA, representative of fungal biomass were taken for DNA isolation. Two independent experiments were performed.

2.3.3 Assessment of plants with *Bipolaris sorokiniana*

To assess the rate of susceptibility of *NH1*-silenced plants to *Bipolaris sorokiniana* surface-sterilized transgenic seeds (as described above) were first germinated for 3 days on water-soaked filter paper and then grown in soil (Fruhsorfer Erde, Hawita Gruppe, vechta, Germany) and kept in climate chamber (Percival scientific, Boone, Iowa, USA) with 16 h light (22°C) and 60% relative humidity. After 7 days, first leaf segments were placed on 0.5% (w/v) water agar containing 20 mg/L benzimidazole and then inoculated by spraying of *B. sorokiniana* spore solution in sterile dionized water containing 2×10^{-3} % Tween20 (with density of 20.000 spores per ml). Consequently, 60 hai the number of

germinated spores developing local lesion was counted under binocular field on the same size of segmented leaves. The means of germinated spores with deveöoping local lesion in 15 individuals of each wild type, negative and positive segregant lines are presented in Fig. 6.

2.4 Histochemical studies of transgenic barley-*Bgh* interaction

To inspect the probable changes in the interaction of *Bgh* and transgenic barley lines' epidermal cells, corresponding primary leaves were inoculated 7 days after germination with *Bgh* spores (with density of 20 conidia per mm^2) by air current dispersion in an inoculation tower and incubated in above-mentioned condition. Histochemical staining was done via 1 mg/ml 3, 3-Diaminobenzidine (DAB) dissolved in water (pH 4, HCl) as described by Hückelhoven *et al.* (2000) and Thordal-Christensen *et al.* (1997). Thirty hours after inoculation bases of leaves were cut off and submersed in solution containing 1mg/ml DAB for 5 h. Afterward, leaves were etiolated in 0.15% trichloroacetic acid dissolved in ethanol: chloroform (4:1 v/v) for 24 h. Etiolated leaves were stored in 50% Glycerol for succeeding microscopy. Fungal structures staining and microscopy were made as described by Hückelhoven and Kogel (1998). Under UV and bright field, the different interactions (haustorium with elongated secondary hyphae, papilla and HR) were counted. Penetration was considered by the detection of initial haustorium.

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

2.5 BTH treatment to induce *Bgh* resistance in *NH1* silenced barley

As it was reported before, salicylic acid and its analogues have positive effect on NPR1 elevation, SAR induction, and disease resistance in plants (Cao *et al.* 1994; Delaney *et al.* 1995; Shah *et al.* 1997). To check the effect of BTH on *NH1* silenced barley plants,(Benzo 1,2,3-thiadiazole-7-carbothioic acid S-methyl ester), 2 days old synchronized germinated transgenic and wild type seeds were grown in 200 gram capacity pots in soil (Fruhsorfer Erde, Hawita Gruppe, vechta, Germany) and kept in climate chamber (Percival scientific, Boone, Iowa, USA) with 16h light and 60% relative humidity (22/18°C day/night cycle) and a photoperiod of 16 h (240 µmol m-2 s-1 photon flux density). Afterwards, 10 ml of 20 ppm BTH (CGA245704, Bion®, Novartis, Basel, Switzerland) formulated as 50% active ingredient with wettable powder (WP) in water was applied to 5-day-old seedlings as soil drench. Control plants were treated with WP. Two days after BTH treatment, first leaf segments were placed on 0.5% (w/v) water agar containing 20 mg/L benzimidazole and, then, inoculated with *Bgh* spores (5 conidia per mm^2 density) as described before and saved in the same climate chamber for 7 days. Then, *Bgh* colony number was counted under binocular filed. The means of colonies in 25 individuals of each wild type, negative and positive segregating lines in treated and control plants are given in Fig. 12.

2.6 RNA extraction and reverse transcription

Leaf samples were grind into fine powder in liquid nitrogen. Total RNA was, then, extracted by Qiagen RNeasy Kit (Qiagen, Hilden, Germany) and the integrity of RNA was examined by electrophoresis on a standard gel. DNA was removed using 1µl DNaseI per µg sample RNA. One µg of RNA was reverse-transcribed using the Promega SuperScript II Reverse Transcriptase Kit (Promega, Mannhein, Germany) according to the manufacturer's instruction. This cDNA was diluted 5-fold and used for genes analyses by quantitative PCR.

2.7 Quantitative assays via real time PCR

2.7.1 Gene expression assays

In the case of *NH1*, *PR1b*, *PR2*, *PR5*, *BI-1* and *MLO* genes, the expression was determined in *Bgh* challenged 7 days old plants using the $2^{-\Delta Ct}$ method (Livak *et al* 2001). Cycles of threshold (Ct) values were generated by deducting the raw Ct values of the candidate genes from the respective raw Ct values of plant-specific ubiquitin. Amplifications were performed in 10 µl of SYBR green JumpStart *Taq* ReadyMix (Sigma–Aldrich, Munich, Germany) with 10 pmol oligonucleotides, using an Mx3000P thermal cycler (Strata gene, La Jolla, CA).

After an initial denaturation step at 95°C for 7 min, 32 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 82°C for 15 s) were performed, and three fluorescent reading were detected at 72°C (once) and at 82°C (twice) in each cycle. Respective melting curves were determined at the end of cycling to ensure amplification of only a single PCR product. Ct values were determined with the Mx3000P V2 software supplied with the instrument.

2.7.2 Genomic DNA isolation and Real-Time PCR

For determination of root colonization by *F. graminearum*, roots of 5 plants were harvested, pooled, frozen and crushed in liquid nitrogen and sampled in 2 ml polypropylene tube. The following DNA isolation was performed according to the manufacturer's instructions (Plant DNeasy Kit). For quantitative PCR, 5-ng of total DNA was used. Amplifications were performed in 10 µl of SYBR green JumpStart *Taq* ReadyMix with 350 nM oligonucleotides, using an Mx3000P thermal cycler. Thermal profile and fluorescence detection were as described above. Ct values were generated by subtracting the raw Ct values of the *F. graminearum* obtained by specific primers Fg16N (Nicholson et al, 1998) from the raw Ct values of plant-specific ubiquitin.

2.7.3 Primers sequences

The sequences of oligonucleotides used in this study are listed in table 1.

Table 1 primer sequences

Primer name	Sequence
HvUbi-For	5'-CAGTAGTGGCGGTCGAAGTG-3'
***HvUbi*-Rev**	5'-ACCCTCGCCGACTACAACAT-3'
***pGY*I-for**	5′-CGTTCCAACCACGTCTTCAA-3′
BamH*I *npr1-rev	5'- GGATCCTAGCAGCGATGTGAAG-3'
***HvNH1*-for**	5'-CAGGTCGACAACCCTTTCAT-3'
***GFP3' BamH*I**	5′- GGATCCTTGTAGAGCTCGTCCAT-3′
***HvNH1*-rev**	5'-TAAATCCGGCAAGCAGTTTC-3'
***HvPR-1b*-for**	5'-GGACTACGACTACGGCTCCA-3
***HvPR-1b* -rev**	5'-GGCTCGTAGTTGCAGGTGAT-3'
***Hv PR-2*-for**	5'-TCTACAGGTCCAAGGGCATC-3
***Hv PR-2*-rev**	5'-CGGAGAGGTCAAAGAGT-3'
***Hv PR* 5 -for**	5'-TAGAGCTTGCAGCAATGTCGACC-3'
***Hv PR* 5 -rev**	5'-CCTGAGCCCAGCTCGAAG-3'
***HvBI-1* -for**	5'-GTCCCACCTCAAGCTCGTTT-3'
***HvBIr-1* -rev**	5'-ACCCTGTCACGAGGATGCTT-3'
***HvMLO* -for**	5'-TCTGCGTGTGGTAGCATTTC-3
***HvMLO* -rev**	5'-AGCCCAGCAACAAGTTCTTC-3'
***Hv BCI4*-for**	5'-TTTTCAAACGGAACAAGGATG-3'
***Hv BCI4*-rev**	5'-GGGTTGTTTGAGGAGGAAGG-3'
***Fg*16N-for**	5'-ACAGATGACAAGATTCAGGCACA-3'
***Fg*16N-rev**	5'-TTCTTTGACATCTGTTCAACCCA-3'

2.8 Statistical analyses

Statistical analyses were done using student's t-test of the Microsoft Excel of Microsoft Office software package for Windows.

3 RESULTS

3.1 Generation of transgenic plants and confirmation of trangene integration

For constitutive expression of *BI-1* in barley, the cDNA sequence of *HvBI-1* was cloned by digestion of *pGYI CaMV35S* (Hückelhoven *et al*. 2003; Eichmann *et al*. 2004) into appropriate sites of the binary vector pLH6000, which was then introduced into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*. 1991) and LBA4404 to transform barley cultivar GP as described before (Tingay *et al.* 1997; Matthews *et al*. 2001). Integration of *GFP::HvBI-1* into the barley genome was confirmed by PCR using *pGY*I and *GFP3' Bam*HI primers. As expected, the *BI-1* fragment of about 950 bp PCR product in agarose gel was detected in transformed barley lines (Fig. 3)**.** Additionally, the fluorescence of *GFP::HvBI-1* at the nuclear envelope and in the endoplasmic reticulum in all transgenic plants used for further analysis, were observed. Expression of GFP in transformed plants was proved by UV light under the normal fluorescence and confocal laser scanning microscopes.

In the case of *35S::NH1* (RNAi), the cDNA sequence of *HvNH1* (RNAi) was cloned downstream of *35S* promoter in the *pGY*I *CaMV35S* (Hückelhoven *et al*. 2003; Eichmann *et al*. 2004) and the resulting cassette was ligated into appropriate sites of the binary vector pLH6000, which was then introduced into *Agrobacterium tumefaciens* to transform the barley.

Integration of *35S::NH1* (RNAi) into the barley genome was confirmed via polymerase chain reaction (PCR) using of *pGY*I and *Bam*HI*npr1* primers. The expected PCR product was detected, in agarose gel, in transgenic plants (Fig. 2)**.** The transformation efficiency was different (5-27%) in three independent events. In the first two transformation events the embryos were obtained from the plants, which were grown in greenhouse; but in the third event, immature embryos were isolated from the plants grown in growth chamber with controlled conditions. In the case of *NH1* (RNAi) construct, the average rate of transformed embryos was 11.5 and 27% in first two and third transformation events, respectively. On the other hand, the transformation

efficiency with *BI-1* construct was 9.5 and 21% in first two and third transformation events, respectively by AGL1 strain. As well, in the case of transformation using LBA4404 strain, transformation efficiency was 8 and 14% in first two and third transformation events, respectively.

Fig. 1. Binary vector of pLH600 containing *the* fragment of *HvNH1* was used for RNAi construct under control of constitutive CaMV35S promoter. A conserved domain of *HvNH1* (AA 204 – 333) was used for construction of hairpin vector for transformation. This fragment was inserted in *Agrobacterium tumefaciens* disarmed Ti plasmid and used for transformation of immature embryos by *Agrobacterium*.

Hygromycine resistant plants were characterized to verify the *35S::NH1* (RNAi) insertion using PCR amplification (Fig. 2).

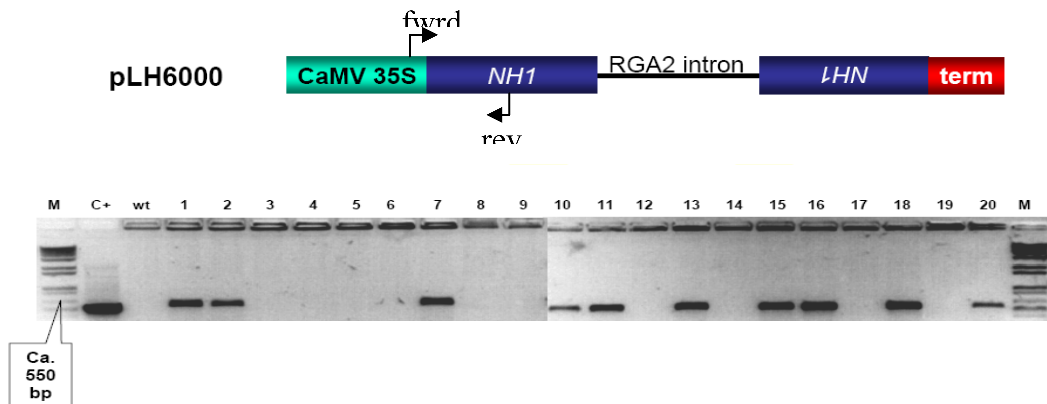

Fig. **2**. Molecular characterization of transgenic lines coding the *35S::NH1*-RNAi was evaluated by genomic amplification of an amplicon containing a part of 35S promoter and *NH1* gene using *pGY*1 and *Bam*H1*npr1* primers. Arrows denote the expected fragment of 550 bp. (**M**) molecular weight marker; (**C**+) plasmid as positive control; (**WT**) Wild-type Golden Promise barley; (**1-20**) putative transgenic plants.

