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Histochemical and molecular studies of the interaction of hop with the hop powdery mildew fungus

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Abbreviations

At	*Arabidopsis thaliana*
Avr	Avirulence
BL	advanced breeding line
Col-0	Columbia 0
co	conidium
cp	conidiophore
cv.	cultivar
dai	days after inoculation
Ec	*Erysiphe cruciferarum*
esh	elongated secondary hyphae
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
f.sp.	forma specialis
GFP	green fluorescing protein
GUS	ß-glucuronidase
hai	hours after inoculation
hau	haustorium
hau pack	haustorium packed in callose
hc	hair cell
HI	haustorial index
Hl	*Humulus lupulus*
HR	hypersensitive reaction
LfL	Bavarian State Research Center for Agriculture (Bayerische Landesanstalt für Landwirtschaft)
LRR-RLK	Leucine-rich repeat receptor-like protein kinases
LRR-RLP	Leucine-rich repeat receptor-like protein
MAMP	microbe-associated molecular pattern
MLO	MILDEW LOCUS O
MTI	MAMP-triggered immunity

n	nucleus
n.v.	no value
NB-LRR	nucleotide-binding site leucine-rich repeat
n.d.	not determined
nec	normal epidermal cell
NO	nitric oxid
no def	no defence
PAMP	pathogen-associated molecular pattern
pap	papillae
pers. comm.	personal communication
Pm	*Podosphaera macularis*
pr Ap	primary appressorium
PRR	pattern recognition receptor
psi	pounds per square inch
res	resistant
R	*RESISTANCE*
RNAi	RNA interference
ROS	reactive oxygen species
SA	salicylic acid
sec Ap	secondary appressorium
SNARE	soluble N-ethylmaleimide sensitive factor attachment protein receptor
spor	sporulation
sus	susceptible
TIGS	transient-induced gene silencing
TM	transmembrane
VIGS	virus-induced gene silencing
Vv	*Vitis vinifera*
WGA-TMR	wheat germ agglutinin-tetramethylrhodamine
WH	wild hops

1 Introduction

Diseases and pests destroy a significant share of the hop crop and influence their quality and quantity. Additionally, financial losses due to the use of pesticides and fungicides and preventive measures like the development of resistant cultivars are caused. The main fungal diseases of hop are downy and powdery mildew, caused by *Pseudoperonospora humuli* [Miyabe et Takahasi] and *Podosphaera macularis* ssp. *humuli* [Braun] (*Pm*), and the verticillium wilt caused by *V. albo-atrum* and *V. dahliae* (Neve, 1991). The powdery mildew is the oldest known fungal disease of hop, the first reports from the UK go back to 1700 (Royle, 1978). The fact, that with an acreage of only 48,528 ha (Barth Report 2011/2012) hops is a special crop, is a recurring challenge for plant pathologists and breeders. In comparison to other crops like barley, little research focuses on hops and fewer pesticides are permitted. In particular, on the molecular level, literature and databases provide very little sequence information and knowledge about resistance-associated genes in hops. Among other things, practical research in hop breeding and hop plant pathology focused/focuses on the establishment of *Agrobacterium*- and particle bombardment-mediated gene transformation methods, the development of (cDNA) AFLP and QTL marker, PCR-based diagnostics of pathotypes and the development of tests systems to select resistant genotypes and to characterize the breeding material. New and deeper insights into molecular and cellular mechanisms conferring resistance are important to develop resistant hop cultivars.

1.1 The special crop hops

1.1.1 Biology and Morphology

The genus *Humulus* contains the species *Humulus lupulus* L., the common hop, *H. japonicus*, the Japanese hop and *H. yunnanensis* a Chinese endemic species, and, together with *Cannabis*, belongs to the family of the *Cannabaceae* (Neve, 1991). Hops is a dioecious and thus highly heterozygous perennial climbing plant that grows in the northern and southern hemisphere between the 35th and 70th lati-

tude. Bines possess hooked climbing hairs and twine clockwise around wires in the hop yards. Only female plants are commercially cultivated and their rootstocks remain in the fields for more than 20 years. The monetary value of hops lies in its usage for brewing beer as lupulin glands of female cones secrete lupulin, a yellow liquid that contains bitter acids, essential oils and phenolic compounds, which impart flavour and bitterness to beer (Neve, 1991; Royle 1978). In recent years, hops became also an important plant in the pharmaceutical and medicinal field as the flavonoid xanthohumol showed health-promoting and anti-carcinogenic effects (Stevens and Page, 2004; Strathmann and Gerhauser, 2012).

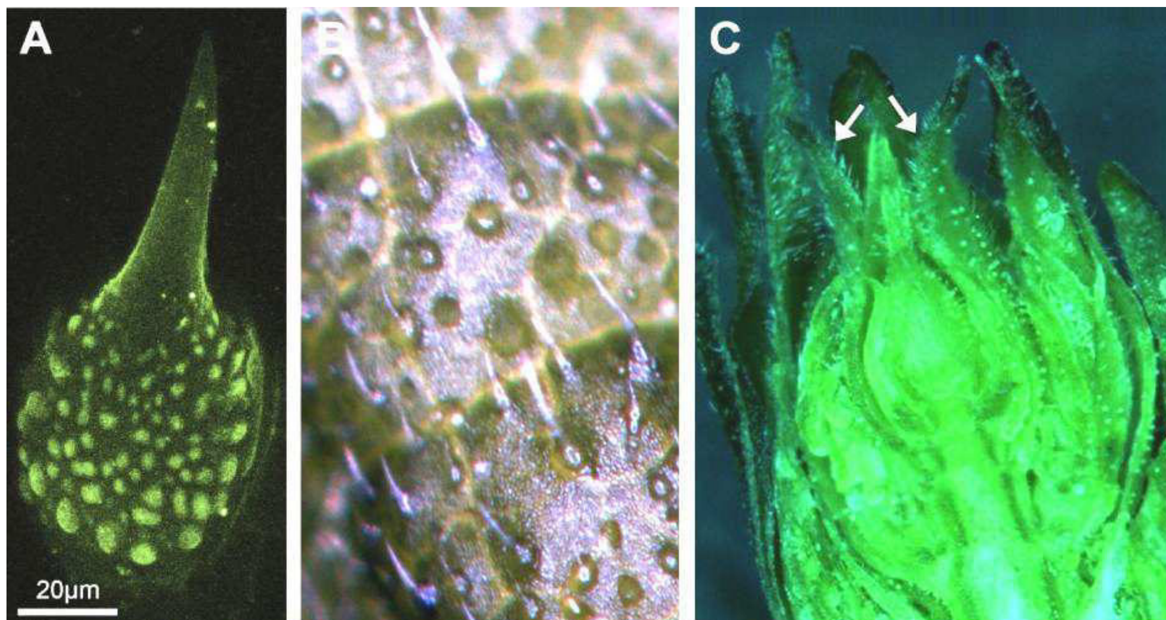

Figure 1: Hop foliage leaf hairs. A, Confocal laser-scanning micrograph of an autofluorescent hop leaf hair with a papillate surface structure. **B**, Photograph of the adaxial side of a hop leaf with numerous leaf hairs. **C**, Section through a developing hop bud. Preformed leaf hairs lie close together (arrows).

Trichomes are an important morphological feature of hops. According to Uphof (1962), the term trichome includes all unicellular or pluricellular outgrowths from the epidermis of leaves, shoots and roots, which can be divided into non-glandular, simple trichomes like hairs, and glandular-secreting trichomes (Uphof, 1962; Wagner et al., 2004). In angiosperms, trichomes occur on diverse plant parts like leaves, petals, stems, petioles, peduncles or seed coats. They possess diverse functions, as they differ greatly in their morphology and the type of tissue on which

they occur (Uphof, 1962; Johnson, 1975; Wagner et al., 2004). On hops, multicellular glandular, lupulin secreting trichomes can be found on bracts and bracteoles as well as on the abaxial side of foliage leaves. In contrast, simple trichomes, i.e. hairs, are present on adaxial and abaxial sides of foliage leaves (Figure 1), whereas two-hooked climbing hairs can be found on the stem (Small, 1978). Due to basal concretions that consist of calcium carbonate and silicic acid, hairs on foliage leaves of hops are also called cystolith hairs (Uphof, 1962; Small, 1978; Evert, 2006).

Despite the fact that in recent years several studies were conducted dealing with trichome patterning (Marks et al., 2009; Grebe, 2012), there is only little new knowledge about the function of simple trichomes. As reviewed in Uphof (1962), Johnson (1975) and Wagner et al. (2004), simple trichomes assist in climbing and play a role in the water balance of leaves and water economies. Apart from that a stress and defence-related role has been ascribed to simple trichomes on leaves. For example, pubescence was shown to reduce insect movement and insect damage, and to diminish fungal infections by reducing the leaf wetness (reviewed in Uphof, 1962; Johnson, 1975 and Wagner et al., 2004; Kivimäki et al., 2007; Kaplan et al., 2009). Furthermore, simple trichomes operate in the elimination of cytotoxins and xenobiotic compounds and prevent high Ca^{2+} accumulation near stomata to maintain stomatal function (reviewed in Wagner et al., 2004; Gutiérrez-Alcalá et al., 2000; de Silva et al., 2001). Transcriptional and metabolite profiling of *Arabidopsis* simple trichomes also revealed a stress- and defence-related role of simple trichomes (Jakoby et al., 2008; Ebert et al., 2010).