For BI-1, several lines were analyzed to confirm the *35S::GFP::BI-1* insertion by corresponding primers using PCR amplification. (Fig. 3)

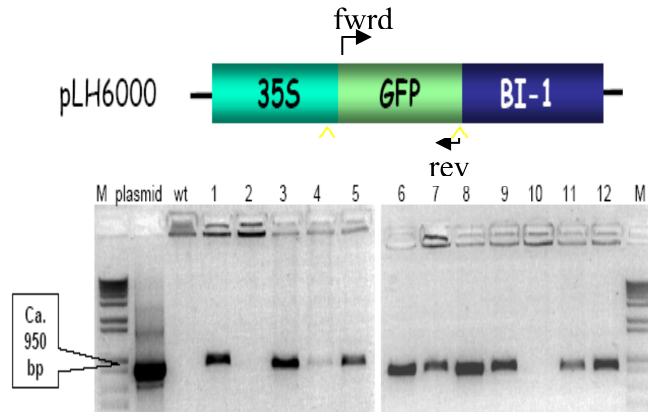

Fig. 3. Molecular characterization of transgenic lines coding *35S::GFP::BI-1* was evaluated by genomic amplification of 35S promoter using *pGY*1 and GFP coding part using *GFP3' Bam*H1 primers. Arrows denote the expected fragment of 950 bp. (**M**) molecular weight marker; (**C**+) plasmid as positive control; (**WT**) Wiled-type Golden promise barley; (**1-12**) putative transgenic plants.

3.2 Increased susceptibility of *NH1* silenced barley to powdery mildew infection

To assess the rate of *NH1*-silenced plants susceptibility to biotrophic pathogen, *Blumeria graminis* f. sp. *hordei* race A6, which is virulent on barley cv. GP, WT and some of *NH1* silenced lines were challenged with the pathogen. The corresponding azygous segregants from independent transformation event were used beside the parental GP as control. In response to *Bgh*, the rate of colonization in some of transgenic azygous control plants were almost identical to parental WT line. In contrast, E11L9 and E7L2 *NH1* silenced barley showed enhanced susceptibility to powdery mildew infection by an average of 29% and 33% when compared with either wild type parental lines or azygous individuals, respectively (Fig. 4. A). In contrast, in the case of *AtNPR1* expressor Bobwhite wheat, transgenic plants showed more resistance to powdery mildew infection by an average of 23 and 31% in 125A and 192D lines, respectively, when compared with WT parents (Fig. 4. B).

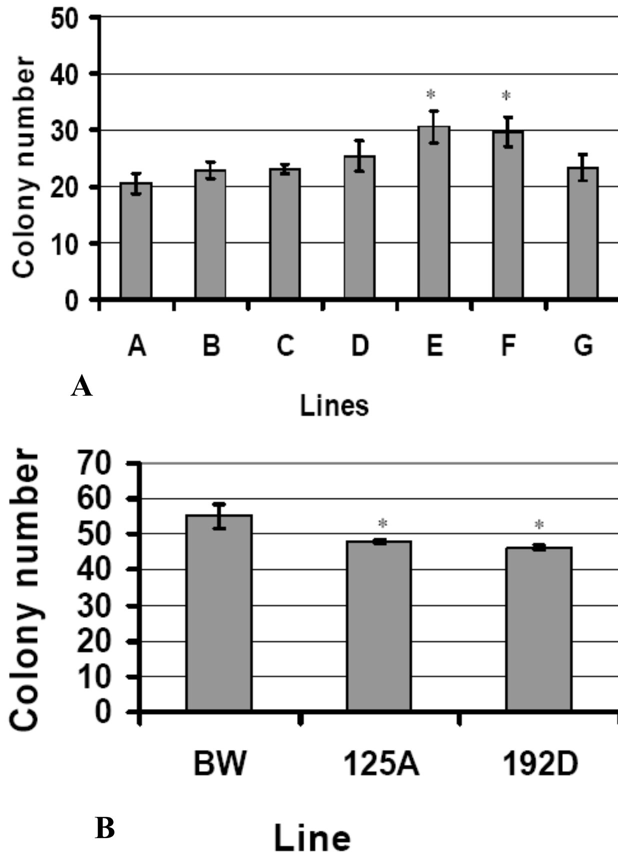

Fig. 4. Rate of susceptibility to powdery mildew. (**A**) The *35S::NH1* (RNAi) barley lines display enhanced susceptibility to fungal invasion and to *Bgh* colony formation. Fungal colonies at 6 days after inoculation of detached leaves of the parent cv. GP, azygous segregants and the *NH1* silenced plants from 5 individual transgenic lines. Error bars represent standard error of the mean in two different experiments. *, indicates P value <0.05 (t test) when compared with WT (control); Azygous plants, (A); WT, (B) Azygous, (C) E5L1, (D) E23L2, (E) E7L2, (F) E11L9 and (G) E23L8 lines.
(**B**) The *Ubi1::AtNPR1*enhanced resistance to *Bgt.* Twenty primary leaves from WT and two positive lines were selected and inoculated with *Bgt* (5 spores per mm^2). Average of fungus colony number (from 3 independent experiments) in transgenic lines (125A and 192D) was significantly reduced when analyzed statically with t test (*, P <0.05).

3.3 *Fusarium graminearum* root rot assessment of *NH1* silenced plants

Susceptibility to biotrophic pathogen is often accompanied by resistance to necrotrophs and vice versa. This provoked us to challenge *35S::NH1*-RNAi plants and corresponding controls with necrotrophic *F. graminearum*. Two weeks after inoculation, the shoot length was measured. Growth reductions of ~44% versus 30% were observed in respective WT and transgenic plants inoculated with *F. graminearum* compared with corresponding mock treated individuals. Biomass of fungus in root was determined by quantitative PCR of fungal DNA. Analyses of shoot length reduction and the *F. graminearum* biomass in infected roots revealed that there were not significant

differences between WT and transgenic line inoculated with *F. graminearum* (Fig. 5. A, B and C).

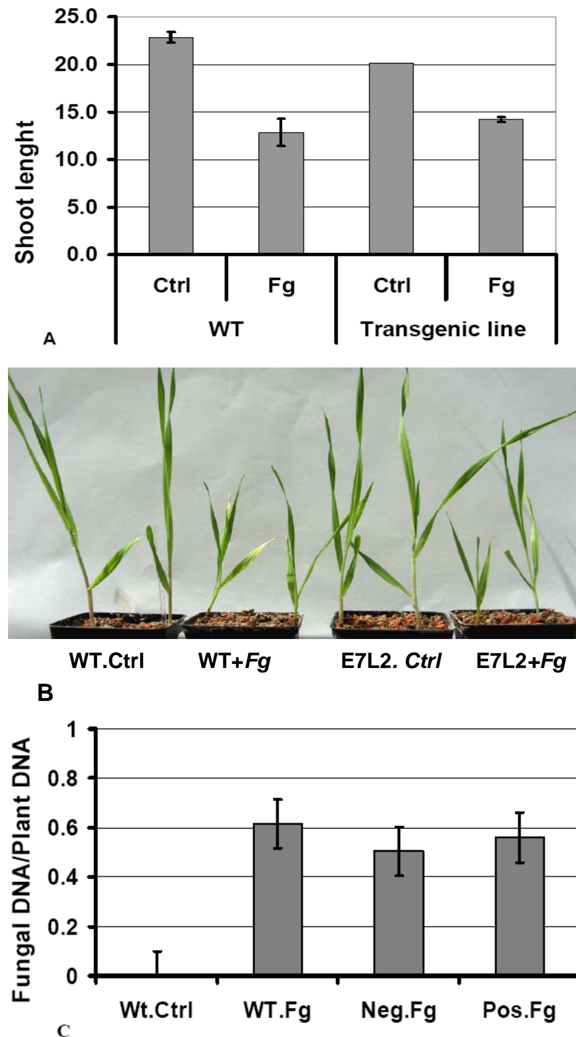

Fig. 5. Barley WT and *35S::NH1* (RNAi) plants show seedling susceptibility to *Fusarium graminearum*. (**A**) The average rate of shoot length of wild type barley and *35S::NH1* (RNAi) transgenic line at 14 days after inoculation were measured. Error bars represent standard errors of the mean in two biological repetitions. (**B**) Appearance of wild type and *NH1* silenced barley plants at 14 days after seedling inoculation with *F. graminearum*. (**C**) Quantitative PCR of fungal DNA in relation to plant DNA in roots at 14 days after inoculation with *F. graminearum*. statistical analysis with t test did not show significant differences among the lines to corresponding controls in both shoot length and *Fg* DNA biomass measurement.

3.4 Assessment of plants susceptibility with *Bipolaris sorokiniana*

For evaluation of transgenic plants susceptibility to necrotrophic pathogen *B. sorokiniana* first leaves of 7-day-old plants from WT, azygous and *NH1* silenced lines were inoculated with 2×10^{4} CFU ml^{-1} spore solution in sterile water with 2×10^{-3} % Tween 20. After 48 hours, penetrated spores, which were associated with necrotic

lesion were counted. The average rate of penetrated cells with necrotic lesions in two independent experiments did not show significant difference between WT, negative and positive lines (Fig. 6).

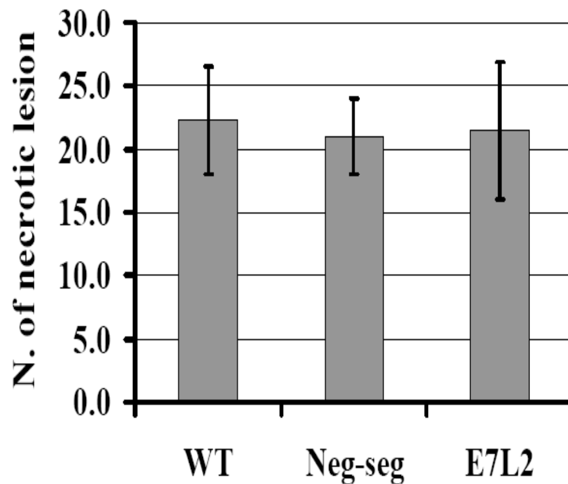

Fig. 6. The rate of susceptibility to *B. sorokiniana*. First leaves of 7-day-old plants of *NH1* silenced, azygous and WT lines were inoculated with *B. sorokiniana* spore solution (see methods). Two days after inoculation, the number of penetrated spores with developed necrotic lesion was counted. Data are the average rate of penetrated spores with necrotic lesions on wild type, *35S::NH1*-RNAi (E7L2) and azygous plants. Error bars represent standard errors of the means in two biological repetitions. Statistical analysis with t test did not show significant differences among the lines ($P>0.05$).

3.5 Histochemical analysis of the barley *Bgh* interaction by DAB staining

Impact of *NH1* silencing on the defense reactions typically exhibited by barley leaves in response to *Bgh* infection was investigated, histochemically. Microscopic inspection, via 3, 3-diaminobenzidine (DAB) staining, of penetrated cells producing haustorium with elongated secondary hyphae, papilla and HR interactions in A, B and C cells were conducted. The rate of penetrated cells increased by 72, 65 and 114% in respective A, B and C cells in *NH1* silenced plants compared that in with WT. In contrast, the rate of cells showing HR interaction in *NH1* silenced plants was suppressed by 23 and 32% in A and B cells, respectively. As well, 18, 26, 49% decreases in papilla numbers in respective A, B and C cells were observed in transgenic plants compared with that in control line (Fig. 7). Therefore, upsurge of penetration rate in *NH1* silenced plants was associated with decreased frequencies of cell wall appositions (CWA) underneath attempted penetration sites and hypersensitive response (HR), resulting more

penetration of attacked cells (Fig. 7). These findings verify that *NH1* has an important role in resistance of barley against biotrophic powdery mildew fungus.

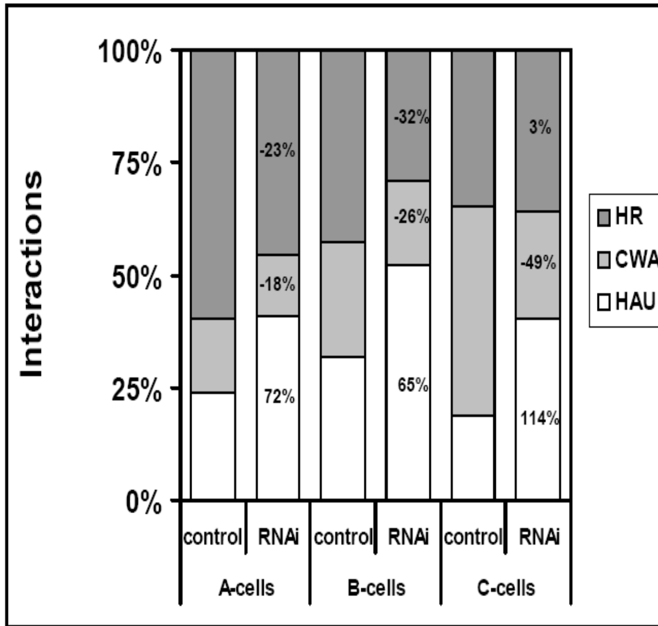

Fig. 7. Interaction phenotype of inoculated plants with *Bgh* at 30 h after inoculation. Primary leaves of E7L2 and WT were inoculated and stained, 30 hours later, with 3,3 diaminobenzidine for 4 h for detection of HR by whole cell DAB staining (see methods). Accessible cells allowed for fungal haustorium formation and development of elongated secondary hyphae (HAU+ESH), papillae (cell wall apposition), at sites of attempted penetration and hypersensitive response (HR) preventing the growth of secondary germ tube were counted by whole cell DAB staining. The experiment was repeated once with comparable results.

3.6 The rate of *NH1* transcripts attenuated in transgenic barley

To show the *NH1* silencing, the rate of *NH1* transcript levels in WT and transgenic plants were examined in both challenged and non-challenged plants with *Bgh*. The rate of *NH1* transcripts decreased in two *NH1* silenced lines E7L2 and E11L9 up to 27% and 38%, respectively, compared to WT in 7-day-old non-challenged plants (Fig. 8.A). On the other hand, plant responses to stress; 7-day-old *NH1* silenced and WT plants were inoculated with *Bgh* and expression levels of *NH1* were monitored at 0, 12, 24, and 48 hai. Expression rates of *NH1* at these time points was 2, 3.7, 2 and 1.8- fold more, respectively, in WT plants compared with those in transgenic plants.

The Rate of *NH1* transcript level in both challenged and non-challenged conditions in WT and transgenic lines were different to each others (Fig. 8).

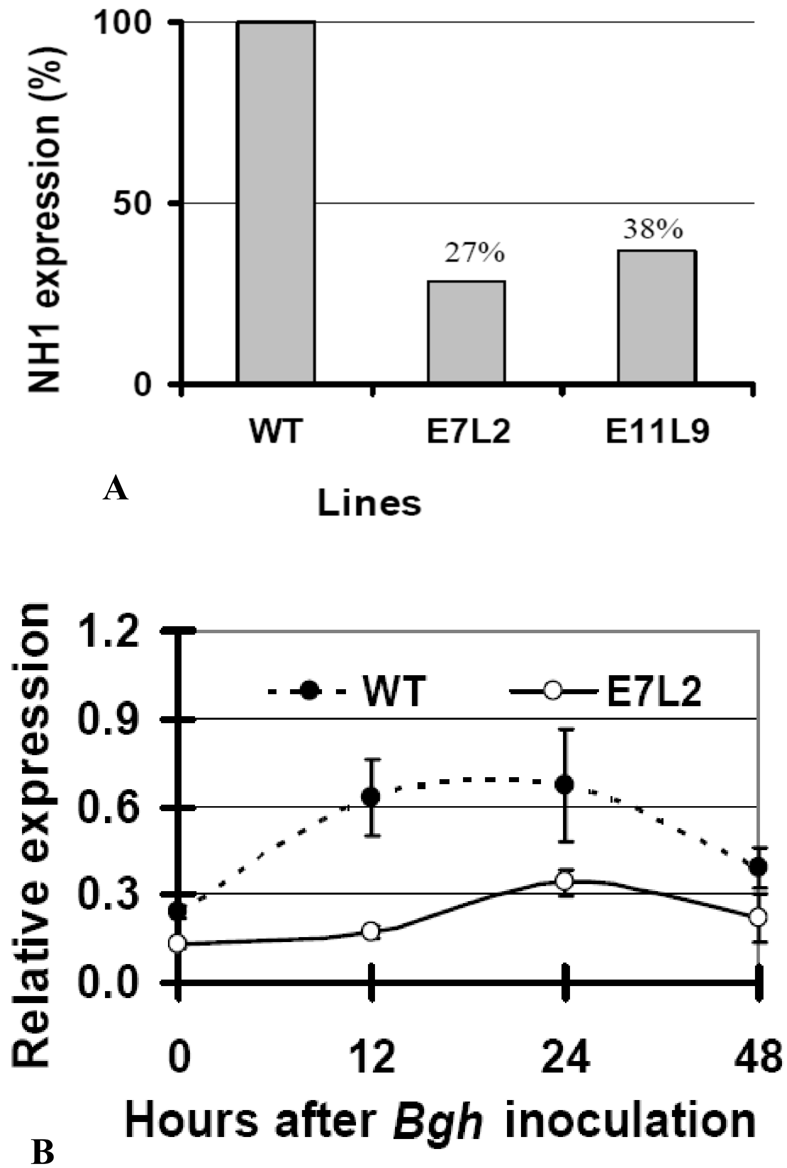

Fig. 8. (**A**) Rate of *NH1* transcript level in two different *35S::NH1*(RNAi) lines and WT barley in 7-day-old plants without pathogen challenge. As it is shown, the *NH1* transcript level was 27 and 38 percent of that in WT plant in E7L2 and E11L9, respectively. (**B**) Rate of *NH1* transcript levels in *NH1* silenced line E7L2, and WT barley. Seven-day-old plants were inoculated with *Bgh* conidia (20 spores/mm^2). As shown in the graph, the value of *NH1* transcript level (average of three independent experiments) was significantly different to that of WT at different time points after inoculation with *Bgh*.

3.7 Effect of *NH1* silencing on expression of pathogenesis-related genes during *Bgh* challenge

To demonstrate the *NH1* silencing effects on plant responses to biotic stress, 7-day-old *NH1* silenced (E7L2 line) and WT plants were inoculated with *Blumeria graminis* f. sp. *hordei* and expression levels of pathogenesis-related (*PR1b*, *PR2*, and *PR5*) genes were monitored at 0, 12, 24 and 48 hours after inoculation. Expression rates of *PR1b*, which is thought to be involved in barley resistance to powdery mildew and in systemic acquired resistance, was found to be induced 35.4-fold in WT at 24 hai, whereas in transgenic plants only a 15.3-fold induction was observed. Even though the expression of this gene leveled off after 24 hai in both lines, but its expression is 5-fold more in WT plants than that in transgenic plants at 48 hai. ß-1, 3- glucanase (*PR2*) that is able to degrade fungal cell walls and to release fragments which may act as endogenous elicitors and suppressors, in WT plants was induced 40.7-fold at 24 hour after *Bgh* attack, while only 18-fold in transgenic plants. The ecpression of this gene in transgenic line decreased harshly, thereafter; whilst it maintained high in WT plants. *PR5*, which is an antimicrobial protein, was induced about 20- and 10-fold at 24 hours after *Bgh* challenge in respective WT and transgenic *NH1* silenced plants. In both WT and transgenic lines expression collapsed to the same amount, at 48 hai. There were no significant differences in the expression of *PR* genes (*PR1b*, *PR2* and *PR5*) between WT and transgenic plants at 12 hai. The expression level of these *PR* genes at 12 hai was almost the as much as that in zero time point (Fig. 9).

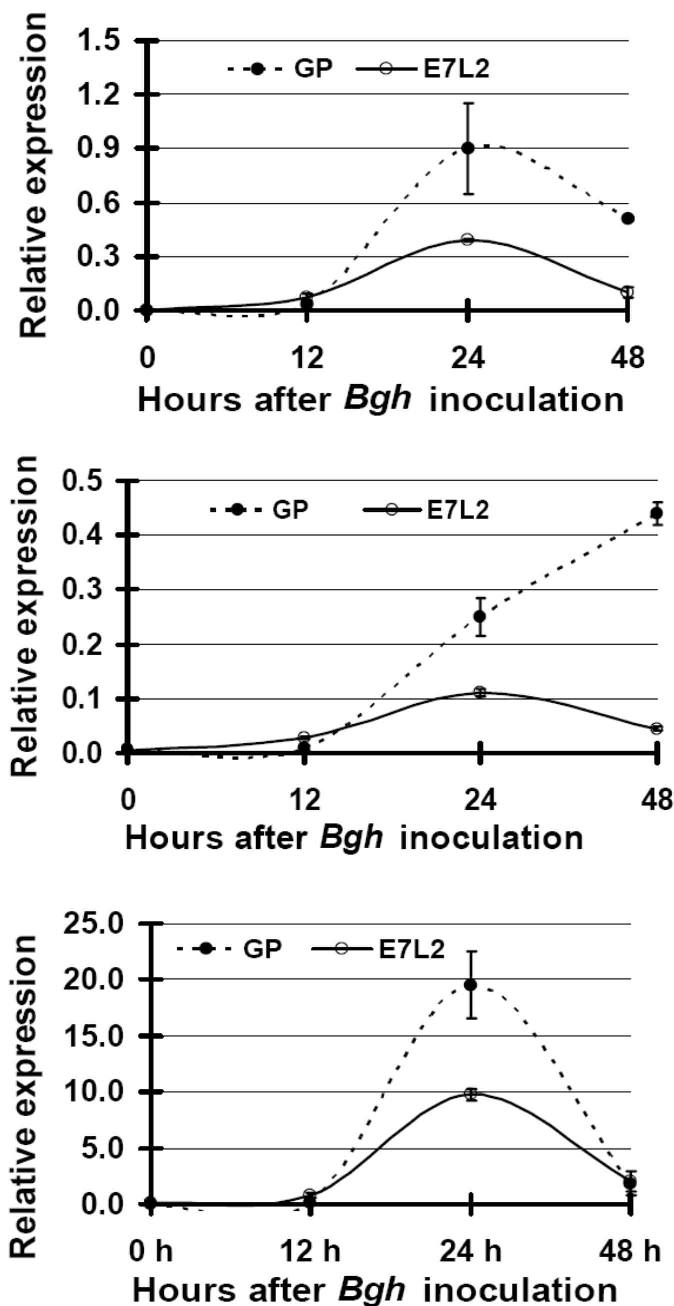

Fig. 9. Relative expression of *PR* genes in barley *NH1* silenced and WT plants after inoculation with *Bgh*. Amounts of specific transcripts were determined relatively to the expression of the *Ubiquitin* as internal control to the same time point transcript using Q-RT-PCR. Each value correspons to the pool of five leaves, assayed by Q-PCR in triplicate. Data are the means of two independent experiments. Y-axis represents relative expression of candidate genes expression in *Bgh* inoculated plants. Error bars show the standard errors of two experiments. upper panel: *PR1b*; middle panel: β-1, 3 glucanase (*PR2*); lower panel: Thaumatin-Like protein (*PR5*).

3.8 Effect of *NH1* silencing on expression of *BI-1* and *MLO* as cell death modulators

To study the relationship between NH1 and cell death modulators, we analyzed *HvBI-1* (*BAX INHIBITOR-1*) and barley Mildew resistance Locus O (*MLO*) genes expression in transgenic and WT plants. In the case of *BI-1* gene, there was no significant difference in *BI-1* gene expression level between WT and transgenic plants after *Bgh* challenge at different time points (Fig. 10). In contrast, *MLO* induction was augmented in *NH1* silenced plants compared with that in WT at 12 hai, which is critical for *Bgh* penetration (Fig. 10). Same pattern was, again, observed at 36 hai (Fig. 11). A detailed time course inspection of MLO transcript levels at 0, 4, 6, 10, 11, 12 hours after inoculation revealed respective 2.18-, 1.5-, 2.63-, 3.5-, 3.26-, 3.38-fold more induction of MLO in transgenic plants compared with those in WT (Fig. 11). These data indicate that barley *NPR1* homologue, *HvNH1*, has regulatory effect on MLO as if its suppression descends the activation threshold of MLO in pathogens challenge.