1.1.2 The powdery mildew disease in hops

Powdery mildew fungi are biotrophic pathogens that grow epiphytically on all green, aerial parts of their host plants and take up nutrients from epidermal cells. They do not kill their hosts, but impair plant vigour and therefore also yield. *Pm*, the powdery mildew of hops causes losses in yield and infected cones negatively influence the quality of beer. Moreover, controlling this disease produces costs for the application of pesticides (Neve, 1991; Krofta and Nesvadba, 2003). The asexual life cycle of powdery mildew fungi begins with the germination of an airborne

conidiospore on its host plant. The germ tube of the powdery mildew spore swells at its tip to form an appressorium, and a penetration peg penetrates the cell wall by enzymatic activity and producing turgor pressure (Pascholati et al., 1992; Pryce-Jones et al., 1999). The fungus invaginates the host plasma membrane, forms a haustorium, which, together with the extrahaustorial membrane that derives from the host cell and the extrahaustorial matrix in-between, constitutes the haustorial complex. Powdery mildew fungi likely induce the transfer of nutrients from the host cytoplasm to the haustorial cytoplasm, then start to grow epiphytically and gain access to nutrients from further epidermal cells (Perfect and Green, 2001; Green et al., 2002). Liyanage (1973) investigated the time course of the early stages of *Pm* development on the susceptible hop cv. Northern Brewer. His results were in accordance with other plant-powdery mildew interactions and revealed that conidia start forming appressoria at 6 hours after inoculation (hai), whereas normal haustoria were visible staring from 12 hai. After about five days, conidiophores arise from the leaf surface and release new spores that start a new infection cycle (Figure 2C). At this stage, fungal growth is macroscopically visible as single pustules or dense mycelium covering larger areas of the plant surface (Figure 2A, B, Glawe, 2008). In hops, *Pm* starts its asexual life cycle from mid May on leaves, and switches to a sexual life cycle at the end of the vegetation period. During the sexual life cycle, cleistothecia are produced, which are resting organs allowing the fungus to overwinter in the fields. *Pm* overwinters also as mycelium in buds, which results in so-called “flag shoots” that are entirely covered by white mycelium in the spring. This form of overwintering is of minor importance in Europe (Liyanage, 1973; Neve, 1991), but is highly significant in the USA (Gent and Nelson, 2009). Hop leaves show an ontogenetic resistance with the first fully expanded leaf pair being most susceptible, whereas flowers and cones are susceptible during all stages of maturity (Figure 2B, Royle, 1978; Seigner et al. 2003).