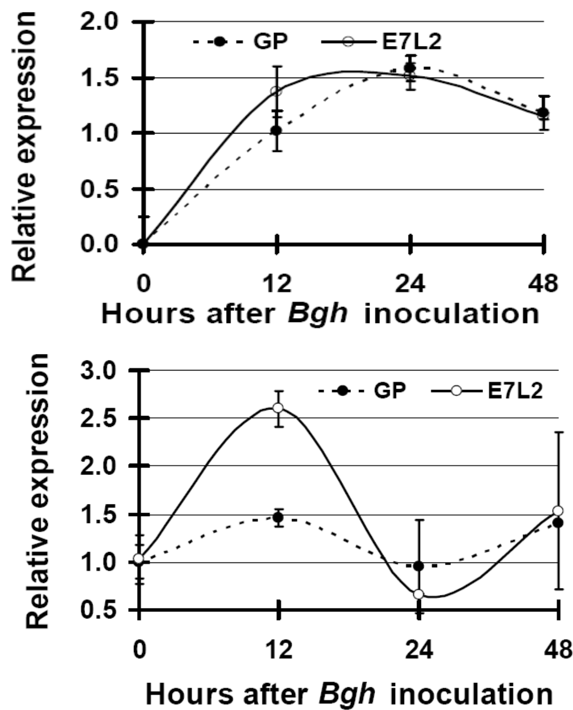

Fig. 10. Expression of cell death suppressors *HvBI-1* (upper panel) and *HvMLO* (lower panel) after inoculation of barley plants with *Bgh*. Amounts of specific transcripts were determined relatively to the expression of the *Ubiquitin* in corresponding time point transcript using Q-RT-PCR. Each value correspons to the pool of five leaves, assayed by Q-PCR in triplicate. Similar results were obtained in an independent experiment. Y-axis represents relative amounts of *BI-1* and *MLO* genes expression. In the case of *HvBI-1* gene, there are no significant changes in its expression rate at different time points between transgenic line and WT plant. Rate of *MLO* transcripts level in response to *Bgh* in WT and *NH1* silenced barley lines, as shown, at 12 hai this gene induced significantly more in *NH1* silenced line than in WT plants.

Detailed time course consideration of *MLO* transcript level verified more expression of this gene in transgenic plants in compare to WT line.

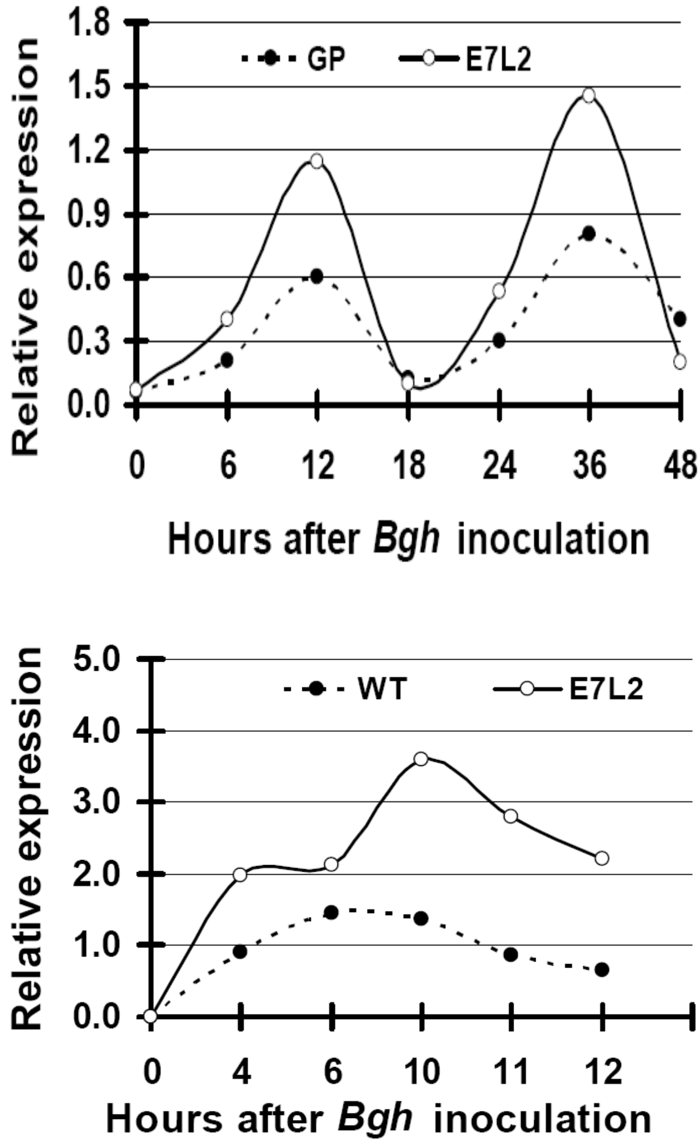

Fig.11. Expression level monitoring of *MLO* in WT and *NH1* silenced plants during challenge with powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*). Rate of *MLO* transcript level in WT and *NH1* silenced plant challenged with *Bgh,* elevated sharply at 12 and 36 hai that are two important time point for penetration of secondaery germ tubes into the epidermal cells of barley leaves (upper panel). In a more detailed time couse of first 12 hai, it has been documented that the *MLO* transcripts level in transgenic line is roughly double of those in WT plants (lower panel).

3.9 SAR induction by BTH in *NH1* silenced plants challenged with *Bgh*

To check whether or not BTH [Benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester)], as an *NPR1* inducer can restore or enhance *NH1* expression in *NH1* silenced plants and, therby confer resistance to *Bgh*, 1 mggr^{-1} (final concentration) of BTH solution was added to pots of 5-day-old plants. Two days after treatment, detached leaves of plants were inoculated with *Bgh* and 6 days later the colony number was counted. The average rate of colony number, in BTH treated WT and in negative segregating plants, reduced 48 and 37%, respectively, when compared with those in corresponding mock treated plants. In contrast, this reduction in *NH1* silenced plants was only 5.5% when compared with that of control treatment. Therefore, BTH could not induce SAR in *NH1* silenced plants.

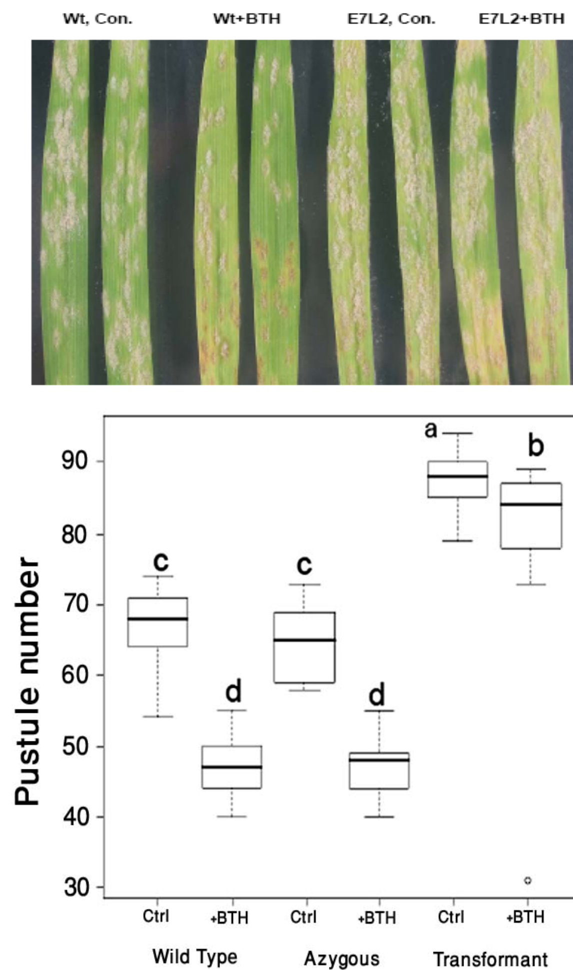

Fig.12. Effect of BTH on induction of resistance in WT and *NH1* silenced barley. Five-day-old plants were treated with 10 ml of 20 ppm BTH (CGA245704, Bion®, Novartis, Basel, Switzerland), formulated as 50% active ingredient with WP in water. Two days later, first leaves of mock and BTH treated plants were inoculated with *Bgh*, and 6 days after inoculation the colony number was determined. Resistance induction is displayed using the average of data from 20 plants. Upper panel: WT plants treated with BTH have shown increased resistance to *Bgh* when compared with WT mock plants. In contrast, there is not any significant changes in *NH1* silenced individuals treated with BTH (reduction: 5.5%) compared with mock treated counterparts. Lower panel: Analysis of the colony number in WT and transgenic barley treated and untreated with BTH; Different letters indicate statistically different groups. The error bars show the standard errors of values from two separate experiments.

4 DISCUSSION

4.1 Generation of *NH1*-silenced barley

In this study, we generated *NH1*-knockdown barley (cv. GP) lines using RNA interference (RNAi) approach to explore the resistance mechanisms that are dependent on *NH1* function in barley. The RNAi construct of *35SHvNH1* was transformed into *Agrobacterium tumefacience* disarmed T_i plasmid and transformed by co-cultivation of *Agrobacterium* and barley immature embryos. Presence of T-DNA was confirmed by PCR using specific primers for *35S* promoter and *NH1* gene, expecting the amplification of a 550 bp amplicon (Fig. 2). *NH1*-silenced plants (T_0) were propagated in greenhouse for obtaining the T_1 and T_2 generations, which were used for succeeding experiments. In transgenic barley plants, no visible detrimental phenotypic was observed during the vegetative growth in greenhouse compared with azygous and WT individuals. The transformation efficacy was different (5-27%) for both *NH1* and *BI-1* in three independent transformation events. In case of *NH1* (RNAi) construct, in two transformation events, the embryos from the plants that were grown in greenhouse with ususal agrochemical application, resulting in 11.5% transformation efficiency. In contrast, in third transformation event, using the embryos from untreated plants (pesticide and fungicide) grown in climate chamber, the transformation rate increased up to 27%. Moreover, the transformation rate for *BI-1* by *Agrobacterium* strain AGL1 was 9.5 and 21% for respective treated and untreated embryo donator plants compared with 8 and 14% for *Agrobacterium* strain LBA4404. In conclusion, it seems that the embryos isolated from untreated plants react properly to transformation procedures. This observation is in accordance with the recently published data indicating the importance of embryo quality as if well-maintained plants with no agrochemical application during growth produce effective embryos that show high competence for transformation (Bartlett *et al.* 2008).

In previous reports, efficiency of *Agrobacterium*-mediated barley transformation could reach to 5.4% (Lange *et al*. 2004), 2.6-6.7% (Shrawat *et al.* 2007), 2–12% (Matthews *et al*. 2001), 4.4-9.2% (Murray *et al.* 2004), and 21.7% (Hensel *et al.* 2008). In this study,

the transformation efficiency of GP line with *BI-1* gene was 14% using LBA4404 strain and 24% using AGL1 versus 16% in Solid CIMT medium (Hensel *et al.* 2008, see Supplementary table1). Additionally, transformation efficiency of 4% was achieved in one extra transformation event with GV3101 strain for *35S::GFP* construct, which is almost the as much as that achieved in GP plant (Murray *et al.* 2004).

4.2 *NH1* transcript is attenuated in transgenic barley

It has been shown that the expression of *NPR1* is blocked or attenuated in *npr1* mutants or in *NPR1*-silenced mono- and dicot plants (Cao *et al.* 1994; Yuan *et al.* 2007; Rayapuram and Baldwin 2007). In this study, to show whether *NH1*-silenced plants lack the *HvNH1* expression the transcript level of *NH1* was quantified during challenge with *Bgh*. In non-challenged condition, *NH1* transcript level showed 73 and 62% reduction in two most promising lines, E7L2 and E11L9, respectively, when compared with that in WT plants (Fig. 8.A). Throughout *Bgh* challenge, *NH1* transcript level in E7L2 transgenic line was significantly different from that in WT plants, i.e., 3.7-, 2-, and 1.8-fold less at respective 12, 24 and 48 hai (Fig. 8.B). Recently, it has been shown that silencing of *NPR1* by RNA interference in rice led to a severe attenuation of *NPR1* transcripts level in transgenic plants (Yuan *et al.* 2007). As well, it was reported that expression level of *NPR1* in *Nicotiana attenuate* was suppressed by ~50% in *NPR1*-silenced line in relation to WT plants (Rayapuram and Baldwin 2007). Accordingly in this study, silencing of *NH1* via RNA interference culminated in suppression of the *NH1* transcription.

4.3 Barley resistance to powdery mildew is dependent on *NH1*

Having confirmed the suppression of *NH1* in transformants, transgenic plants were challenged with obligate biotrophic *Blumeria graminis* f. sp. *hordei* (*Bgh*). Powdery mildew assay showed different rates of susceptibility in different *NH1*-silenced lines. However, *Bgh* bioassay revealed significant increase in susceptibility of two transgenic lines to the extents of 33 and 29%, when compared with azygous segregants (Fig. 4.A).

Additionally, wheat expressors of *AtNPR* (lines 125A; 192D), which were already shown to be more resistant against *F. graminearum* (Makandar *et al*. 2006), were analyzed. These transgenic lines represented significant resistance up to 23 and 31% when challenged with wheat powdery mildew, *Blumeria graminis* f. sp. *tritici* (*Bgt*) (Fig. 4.B). These findings extend the accuracy of earlier results achieved in dicots to monocots, which declare the involvement of *NPR1* in powdery mildew resistance (Cao *et al.* 1998; Vogel and Somerville 2000 and Reuber *et al.* 1998; Chern *et al*. 2001, 2005; Fitzgerald *et al.* 2004; Friedrich *et al.* 2001; Lin *et al*. 2004; Makandar *et al*. 2006; Malnoy *et al*. 2007).

4.4 Histochemical studies of barley-*Bgh* interaction revealed suppression of defense responses in *NH1*-silenced plants

Previous studies on *NPR1* function have revealed its regulatory mechanisms in plants, such as activation of SAR by induction and folding of some proteins during the SAR activation and induction of cell death in response to pathogen attacks (Cao *et al.* 1998; Makandar *et al*. 2006; Vitale and Denecke 1999*;* Trombetta and Parodi 2003; Fu and Kreibich 2000; Wang *et al.* 2005; Vanacker *et al.* 2001 and Chern *et al.* 2005).

Higher susceptibility to *Bgh* in *NH1*-silenced barley has been justified in association with suppression of plant defense responses. To check this hypothesis, the defense reactions during barley-*Bgh* interaction were monitored with DAB staining in WT and transgenic barley plants. Microscopic inspection showed that the frequencies of penetrated *Bgh* spores with developed haustorium in *NH1*-silenced plants increased 72, 65 and 114% in A, B and C leaf epidermal cells, respectively, compared with those in WT (Fig. 7). In contrast, the rate of hypersensitive response (HR) in transgenic plants reduced 23 and 32% in respective A and B cells, when compared with those in WT. Likewise, papilla formation decreased in transgenic plants by 18, 26 and 49% in respective A, B and C cells, compared with those in WT plants (Fig. 7). Hence, *NPR1* plays different roles concerning different defense responses in plants. As reported before in *Arabidopsis*, NPR1 as a key SA signaling protein is essential for regulating

defenses, cell death, and cell division (Vanacker *et al.* 2001). Besides, in *Arabidopsis npr1* mutants, which are partially blocked in SA signaling, cell death is reduced and delayed (Cao *et al.* 1994; Rate *et al.* 1999). *NPR1* positive regulatory function of cell death has been shown in rice by overexpression of rice *NPR1* homologue *NH1* (*NH1ox*) and expression of *AtNPR1*, that triggered a lesion-mimic/cell death (LMD) phenotype and, subsequently, conferred resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Chern *et al.* 2005; Fitzgeraled *et al.* 2004). It is well known that *NPR1* negatively regulates HR in *Arabidopsis* infected with *P. syringae* carrying avrRpm1 and, in contrast, positively regulates cell death in Aberrant Growth and Death 2 (agd2) and accelerated cell death (acd5 and acd6-1) mutants (Rate and Greenberg 2001; Greenberg *et al.* 2000 and Rate *et al.* 1999). Resembling rice and *Arabidopsis*, *NH1* in barley has positive regulatory effect on cell death in response to *Bgh* infection.

Expression of SAR-involved *PR* genes elevate at the time of papilla formation and HR in barley challenged with *Bgh* (Thordal-Christensen *et al.* 1992; Clark *et al.* 1994, 1995; Zhou *et al.* 1998). The role of PR1 proteins in effective papillae formation is verified in barley (Schultheiss *et al.* 2003; Gjetting *et al*. 2004). Present results suggest that silencing of *NH1* in barley, through direct or indirect effects, attenuates the expression *PR* genes and, in that way, leads to suppression of HR and papilla formation (Fig. 7).

4.5 *Pathogenesis-related* (*PR*) genes are downstream of *HvNH1*

It is well known that *NPR1* regulates the expression of *PR* genes in response to pathogens and other SAR inducers (Cao *et al.* 1994; Shah *et al.* 1997; Malnoy *et al*. 2007). Studies on *npr1* mutants and *NPR1*-silenced plants revealed the roles of *NPR1* as a battery of downstream *PR* genes (Cao *et al.* 1998; Shah *et al.* 1997; Rayapuram and Baldwin 2007; Yuan *et al.* 2007). To substantiate this role for barley *NH1*, PR genes expression during powdery mildew challenge was monitored in *NH1* silenced and WT plants. As shown in Fig. 9, the expression of *PR-1b*, *PR-2* and *PR-5* genes, which are previously shown to be involved in SAR did not change, considerably, in the first 12 hours after *Bgh* inoculation in both *NH1*-silenced and WT plants. Interestingly, at 24

hai, the expression of these genes reduced, significantly, in transgenic plants with regard to those in WT individuals. This retardation in *PR* genes activation during the first 12 hours after pathogen attack points out the dependency of *PR* genes expression on *NPR1*. Activation of NPR1 is accompanied by redox changes in attacked cells owing to NPR1 monomerization and subsequent nuclear localization (Mou *et al.* 2003; Kinkema *et al.* 2000, Tada *et al.* 2008) which *per se* postpones the *PR* genes induction. Except *PR-5* that was attenuated strongly at 48 hai, both *PR-1b* and *PR-2* were significantly different in their expression pattern (Fig. 9). In accordance with results of previous studies (Cao *et al.* 1994; Cao *et al.* 1998; Fitzgerald *et al.* 2004; Chern *et al*. 2001, 2005; Fitzgerald *et al.* 2004; Friedrich *et al.* 2001; Lin *et al*. 2004; Makandar *et al*. 2006; Malnoy *et al*. 2007), this observation validates the regulatory function of *HvNH1* on the expression of *PR* genes in response to pathogens. Consequently, silencing of this regulatory molecule in barley via RNA interference led to suppression of *PR* genes, which is in accordance with the recent reports in rice and tobacco that silencing of *NPR1*, using RNA interference, resulted in suppression of *PR-1b* expression (Rayapuram and Baldwin 2007; Yuan *et al.* 2007).

4.6 ***NH1* has negative regulatory effect on *MLO* expression but not on *BI-1***

It is plausible that any specific pathogen has evolved its own specific means to suppress and defeat general or specific plant host defense mechanisms. For instance, the causal agent of cowpea rust, *Uromyces vignae*, seems to hamper formation of thin cytoplasmic plasma membrane cell wall adhesion sites (Hechtian strands) for the host cell invasion (Mellersh and Heath 2001). In contrast, Hechtian strands formation was not decreased at penetrated sites of compatible or incompatible powdery mildew species, suggesting that an induced reduction in Hechtian strands at penetration sites might be a specific pathogenic strategy of the cowpea rust fungus (Mellersh and Heath 2001). Correspondingly, powdery mildew fungi appear to have specialized seven transmembrane domain protein (MLO), which is involved in repairing of cell wall against cell wall apposition and protecting cells from cell death induced by powdery

mildew stress (Panstruga 2005; Peterhänsel *et al.* 1997). MLO proteins play as a pathway for host cell entry. Barley plants lacking the functional MLO protein are almost completely resistant to penetration attempts by all strains of powdery mildew agent (Wolter *et al.* 1993; Peterhänsel *et al.* 1997; Schulze-Lefert and Vogel 2000). Loss of MLO function in barley leads to induced cell death, papillae formation and earlier senescence in mutated barley in response to pathogen and abiotic stress (Piffanelli *et al.* 2002; Jørgensen 1992; Peterhänsel *et al.* 1997). As well, it has been shown the rate of *PR* genes expression in susceptible *Mlo* plants is lower than that in *mlo-5* mutant line. This result indicates that *PR* genes are associated with *mlo* powdery mildew resistance (Peterhänsel *et al.* 1997) and, thus, there might be a relationship between *MLO* and *NPR1* as *PR* genes regulator, which was investigated in this study. On the other hand, like *BI-1* expressed in barley, wheat and rice, MLO expression led to restoring of susceptibility to powdery mildew in resistant barley *mlo5* mutants and to increasing of haustorium development of non-specific pathogen, *Bgt* (Kim *et al.* 2002b; Shirasu *et al.* 1999; Elliott *et al.* 2002; Hückelhoven *et al.* 2003; Eichmann *et al.* 2006). Interestingly, the rate of penetration in *MLO* expressor lines increased, significantly, when compared with *BI-1* expressing plants (Eichmann *et al.* 2006). Therefore, it seems that the MLO protein is more effective than BI-1 in suppression of defense response against *Bgh* in barley. Furthermore, it has been shown that reminiscent of RAC/ROP G-proteins, MLO is involved in the modulation of actin reorganization and cell polarity in barley-*Bgh* interaction. Some findings also showed that overexpression of RAC/ROP or MLO delay reorganization of actin filaments (AFs). As well, accumulation of AFs in response to *Bgh* in *mlo5* plants was much higher than that in *MLO*-barley (Opalski *et al.* 2005). Suspension of actin filaments reorganization by *MLO* causes interruption of AFs functions in vesicle delivery to the plasma membrane as well as local accumulation of phenolic compounds and H_2O_2, which hinge on intact actin cytoskeleton (Gross *et al.* 1993; Mellersh *et al.* 2002; Skalamera and Heath 1998). Therefore, MLO most probably support the formation of penetration barriers by recruiting defense-related products, specifically, to the sub-cellular site of fungal attack.

These findings substantiate that presence of the MLO protein is absolutely required for successful penetration, into the host cell wall, of the powdery mildew agent, *Bgh* (Panstruga 2005). The role of *NPR1* in plant disease resistance was identified well using *npr1* mutants and *NPR1*-silenced plants as if it increases the production of pathogenesis related (PR) proteins (Cao *et al.* 1998; Fitzgerald *et al.* 2004; Chern *et al*. 2001, 2005; Fitzgerald *et al.* 2004; Friedrich *et al.* 2001; Lin *et al*. 2004; Makandar *et al*. 2006; Malnoy *et al*. 2007). Appropriately, in this study, silencing of *HvNH1*, *NPR1* homolog in barley, led to significant elevation of MLO transcript in response to *Bgh* at 12 hai, which is a crucial time point for powdery mildew penetration (Fig. 10). This observation was confirmed in a more detailed time course, showing that MLO transcript was induced 2-, 2.6-, 3.3-, and 3.8-fold more in *NH1* silenced at 4, 10, 11, and 12 hai, respectively, compared with that in WT (Fig. 10). Therefore, silencing of *NH1* in barley increased rate of susceptibility of host plant not only by suppression of *PR* genes in association with SAR activation, HR and papillae formation, but also by regulation of MLO expression as a susceptibility factor to *Bgh*. As a result, beside recognition of *NH1* functions in disease resistance, results of this study demonstrate that *NH1* also regulates the *MLO* gene expression for managing the defense response against *Bgh*.

The negative regulatory function of *NH1* on *MLO* is, appropriately, mirrored in higher abundance of MLO transcript level in the first 12 hours of challenge with *Bgh* in *NH1*-silenced plants compared with that in WT counterparts. In other words, higher transcription of MLO gene in *NH1*-silenced plants is due to the constant low expression of *NH1*, while in WT plants *NH1* expression is induced 2.5-fold at 12 hai relatively to that of zero time point, which does not let the *MLO* gene be transcribed robustly.