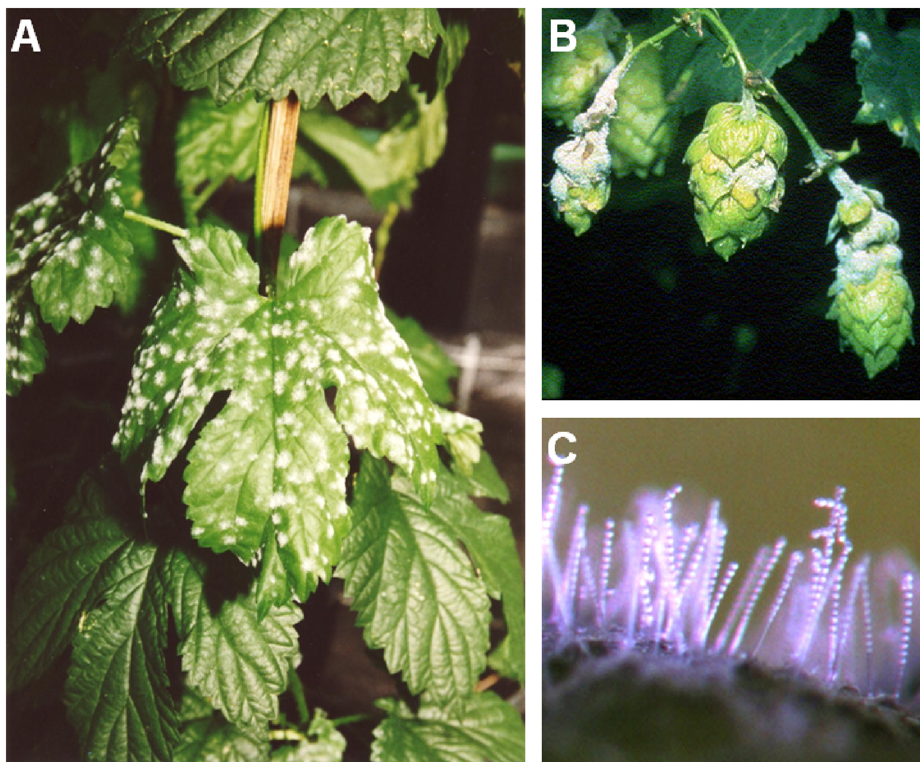

Figure 2: Powdery mildew symptoms on hop plants. A-B, Macroscopically visible symptoms of *Pm* on a hop leaf (A) and on hop cones (B). **C,** Conidiophores of *Pm* on a hop leaf. Pictures A, B: B. Engelhard, LfL (Freising, Germany).

Until the late 1990s, most hop cultivars grown in Europe were susceptible to the hop powdery mildew fungus. Only in the UK, enhanced efforts in breeding for *Pm* resistance were already taken as of the 1920s which resulted in the incorporation and characterization of seven dominantly inherited RESISTANCE (R) genes termed R1-R6 and RB (Neve, 1991; Darby, 2001; Darby, 2005). These R-genes act race-specifically according to the gene-for-gene hypothesis of Flor (1971). In 1972 the fully *Pm* resistant cv. Wye Target which carries the R2 gene as well as minor genes all contributing to its resistance was released in the UK. Up to now, the cv. Wye Target, is the most important source for *Pm* resistance in the German breeding program of the Bavarian State Research Center for Agriculture (LfL, Freising, Germany). Therefore, many breeding lines carry this resistance (E. Seigner, LfL, Freising, Germany, pers. comm.). However, it is probably only a matter of time until *Pm* pathotypes will overcome this resistance as it already happened in the UK (Darby, 2005). To incorporate new resistance traits in future cultivars, Seigner et al. (2006) screened more than 25,000 wild hops, which were collected

from Europe, Asia, Australia and Northern America. This screening, which was performed under controlled green house conditions followed by a detached leaf assay in the laboratory using eight *Pm* isolates containing the virulence genes v1-v6 and vB, identified 54 broad-spectrum powdery mildew resistant genotypes. These genotypes showed no visible symptoms after powdery mildew infection and are currently used as crossing partners to incorporate *Pm* resistance in the hop germplasm of the Hop Research Center Hüll (LfL, Freising, Germany).

1.2 Plant-pathogen interactions

1.2.1 The plant immune system

The zigzag model of Jones and Dangl (2006) explains the current model of the plant immune system by proposing a stepwise co-evolution of plants and pathogens.

The first hurdles for pathogens are preformed defence barriers of the plant such as secondary antimicrobial metabolites or physical barriers (Osbourn, 1996). If pathogens overcome these barriers, plants are able to recognize pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) by transmembrane pattern recognition receptors (PRRs) to trigger defence reactions (Zipfel, 2008). These MAMPs play a crucial role for the fitness and the survival of the pathogens and therefore are evolutionary conserved (Nürnberger et al., 2004; Thomma et al., 2011). Fungal MAMPs are for example protein motifs like Pep-13 and oligosaccharides like ß-glucan fragments or chitin (Brunner et al., 2002; Fliegmann et al., 2004; Kaku et al., 2006). PRRs are membrane anchored leucine-rich repeat receptor-like kinases (LRR-RLKs) or leucine-rich repeat receptor-like proteins (LRR-RLPs). LRR-RLKs and LRR-RLPs have an extracellular receptor domain for the perception of MAMPs. LRR-RLKs also possess a cytoplasmic kinase domain for intracellular signalling (Altenbach and Robatzek 2007; Monaghan and Zipfel 2012). Examples for PRRs are the chitin elicitor-binding protein (CEBiP) or the chitin elicitor receptor kinase 1 (CERK1) receptors that recognize chitin oligosaccharides (Kaku et al., 2006; Miya et al., 2007; Petutschnig et al., 2010; Shimizu et al. 2010). Plants that recognize MAMPs by PRRs and successfully trigger defence

reactions to restrict pathogens growth are non-hosts for this pathogen, and this kind of resistance is called MAMP-triggered immunity (MTI) or non-host resistance (Jones and Dangl 2006).

As an evolutionary consequence of MTI, pathogens have evolved effectors to suppress MTI and to promote infection (Göhre and Robatzek, 2008). These effectors act in the plant apoplast or may reach the cytosol (de Jonge et al., 2011). They circumvent or suppress MTI by acting upstream of PRRs, interfering with downstream defence signalling of PRRs or facilitating the accommodation and the nutritional supply of the pathogen (de Jonge et al., 2010; Bozkurt et al., 2011; Hemetsberger et al., 2012). Examples for effectors, which act in the apoplast are Ecp6 and Avr4 that prevent the recognition of the MAMP chitin by PRRs and therefore also defence signalling and reactions. In detail, Avr4 and Ecp6 prevent the release of chitin fragments by plant chitinases by catching chitin and binding to fungal cell walls, respectively (van den Burg et al., 2006; de Jonge et al., 2010). No secretion system analogous to the needle-like type three secretion system of bacteria has been found in fungi. Despite the fact that fungal effectors may enter the cytoplasm via the standard endomembrane pathway the process of effector delivery into plant cells remains largely unclear (Kale et al., 2010; Dean, 2011; Koeck et al., 2011; Rafiqi et al., 2012). An example for an effector acting in the cytoplasm is SP7 of the fungal symbiont *Glomus intraradices* that interacts with a transcription factor in the plant nucleus and thus suppresses defence-related gene expression (Kloppholz et al., 2011). The above described form of susceptibility caused by pathogens that successfully circumvent or suppress MTI by their effectors is termed effector-triggered susceptibility (ETS) (Jones and Dangl, 2006).

As a response to ETS, plants have evolved R proteins, intracellular nucleotide-binding site leucine-rich repeat (NB-LRR) proteins, which recognize pathogen effectors and trigger defence reactions (Tör et al., 2009). Direct interactions between R proteins and effectors like in the flax-flax rust L567/AvrL567 system are apparently rather an exception (Ellis et al., 2007). The guard hypothesis assumes that R proteins monitor the status of host effector targets (Dangl and Jones, 2001). For example, in the interaction of *Pseudomonas syringae* and *Arabidopsis,* the effectors AvrRpm1 and AvrB phosphorylate the host protein RIN4, whereas the effector

AvrRpt2, a cysteine protease, cleaves RIN4 at two sites. These modifications of the effector target RIN4 probably activate the RPM1 and RPS2 NB-LRR proteins (Mackey et al., 2002; Axtell and Staskawicz 2003; Mackey et al., 2003; Kim et al., 2005). Plants that recognize effectors or effector activity by their R proteins and successfully trigger defence responses exhibit effector-triggered immunity (ETI). During evolution, pathogens may alter their effectors, loose them or develop new effectors to overcome ETI and to avoid R protein-mediated triggering of defence reactions. In turn, plants may restore ETI by adapting their R proteins again (Chisholm et al., 2006; Jones and Dangl, 2006). Another important term in plant-pathogen interactions is basal resistance, which is defined as the remaining level of MTI and ETI during ETS (Jones and Dangl, 2006).

1.2.2 Plant defence reactions

In plant-powdery mildew interactions, plant cells can restrict fungal growth at the pre- or post-invasive stage. Pre-penetration resistance is often achieved or accompanied by the formation of cell wall appositions which are also termed papillae and which likely constitute a physical and chemical barrier to resist fungal penetration. This defence mechanism is often observed during MTI (Pascholati et al., 1992; Pryce-Jones et al., 1999; Hückelhoven 2007; Underwood, 2012). Papillae are composed of the ß-1,3-glucan polymer callose, suberin, lignin, cellulose, cell wall polymers like pectin, and cell wall structural proteins (Aist, 1976; Underwood 2012). Furthermore, secondary metabolites like phenolics or glucosinolates accumulate in papillae (von Röpenack et al., 1998; Clay et al., 2009). Oxidative cross-linking of structural proteins, which might partly be mediated by hydrogen peroxide (H_2O_2), probably fortifies papillae and therefore contributes to penetration resistance (Bradley et al., 1992; Brisson et al., 1994; Thordal-Christensen et al., 1997; Hückelhoven et al., 1999; Hückelhoven, 2007).