As depicted in Fig. 10 and 11, in both WT and *NH1*-silenced barley, MLO transcript levels attenuate, gradually, after 12. Apparently, that attenuation corresponds to the rest time after formation of penetration peg from appresorial germ tube (Thordal-Christensen *et al.* 2000). Precisely, when penetration peg forces to breach the cell wall MLO induces, and levels off thereafter. Fittingly, the second peak of MLO expression at 36 hours after inoculation is interpreted in favor of the pressure of penetration peg

originated from elongated secondary hyphae to penetrate the neighboring cells. Then, MLO expression down-regulates at 48 hai, which the new penetration pegs, passed through the new infected cell walls (Fig. 11).

Regardless of negative regulatory effect of *NH1* on expression of *MLO* gene as a cell death regulator, results of this study revealed that silencing of *NH1* influences neither positively nor negatively the expression of *BI-1* gene (Fig. 10). Even though the *BI-1* gene transcription increased by 34% at 12 hai in transgenic plants compared with that in WT, but statistical analysis did not confirm significant differences in the rate of BI-1 expression in *NH1*-silenced and WT plants at different time points (Fig. 10). Overall, these results imply that cell death suppressor *BI-1* plays no role in cell death suppression in *NH1*-silenced barley as well as its function is independent of *NH1* in barley.

4.7 BTH failed to provoke disease resistance against *Bgh*

It is evident that salicylic acid and its analogues such as 2, 6-Dichloroisonicotinic acid (DCINA) and BTH act as inducers of NPR1. Those compounds increase the disease resistance by elevating of PR proteins production. This leads to induction of both local and systemic acquired resistances in a wide range of plant species in monocots and dicots (Kogel *et al.* 1994; Görlach *et al*. 1996; Beßer *et al*. 2000; Rohilla *et al.* 2002; Schweizer *et al.* 1999). On the other hand, these compounds are not able to activate *PR* genes expression in *Arabidopsis npr1* mutants and, thereby, fail to induce disease resistance (Cao *et al.* 1994; Delaney *et al.* 1995; Shah *et al.* 1997). To show the capability of BTH in induction of SAR in *NH1*-silenced barley plants, 5-day-old plants were treated with BTH, inoculated with *Bgh*, and then assayed for mildew colony formation, as described. The outcome showed that BTH induces disease resistance against powdery mildew agent, *Bgh*, in WT and azygous counterparts by 48 and 37%, respectively, compared with the relevant mock treated plants. This is in accordance with the prior findings that BTH can induce powdery mildew resistance in barley (Görlach et al. 1996). Quite the opposite, BTH was unable to breakdown the susceptibility to *Bgh* in

NH1-silenced plants. In fact, the average rate of colony number decreased 5.5% in transgenic plants in relation to corresponding control individuals (Fig. 12). In spite of statistically meaningful dissimilarity of BTH-treated and non-treated transgenic plants with regard to induced resistance, the difference, i.e., 5.5% is very low relatively to those observed in WT (48%) and negative segregants (37%). Therefore, in accordance with previous findings on *npr1*, *eds* (enhanced disease susceptibility) and *sai1* (salicylic acid–insensitive) *Arabidopsis* mutants, which are all involved in SA signaling pathway (Cao *et al.* 1994; Delaney *et al.* 1995; Shah *et al.* 1997), BTH did not succeed in activation of *NH1* and subsequent induction of SAR in *NH1*-silenced barley.

4.8 Influence of *NH1* silencing on barley interaction with hemibiotrophic and necrotrophic pathogens

There are several reports indicating that overexpression of *NPR1* confers disease resistance against broad range of biotrophic, necrotrophic and hemibiotrophic pathogens, namely, *Erysiphe chicoracearum, Fusarium graminearum*, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, *Xantomonas oryzae* pv. *oryzea*, *Peronospora parasitica*, *Erwinia amylovora* and *F. culmorum* on wheat, *Arabidopsis,* rice, apple and banana (Makandar et al., 2006, 2000; Cao *et al.* 1998; Chern *et al*. 2001, 2005; Fitzgerald *et al.* 2004; Friedrich *et al.* 2001; Lin *et al*. 2004; Cuzick *et al.* 2008). The resistance is accompanied by stronger and faster expression of *NPR1* and subsequently, elevation of pathogenesis related proteins translation (Cao *et al.* 1998; Friedrich *et al*. 2001; Makandar et al. 2006; Malnoy *et al*. 2007). In this study, to assess the susceptibility of transgenic plants to necrotrophic pathogen *F. graminearum* (*Fg*), the major casual agent of barley head blight and root rot diseases, 3-day-old germinated seedlings were dip-inoculated with fungal spore solution, incubated in climate chamber, and assayed for shoot length and biomass as well as fungal biomass in the infected root tissue (see materials and methods). Surprisingly, shoot growth reductions up to 44% versus 30% were observed in respective WT and transgenic plants upon inoculation with *F. graminearum* compared with corresponding mock treated individuals.

Moreover, shoot length reduction (descriptive of susceptibility to *Fg*) in transgenic plants was not significantly different from that in WT (Fig. 5.A and C). Additionally, in the case of hemibiotrophic pathogen *Bipolaris sorokiniana*, the incidence of spore penetration and necrotic lesion development was impossible to tell apart in *NH1*-silenced and WT barley during 48 hai (Fig. 6). Albeit silencing of *NH1* culminated in down-translation of PR proteins, it could not influence, meaningfully, the interaction of transgenic plants with *F. graminearum*, head blight and root rot agent as well as with *B. sorokiniana* , leaf spot agent.

Several reports have shown that the endogenous application of phytohormones salicylic acid (SA) and jasmonic acid (JA), whose levels increase on pathogen infection, induce separate sets of genes encoding antimicrobial proteins in different plants. As well, it is obvious that pathogen-inducible genes *PR-1*, *PR-2* and *PR-5* require SA-signaling for activation whereas the plant defensin gene *PDF* (*Protodermal factor*), along with a *PR-3* and *PR-4* genes, are activated by pathogens via an SA-independent but JA-dependent pathway (Glazebrook *et al.* 2005; Thomma *et al.* 1998, 2001; Jones and Dangl 2006; Van Loon and Van Strien 1999; de Wit 2007; Koornneef and Pieterse, 2008; Norman-Setterblad 2000). Previous findings showed SA induced SAR is effective against biotrophic pathogens, whereas JA/ET-mediated signal transduction provides resistance against necrotrophic fungi (Thomma *et al.* 2001). For instance, infection with necrotrophic fungus *Alternaria brassicicola* was shown to cause rapid increase of JA production in infected plant and induction of defensins (PDF 1.1 and PDF 1.2). Furthermore, defensins were shown to be inducing by exogenous application of JA but not SA (Thomma et al. 1998). It has been, also, reported that *coi1* mutation in *Arabidopsis*, which blocks JA signaling, severely compromised the resistance to *A. brassicicola* but had no effect on resistance to biotrophic *Peronospora parasitica* (Thomma *et al.* 1998). Conversely, the *npr1* mutant and transgenic expression of the bacterial gene salicylate hydroxylase (NahG), which blocks SA signaling resulted in loss of resistance to *P. parasitica* but had no effect on resistance to *A. brassicicola* (Thomma et al. 1998). These data point out to the existence of, at least, two separate

hormone-dependent defense pathways in *Arabidopsis* that contribute to resistance against distinct microbial pathogens.

A recent investigation on *Arabidopsis npr1* mutant, demonstrated that the development of hemibiotrophic *colletotrichum higginisianum* on detached leaves was identical in both WT and *npr1* mutant (Liu *et al.* 2007). As well, it was claimed that silencing of *NH1* in rice does not result in more susceptibility to the hemibiotrophic *Magnaporthe grisea* when compared with WT (Yuan *et al.* 2007). Accordingly, *NH1*-silenced barley does not represent any alteration in susceptibility rate to necrotrophic and hemibiotrophic pathogens, demonstrating that *NPR1* is unconvinced to be involved in plant interaction with necro- and hemibiotrophic fungi.

It is acknowledged that compromised *MLO* pathway in barley enhances the plant susceptibility to fungal pathogens like *M. grisea* and *B. sorokiniana* due to induction of cell death in mutated plants (Jarosch *et al.* 1999; Kumar *et al.* 2001). On the other hand, comprehensive examination of *F. graminearum* development on different cultivars of wheat and barley revealed that *mlo* lines were much more susceptible and the fungus developed deeper in kernel with regard to other genotypes (Jansen *et al.* 2005). These findings have proven the involvement of *MLO* gene in regulating of susceptibility to necrotrophic pathogens like *Fusarium* and *Bipolaris* as well as in biotrophic powdery mildew agent, *Bgh*. Hence, a perceptible interpretation is that the attenuation of *PR* genes, which might distress the resistance of *NH1*-silenced plants to necrotrophic fungi, is counterweighed by augmented *MLO* expression that *per se* inhibits the incidence of HR elicited by those pathogens, and, overall, no change is observed in the interaction of *NH1* knock-out plants with necrotrophic and hemibiotrophic fungi. Nevertheless, due to the fully susceptible background of barley cultivar GP (Babaeizad *et al.* 2009) that was used for generation of *NH1*-silenced plants, more data are needed to determine, definitely, the roles of *NH1* in susceptibility to necrotrophic and hemibiotrophic pathogens.

5 SUMMARY

In plant-pathogen interaction, both host and pathogen have evolved very sophisticated strategies to survive. Plants need to resist infection and pathogens require colonizing their hosts to attain nutrients for reproduction. Plants have evolved several mechanisms to resist pathogen invasion that consists of several defence layers. Meanwhile, *non-expressor* of *pathogenesis-related* genes *1* (*NPR1*) plays in the model plant Arabidopsis an essential role in systemic resistance against pathogen infection. Previous reports and recent findings have indicated a similar function for it's homologous in rice. The significant of *NPR1* in disease resistance have been proven by transient transformation of the Arabidopsis gen *AtNPR1* in barley (*Hordeum vulgare*)-powdery mildew (*Blumeria graminis* f.sp. *hordei, Bgh*) interaction. In this respect, silencing of the *NPR1* homologous gen *HvNH1* using RNA interference method led to suppress NH1 transcript up to 3.7-fold at 12 hours after inoculation (hai) in challenging to *Bgh*. As well, in transgenic lines, the expression levels of pathogenesis-related proteins (PR-1b, PR-2 and PR-5) attenuated between 50-100% in compare to WT barley at 24 hai. The rate of susceptibility to *Bgh* in *NH1*-silenced lines increased about 29-33%. In contrary, wheat expressing *AtNPR1* showed 31% increased resistance to *Blumeria graminis* f.sp. *tritici*. Histochemical observation showed suppression of hypersensitive response (HR) and cell wall apposition (CWA) by 28 and 22% in epidermal cells, respectively in *NH1*-silenced lines, resulted in increased fungal penetration rate. Application of systemic acquired resistance inducer, benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) induced up to 48% resistance against *Bgh* in WT plants, whereas this rate in transgenic plants was only 5.5%. Therefore, BTH failed in breaking down the susceptibility to *Bgh* in *NH1*-silenced line. Consistently, *MLO (*Mildew resistance Locus O*)* as a negative cell death regulator was induced up to 63-64% more in *NH1*-silenced lines than in WT plants at 12 and 36 hai, respectively. However, the expression level of *Bax inhibitor 1* as a cell death suppressor was not affected in *NH1*-silenced plant. On the other hand, *NH1*-silenced barley had shown no difference in susceptibility

to necrotrophic pathogen *Fusarium graminearum*, the causal agent of barley root rot. Similarly in response to leaf spot fungus, *Bipolaris sorokiniana*, the rate of spore penetration associated with necrotic lesions was not significantly changed in transgenic lines in compared with WT plants at 60 hai. The described phenotypes in *NH1*-silenced barley are inheritable and stable in different generations. In conclusion, silencing of *NH1* conferred susceptibility to *Bgh* but not to necrotrophic and hemibiotrophic fungi. The probable mechanisms behind *Bgh* susceptibility in *NH1*-silenced plants are MLO induction as well as suppression of PR proteins.