R protein-mediated recognition of effector activity during ETI usually triggers the hypersensitive reaction (HR), a rapidly executed form of programmed cell death that restricts fungal growth at the post-invasive stage (Heath, 2000; Mur et al., 2008). However, in this context it is discussed whether the HR is a defence mechanism itself or only a consequence of defence reactions as death signals like

reactive oxygen species (ROS), nitric oxide (NO) and salicylic acid (SA) trigger resistance also in the absence of the HR (Mur et al., 2008). HR signalling is accompanied by a Ca^{2+} influx into the cell (Xu and Heath, 1998; Grant et al., 2000; Ali et al., 2007). The stepwise execution of the HR includes a stop in cytoplasmic streaming, a nuclear collapse, the loss of plasma membrane permeability followed by protoplast collapse and in penetrated cells almost simultaneously the fungal collapse (Heath, 2000). HR cells often accumulate callose and oxidized phenolic compounds, the latter being responsible for the frequent brown and necrotic appearance of these cells and for autofluorescence (Heath, 2000). Another strategy of attacked cells to stop fungal growth at the post-invasive stage is the packing of fungal haustoria into callose-like material. Often an HR follows this defence reaction (Lipka et al., 2008; Micali et al., 2011; Wen et al., 2011).

Despite the fact that callose is omnipresent in different kinds of defence reactions like in papillae, the HR and haustorial encasements, the role of callose is not yet fully understood. Probably depending on the MAMP and environmental conditions, different pathways lead to callose depositions (Luna et al., 2011). Unexpectedly, a few studies suggest that callose does not play an important role as a structural barrier in plant-powdery mildew interactions (Underwood, 2012). Nishimura et al. (2003) showed that *Arabidopsis* loss-of-function mutants of the callose synthase PMR4 (GSL5) lack pathogen-induced callose depositions in papillae and become more resistant to the powdery mildew fungus in comparison to the susceptible wild-type. Here, enhanced resistance in *pmr4* (*gsl5)* plants probably is based on an upregulation of SA. Loss-of-function of this *Arabidopsis* callose synthase PMR4 (GSL5) led only to a minor increase in the penetration rate of the non-adapted barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei*, but interestingly growth of normally virulent powdery mildew species and *Peronospora* was stopped (Jacobs et al., 2003). Therefore, Underwood (2012) suggested that callose accumulating in papillae, HR cells, haustorial encasements and in plasmodesmata rather serves as a barrier protecting plant cells from toxic compounds or alternatively, that callose affects the diffusion of effectors and thus the activation of defence pathways. Another structural function of callose is probably the formation of a matrix for the deposition of antimicrobial compounds (Hardham et al., 2007).

Defence reactions of plant cells also include the rearrangement of the cytoskeleton and the endomembrane system, as successful defence reactions depend on effective transport processes (Kobayashi et al., 1997; Opalski et al., 2005; Day et al., 2011; Hückelhoven and Panstruga 2011). Furthermore, the secretion machinery is activated to provide material to strengthen the cell wall and to execute immune responses in the apoplast like the release of inhibitors of fungal derived cell wall-degrading enzymes (Collins et al., 2003; Brutus et al., 2005; Hückelhoven, 2007; Kwon et al., 2008). Other crucial events during defence responses are the transcriptional activation of the synthesis of antifungal compounds like phytoalexins that accumulate at sites of fungal infection, but might also act as a signal involved in the HR (Snyder and Nicholson, 1990; von Röpenack et al., 1998; Chang et al., 2011). In addition, ROS are an important part of the plant immune response. An oxidative burst occurs at the pre-invasive stage in the apoplast as well as before and during the early stages of HR (Doke and Ohashi, 1988; Levine et al., 1994; Thordal-Christensen et al., 1997; Lamb and Dixon, 1997; Hückelhoven and Kogel, 2003; Daudi et al., 2012). Besides this, SA and NO also play important roles in HR signalling. SA might function in stimulating the production of ROS, whereas NO might interact with ROS in triggering the HR (Lamb and Dixon, 1997; Delledonne et al., 2001; Mur et al., 2008).

1.2.3 *mlo*-mediated powdery mildew resistance

The *MILDEW LOCUS O* (*MLO*) gene was originally discovered in barley as induced loss-of-function mutations conferred recessively inherited broad-spectrum powdery mildew resistance (Jørgensen, 1992). Since then, a couple of studies also reported naturally occurring loss-of-function mutations in *MLO* genes of barley, tomato and pea, as well as induced loss-of-function mutations in *Arabidopsis MLO* genes, all conferring broad-spectrum powdery mildew resistance (Jørgensen, 1992; Consonni et al., 2006; Bai et al., 2008; Humphry et al., 2011; Pavan et al., 2011). This broad occurrence of *mlo* resistance in the plant kingdom may account for an evolutionary conserved mechanism, at least since monocots and dicots split. However, although naturally occurring powdery mildew resistance exists, pleiotropic effects like spontaneous cell death in plants lacking MLO wild-type

proteins represent a disadvantage in nature (Peterhänsel et al., 1997; Piffanelli et al., 2002). MLO proteins are located in the plasma membrane and the endoplasmic reticulum and possess seven hydrophobic membrane-spanning helices (Devoto et al., 1999). The cytoplasmic C-terminus has a calmodulin binding domain that serves as a cytoplasmic calcium sensor and is important for full functionality of the MLO protein. The N-terminus is located extracellularly (Devoto et al., 1999; Kim et al., 2002).

MLO proteins are transcriptionally up-regulated in response to powdery mildew fungi in barley, *Arabidopsis,* tomato and grapevine, and focally accumulate at sites of attempted penetration (Piffanelli et al., 2002; Bhat et al., 2005; Chen et al., 2006; Bai et al., 2008; Feechan et al., 2008). In barley and *Arabidopsis*, *mlo* resistance relies on the SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) proteins ROR2 and PEN1, respectively (Consonni et al., 2006; Collins et al. 2003). It is assumed that MLO proteins regulate SNARE protein-mediated membrane fusion events during which vesicle cargo is secreted as a part of the plant defence reaction. In compatible plant-powdery mildew interactions, the powdery mildew fungus might exploit the SNARE complex to deliver plasma membrane material by exocytosis for its accommodation in the plant cell (Panstruga, 2005).

mlo resistance is probably related to MAMP-triggered immunity, as both resistance types are based on pre-invasive penetration resistance and confer broad-spectrum powdery mildew resistance relying on SNARE proteins, at least in barley and *Arabidopsis* (Humphry et al., 2006; Hückelhoven and Panstruga, 2011).

1.2.4 Organ, tissue and cell type-specificity of plant-pathogen interactions

In plant-pathogen interactions, specificity occurs in the form of host and non-host interactions, narrow and broad host ranges and on the host level in the form of ETI or ETS. Furthermore, in compatible interactions, organ and tissue specificity exists (Hermanns et al., 2003). For example, powdery mildew fungi occur most frequently on the upper side of leaves, *Verticillium* spp. only infect roots and the vascular system, whereas *Xanthomonas* spp. colonize the xylem and the intercellular

spaces of nonvascular, mesophyll tissues (Agrios, 2005; Klosterman et al., 2009; Bogdanove et al., 2011). There are also indications that host and non-host resistance might only be active in certain tissue types. For example, in the interaction of *Arabidopsis* and the leaf pathogen *Hyaloperonospora parasitica*, race-specifically resistant *Arabidopsis* plants restrict growth of the oomycete on leaves by HRs. In contrast, roots of these resistant plants do not execute an HR and are susceptible to the oomycete (Hermanns et al., 2003). In a similar study, Schreiber et al. (2011) reported that non-host resistance of *Arabidopsis* to *Magnaporthe oryzae* is not active in roots. Besides organ and tissue specificity in plant-pathogen interactions, also cell type-specificity occurs. In barley, *B. graminis* f.sp. *hordei* forms more haustoria in short leaf cells than in long cells, indicating a correlation of haustorium formation and cell morphology (Lin and Edwards, 1974; Jørgensen and Mortensen, 1977; Koga et al., 1990). Furthermore, subsidiary cells of mlo-resistant barley seedlings show a cell type-specific susceptibility to *B. graminis* f.sp. *hordei* (Jørgensen, 1992).

Despite these phytopathological observations on organ-, tissue- and cell type-specificity, to date little is known about the molecular and biochemical basis of these phenomena. Recently, the fact that *Ustilago maydis*, the maize corn smut fungus, expresses tissue specific effectors threw light on this question (Skibbe et al., 2010). In another study, Catoni et al. (2009) reported of organ-specific gene expression patterns of tomato in the interaction with the tomato spotted wilt virus.