5 ZUSAMMENFASSUNG

Für Pflanze-Pathogen-Interaktionen haben beide Wechselwirkungspartner ausgeklügelte Überlebensstrategien entwickelt. Pflanzen müssen sich einer Infektion erwehren, wohingegen Pathogene ihre Wirtspflanzen erfolgreich befallen müssen um an die lebensnotwendigen Nährstoffen zu gelangen. Pflanzen haben diverse Schutzmechanismen gegen einen Pathogenbefall entwickelt. Für die systemische Resistenz gegen den Pathogenbefall spielt das Gen *non-expressor of pathogenesis-related genes 1* (*NPR1*) in der Modellpflanze Arabidopsis eine essenzielle Rolle. Frühere und neuste Forschungsergebnisse haben eine ähnliche Funktion für dessen Homolog in Reis gezeigt. Die Bedeutung von *NPR1* für die Krankheitsresistenz konnte mit Hilfe einer transienten Transformation des Arabidopsis Gens *AtNPR1* auch in der Gerste (*Hordeum vulgare)* - Echter Mehltau (*Blumeria graminis* f.sp. *hordei, Bgh*) Interaktion gezeigt werden. Übereinstimmend konnte in stabil transgenen Gerstenpflanzen durch eine RNA-Interferenz vermittelte Suppression des *NPR1*-homologen *HvNH1* Gens 12 Stunden nach der Inokulation (hai) mit *Bgh* eine 3.7-fache Unterdrückung der *NH1* Transkripte gezeigt werden. Ebenfalls konnte nach einer 24-stündigen Inokulation gezeigt werden, dass sich das Expressionsniveau der *pathogenesis-related* Proteine (PR-1b, PR2 und PR-5) in den transgenen Linien zwischen 50-100% im Vergleich zu der Wildtyp- (WT) Gerste bewegte. In den *NH1*-unterdrückten Linien konnte eine 29-33%ige Anfälligkeitszunahme gegen *Bgh* festgestellt werden. Im Gegensatz dazu zeigte *AtNPR1* exprimierender Weizen eine um 31% erhöhte Resistenz gegen *Blumeria graminis* f.sp. *tritici*. Histochemische Beobachtungen an den *NH1*-unterdrückten Linien wiesen eine Suppression der hypersensitiven Reaktion (HR) und von Zellwand Appositionen (CWA) in der Höhe von 28 und 22% in den epidermalen Zellen auf, die in einer erhöhten pilzlichen Penetration resultierten. Durch die Anwendung eines Induktors für die systemisch erworbene Resistenz, benzo-(1,2,3)-thiadiazole-7-carbothioic Säure S-Methylester (BTH), wurde in den WT Pflanzen eine 48%ige Resistenzerhöhung gegen *Bgh* erzielt,

wobei diese Erhöhung in den transgenen Pflanzen nur 5.5% betrag. Demzufolge konnte BTH in den *NH1*-unterdrückten Linien nur eine geringe Anfälligkeitsminderung gegen *Bgh* hervorrufen. Entsprechend konnte gezeigt werden, dass die Expression des Gens *MLO (Mehltauresistenz Locus O)* als ein negativer Zelltodregulator bis zu 63-64% höher nach 12 und 24 hai in den *NH1*-unterdrückten Linien induziert war als in den WT Pflanzen. Jedoch war in den *NH1*-unterdrückten Pflanzen das Expressionsniveau von *Bax Inhibitor* 1 als Zelltodsuppressor nicht beeinflusst. Auf der anderen Seite zeigten die *NH1*-unterdrückten Gerstenpflanzen keinen Unterschied in der Anfälligkeit gegen das nekrotrophe Pathogen *Fusarium graminearum*, das als kausaler Erreger der Wurzelfäule bei Gerste bekannt ist. Ähnliche Ergebnisse gab es mit dem Erreger der Braunfleckigkeit, *Bipolaris sorokiniana*. Hierbei gab es zum Zeitpunkt 60 hai in der mit nekrotischen Lessionen verbundenen Sporenpenetrationsrate keinen signifikanten Unterschied zwischen den transgenen und WT Pflanzen. Die beschriebenen Phänotypen von der *NH1*-unterdrückten Gerste sind vererbbar und stabil über mehrere Generationen. Zusammengefasst kann gesagt werden, dass die Unterdrückung des *NH1* Gens Anfälligkeit gegen *Bgh* verursacht aber nicht gegen nekrotrophe und heminekrotrophe Pilze. Die wahrscheinlichen Mechanismen der beobachteten *Bgh*-Anfälligkeit in den *NH1*-unterdrückten Pflanzen beruhen auf der *MLO* Induzierung wie auch auf der Unterdrückung der PR-Proteine.

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7 SUPPLEMENTARY DATA

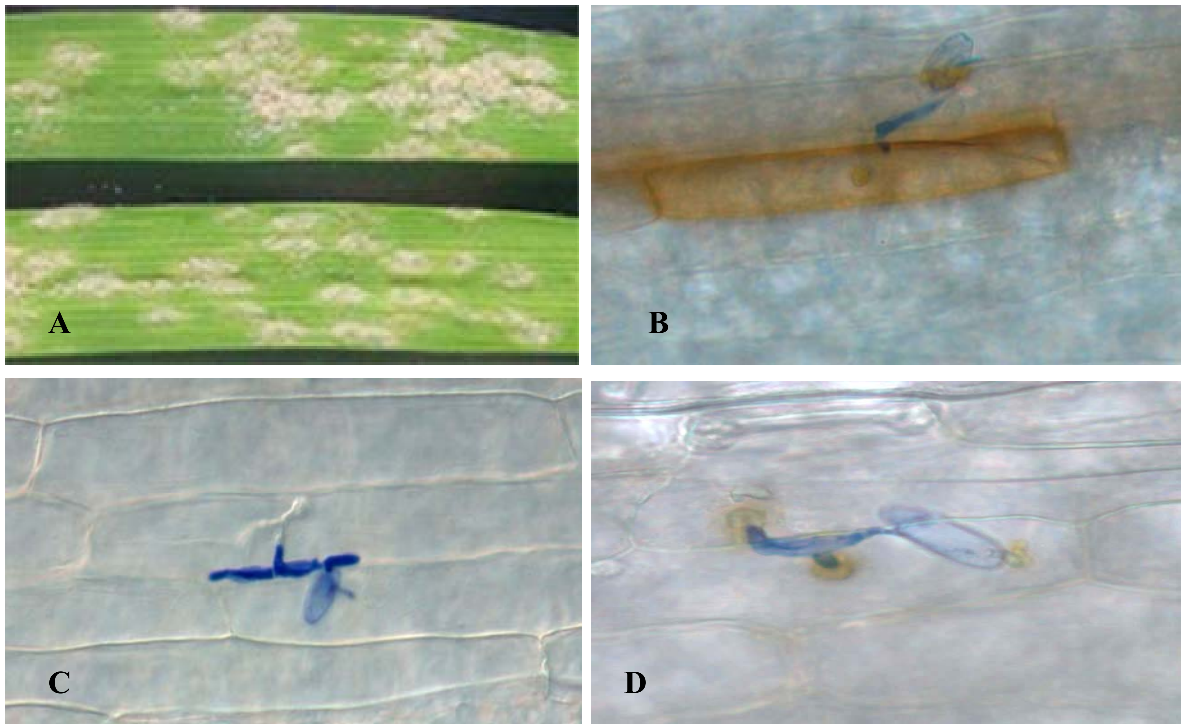


Fig. 1. Interaction of barley with the powdery mildew fungus (*Bgh* race A6). (A) Barley leaf segments showing symptoms of the powdery mildew disease. Surface mycelium and conidiophores form typical velvety pustules. (B) Hypersensitive response (HR) of a barley epidermal cell attacked by *Bgh*. Accumulation of brownish polymers indicating hydrogen peroxide production. (C) Haustorium within a barley epidermal cell continuing its extracellular growth by developing elongated secondary hyphae (ESH). (D) A cell wall apposition or papilla (PAP) preventing *Bgh* from penetrating the host epidermal cell.

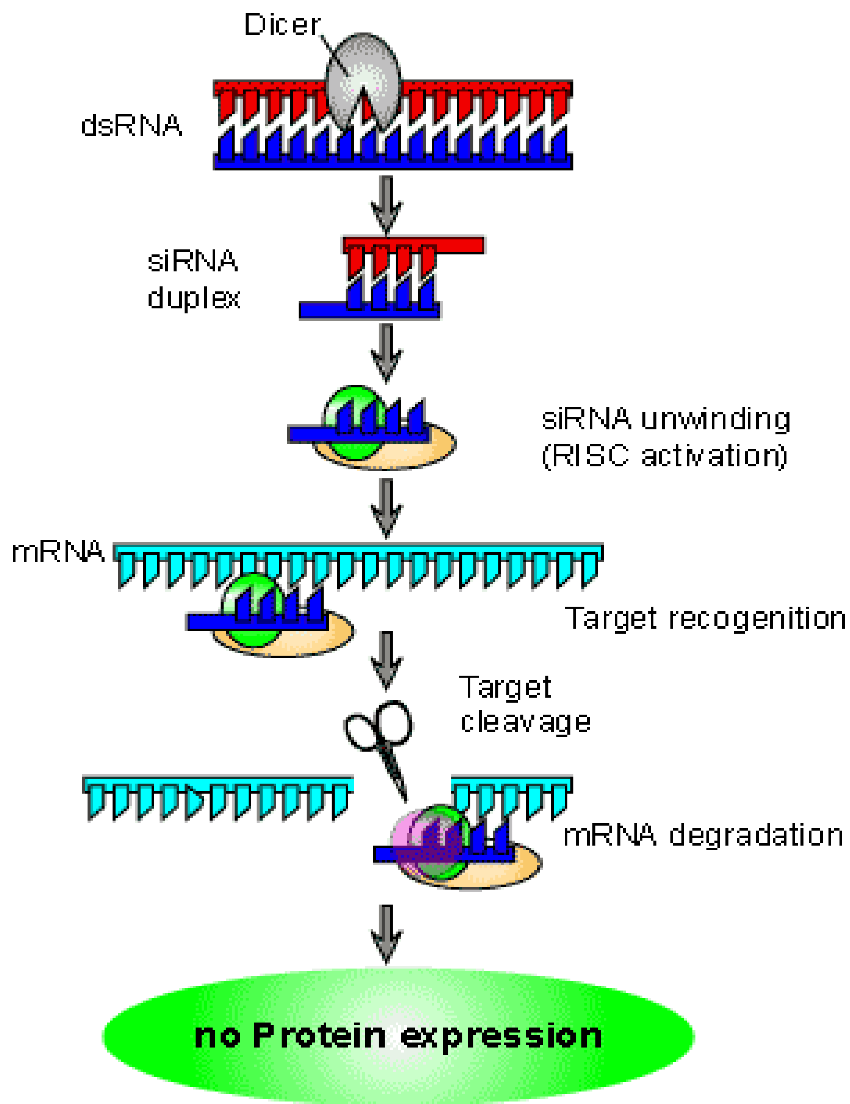
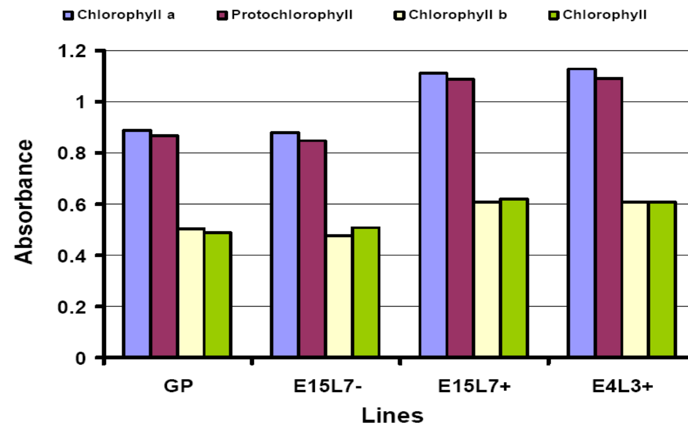
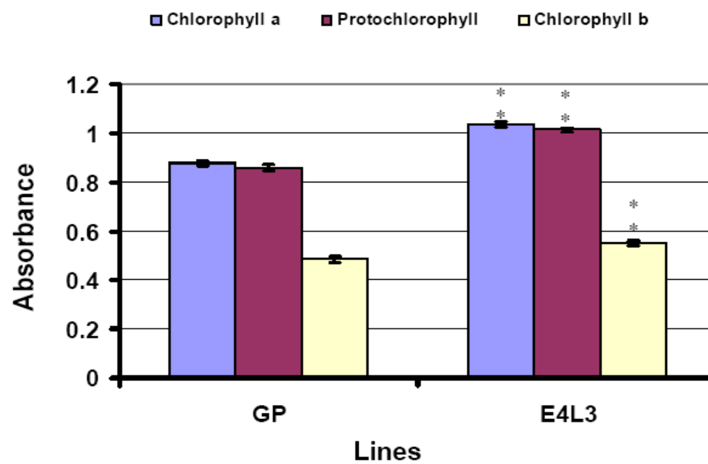


Fig. 2. Mechanism of action of RNAi. Double stranded RNA is introduced into a cell and gets sliced by the enzyme dicer to form siRNA. siRNA then binds to the RISC complex. The anitsense RNA complexed with RISC binds to its corresponding mRNA, which is cleaved by the enzyme slicer rendering it inactive. (for further details referto www.scq.ubc.ca/antisense-rna/).



A



B

Fig. 3. Chlorophyll content in WT and *HvBI-1* overexpressor lines. Seven-day-old plants were inoculated with dense *Bgh* spores, and after 48 hours leaves were sampled. Afterward, 10 disks (0.5 cm in diameter) of each sample were used for chlorophyll extraction using 5 ml of 80% acetone at 4°C and darkness. Different types of Chlorophylls were measured using Beckman UV-DU 7400 Spectrophotometer with following wavelengts:

432 nm	435 nm	459 nm	664 nm
Chlorophyll a	Protochlorophyll	Chlorophyll b	Total Chlorophyll

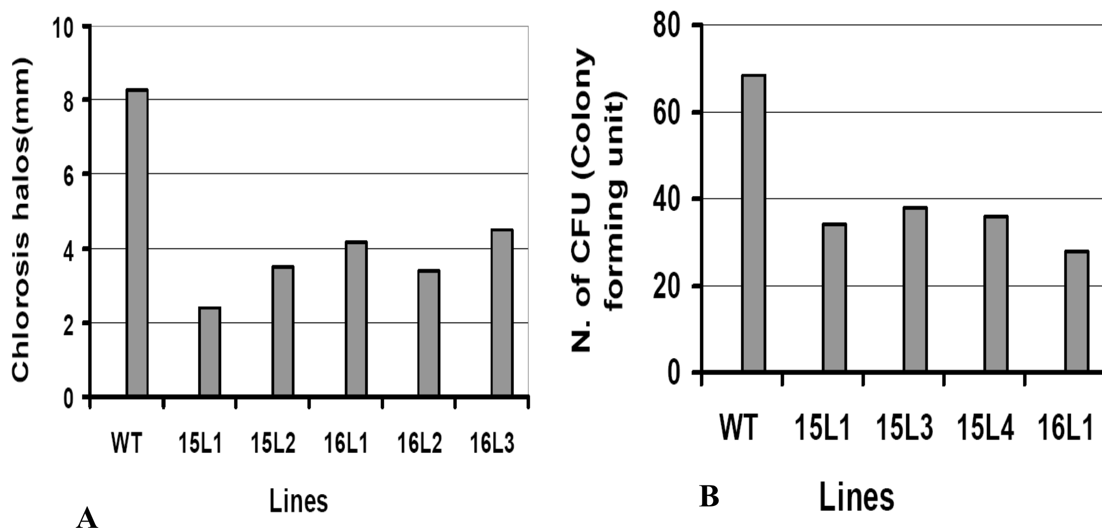


Fig. 4. **Evaluation of tobacco *HvBI-1* expressing lines after challenge with *Pseudomonas syringae* pv. *tabaci*.** Fully-expanded leaves of 7-week-old tobacco plants were used for inoculation with *Pst*, the causal agent of tobacco wildfire. Pure culture of *Pst* (strain 50312 from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was grown for 24 h in sucrose yeast extract nutrient agar medium (10 g l^{-1} sucrose, 5 g l^{-1} yeast extract, Nutrient broth 8 g l^{-1}) at 28°C. The bacterial culture was diluted with a solution of 0.9% NaCl to obtain a final inoculum concentration of 10^6 cells ml^{-1} and used for infiltration into detached tobacco leaves. Inoculated leaves were kept on 0.5% water agar supplemented with 20 ppm benzimidazole in closed plastic boxes. (**A**) The development of disease (diameter of chlorosis halos) around inoculated points was determined 10 days after inoculation. (**B**) To examine bacterial growth, leaf disks of 1 cm diameter were punched from the initially infiltrated area and the adjacent area with a cork borer at 5 days after inoculation. Leaf discs were surface-sterilized in 70% ethanol and then homogenised in sterile 0.9% NaCl solution. Bacterial populations were measured by the standard plate-dilution method, on sucrose yeast extract nutrient agar medium plates. Colony-forming units (CFU) were counted after 48 h of incubation at 28°C.

Agro strain/construct	**Embryos**	**Transfomations efficiency**
AGL1 pLH6000 35S::NH1(RNAi)		
1.Transformation	100	12
2.Transformation	100	11
3.Transformation	100	27
AGL1 pLH6000 35S::GFP-BI-1		
1.Transformation	100	5
2.Transformation	100	14
3.Transformation	100	21
LBA4404 pLH6000 35S::GFP-BI-1		
1.Transformation	100	11
2.Transformation	100	5
3.Transformation	100	14
GV3101 pGE2 35S::GFP		
1.Transformation	100	4

Table 1. Results of 3 transformation events of *NH1* (RNAi), *BI-1* and one transformation event of GFP constructs.

Line	T_1		T_2	
	+ (%)	- (%)	+ (%)	- (%)
E4L3(*35S:: GFP–HvBI-1*)	100	0	100	0
E7L6(*35S:: GFP–HvBI-1*)	69	31	79	21
E8L1(*35S:: GFP–HvBI-1*)	68	32	77	23
E15L7(*35S:: GFP–HvBI-1*)	73	27	83	17
E14L1(*35S:: GFP–HvBI-1*)	66	34	78	22
E7L2(dsRNA-*NH1*)	72	28	82	18
24E11L9(dsRNA-*NH1*)	65	35	81	19
E23L2(dsRNA-*NH1*)	62	28	-	-
E5L1(dsRNA-*NH1*)	74	26	-	-

Table 2. Efficiency of stable transformation in dsRNA-*NH1* and *35SGFP::HVBI-1* barley lines. In case of *35S::GFP–HvBI-1* line, integration of transgene into the barley genome was confirmed by PCR using *pGYI* and *GFP3′ Bam*HI primers. As well, integration of *35S::NH1* RNA interference into the barley genome was confirmed via polymerase chain PCR using of *pGY*I and *Bam*HI*npr1* primers (see materials and methods).

Family	Type member	Properties
PR-1	Tobacco PR-1a	Unknown
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase class I, II, IV-VII
PR-4	Tobacco R	Chitinase class I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato inhibitor I	Proteinase-inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase class III
PR-9	Tobacco-lignin-forming peroxidase	Peroxidase
PR-10	Parsley "PR-1"	Ribonuclease-like
PR-11	Tobacco ''class V"chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-oxidase-like
PR-17	Tobacco PRp27	Unknown

Note: Further details can be found at *http://www.bio.uu.nl/~fytopath/PR-families.htm*

Table 3. Recognized families of pathogenesis-related proteins (modified from van Loon et al., 2006).

Family	Class	Induced by
PR-1	Basic	Pathogen
PR-2	Glucanase	Pathogen & developmental
PR-3	Chitinases I & II	Pathogen & developmental
PR-4	Chitin binding	hevein Pathogen
PR-5	Thaumatin-like	Pathogen & developmental
PR-6	Thaumatin-like	Pathogen
PR-8	Chitinase III	Pathogen
PR-13	Thionin	Pathogen & developmental
PR-14	Lipid-transfer protein	Pathogen
PR-15	Oxalate oxidase	Pathogen
PR-16	Oxalate-oxidase-like	Pathogen
PR-17	Aminopeptidase-like	Pathogen

Table 4. Pathogenesis-related proteins in barley (modified from Muthukrishnan *et al.*, 2001)

Fragment	Sequence
Sense *Sma*I-*Bam*HI	CCCGGGCAACAAATCTTGCGTGAAACTGTTCGAGAGA TGCCTGGAGAGGGTAGTCCGGTCAGACCTTGACATGAT TACTCTTGATAAAGCATTGCCTCTAGATGTTATCAAGC AAATTATTGATTCACGGATAACTCTTGGATTAGCTTCA CCCGAAGACAATGGTTTTCCTAACAAGCACGTAGGAA GGATACTCAGCGCACTTGATTCTGATGATGTGGAGCTA GTCAGGTTGCTGCTCAAAGAAGGGAAGACTAACCTTG ATGATGCATTTGCATTGCACTATGCTGTAGAACACTGT GACTCCAAAATTACAACAGAACTTCTGGACATCGCACT TGCAGATGTTAATCTCAGAAACCCAAGAGGTTATACTG TTCTTCACATCGCTGCTAGGATCC
Antisense *Spe*I-*Sph*I	GCATGCAACAAATCTTGCGTGAAACTGTTCGAGAGAT GCCTGGAGAGGGTAGTCCGGTCAGACCTTGACATGATT ACTCTTGATAAAGCATTGCCTCTAGATGTTATCAAGCA AATTATTGATTCACGGATAACTCTTGGATTAGCTTCAC CCGAAGACAATGGTTTTCCTAACAAGCACGTAGGAAG GATACTCAGCGCACTTGATTCTGATGATGTGGAGCTAG TCAGGTTGCTGCTCAAAGAAGGGAAGACTAACCTTGA TGATGCATTTGCATTGCACTATGCTGTAGAACACTGTG ACTCCAAAATTACAACAGAACTTCTGGACATCGCACTT GCAGATGTTAATCTCAGAAACCCAAGAGGTTATACTGT TCTTCACATCGCTGCTAGGACTAGT

Table 5. The sense and antisense fragments sequences of *HvNH1*.

Primer	Sequence
*Sma*I (sense)	CCCGGGAACAAATCTTGCGTGAAA
*Bam*HI (as)	GGATCCTAGCAGCGATGTGAAGAA
*Spe*I (as)	ACTAGTCCTAGCAGCGATGTGAAGAA
*Sph*I (sense)	GCATGCAACAAATCTTGCGTGAAA

Table 6. The PCR fragment covers nucleotides from position 3671 to 4026 (355 bp) of the genomic *Hv NH1* sequence (AM050559). Underlined sequences indicate the restriction sites.

LIST OF ABBREVIATIONS

acd	Accelerated cell death
agd	Aberrant growth and death
Bgh	*Blumeria graminis* f.sp. *hordei*
Bgt	*Blumeria graminis* f.sp. *tritici*
BTH	Benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester
Cep	Constitutive expression of PR genes
coi1	Coronatine insensitive1
CPMP	Coat protein mediated protection
dai	Day after inoculation
dsRNA	Double-stranded RNA
eds	Enhanced disease susceptibility
ein2	Ethylene insensitive2
GAL4	Galactose utilization4
GUS	ß-glucuronidase
hpRNA	Hairpin RNA
HR	Hypersensitive response
ICS	Isochorismate synthase
IPL	Isochorismate pyruvate lyase
JA	Jasmonic acid
jar1	JA-insensitive1
LMD	Lesion-mimic/cell death
LRR	Leucine-rich repeat
Lsd	Lesion simulating disease
MeJA	Methyl jasmonate
miRNA	Micro RNA
NahG	Salicylate hydroxylase gene
NBS	Nucleotide-binding site
ndr1	Non-race-specific disease resistance
nim1	Non-inducible immunity

NPR1	Non-expressor of PR1
pad4	Phytoalexin deficient4
PAL	Phenylalanine ammonia–lyase
PDF	Protodermal factor
PR	pathogenesis-related
PTGS	Post-transcriptional gene silencing
R	Resistance gene
RG-PtoR	Rio Grande-PtoR
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
SA	Salicylic acid
sai1	Salicylic acid– insensitive
SAR	Systemic acquired resistance
sfd1	Suppressor of fatty-acid-desaturase deficiency1
SID2	Salicylic acid induction deficient2
siRNA	Small interfering RNA
SNAR	Soluble N-ethylmaleimide–sensitive factor attachment protein receptor
ssi2	Suppressor of SA-insensitivity2
TGA	TGA-element binding protein
TIR	Toll-interleukin-2 receptor
UAS	Upstream activation sequence
UTR	Untranslated region
VIGS	Virus-induced gene silencing

Declaration

Ich erkläre:

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

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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