1.3 Functional analysis of defence-associated genes using transient transformation assays

Stable transformation of hops based on *Agrobacterium*-mediated transformation or particle bombardment is established, but still remains time and cost intensive (Horlemann et al., 2002; Batista et al., 2008). In several plant species, the ability of elicitors to trigger HRs was investigated by *Agrobacterium*-mediated transient transformation of leaf tissue (Scofield et al., 1996; Takken et al., 2000; Vleeshouwers et al., 2006). This transformation method was also applied with promoter-reporter gene fusions to analyze promoter activity after biotic stress (Yang et al.,

2000; Xu et al., 2010). Functional assessment of defence-associated genes in leaf tissue during pathogen infection was achieved by virus-induced gene silencing (VIGS) in different plant species such as barley, potato, tobacco, wheat and maize (Brigneti et al., 2004; Borrás-Hidalgo et al., 2006; Cakir et al., 2010; van der Linde et al., 2011). Although VIGS became a widely used tool for the functional analysis of defence-associated genes one disadvantage is that VIGS itself might induce stress responses. In the pathosystems wheat-*B. graminis* f.sp. *tritici* and barley-*B. graminis* f.sp. *hordei,* a transient transformation system based on particle bombardment of single leaf epidermal cells routinely identifies genes related to pathogen resistance or susceptibility (Nielsen et al., 1999; Schweizer et al., 1999; Shirasu et al., 1999; Douchkov et al., 2005). For example, in barley the function of MLO and BAX INHIBITOR-1 as susceptibility factors and the function of the barley pathogenesis-related protein PR-1b were confirmed or discovered with this assay (Shirasu et al., 1999; Hückelhoven et al., 2003; Schultheiss et al., 2003). In this assay, gold or tungsten particles are usually coated with a reporter gene construct (for the expression of the *green fluorescing protein* (*GFP*) or *β-glucuronidase* (*GUS*) gene) and an overexpression or silencing construct of the gene of interest. Then, particle bombardment delivers these particles into single epidermal cells of detached leaves, which are subsequently inoculated with spores of the powdery mildew fungus. Spores germinate and may penetrate transformed cells followed by the establishment of a haustorium for nutrient transfer from the host or may fail to do so because of successful defence reactions of transformed cells. Finally, the impact of the test gene construct on haustoria formation in transformed cells is determined relative to the (empty vector) control. The availability of microscope robotics has turned this transient transformation assay into a high throughput technology (Douchkov et al., 2005; Ihlow et al., 2008).

1.4 Objectives

In addition to yield and brewing quality, resistance to the powdery mildew fungus *Podosphaera macularis* (*Pm*) is a major objective in hop breeding. In hop cultivars, powdery mildew resistance relies, on the presence of one or several of seven, dominantly inherited *RESISTANCE* (*R*) genes. None of these *R* genes has been described on the molecular or biochemical level yet. However, since certain *Pm* pathotypes were able to overcome these resistances, the German breeding program needs to introduce new resistance carriers.

To support this breeding strategy, this study aimed at characterizing the cellular basis of resistance in twelve hop genotypes of the German germplasm to provide new insights for the breeding process. For example, different resistance mechanisms might be combined in new cultivars to achieve long-term resistance. This study was of special interest because information about resistance mechanisms and defence reactions is completely lacking for the German hop germplasm.

Hop hair cells show a cell type-specific susceptibility to adapted and non-adapted powdery mildew fungi. So far, the literature provides little information about tissue- and especially cell type-specific susceptibility in fungal-plant interactions. To extend this sparse knowledge and to provide new insights into the hop-powdery mildew interaction the cell type-specific susceptibility of hop hair cells was investigated.

Another part of this work aimed at supporting the German breeding program by establishing a transient transformation assay for hops. The transient transformation assay is routinely applied in barley and wheat and is a fast and cost-effective method for the functional assessment of resistance-associated genes, based on the biolistic transformation of single epidermal cells of detached leaves. In hops, genes with a confirmed function in the hop-powdery mildew interaction could be used for transgenic approaches or serve for the development of molecular markers. The establishment of the transient transformation assay included the investigation and optimization of different parameters as well as proof of concept by transient-induced gene silencing (TIGS).

2 Material and Methods

2.1 Maintenance of plant material

For all experiments except the gene expression studies, hop plants were obtained as root cuttings from January to March from the Hop Research Center Hüll of the LfL in Germany. To have fresh plant material available for a longer time period, a system was re-established, in which root cuttings were covered with soil and stored in a refrigerator at 4°C for up to six months. When required, root cuttings were potted and placed in a growth chamber at 20°C with a night setback of 8°C, 70% relative humidity, a photoperiod of 16 h, and a light intensity of 110 µmol $m^{-2}s^{-1}$. Experiments were performed with first fully expanded leaf pairs from a vigorously growing shoot. These leaf pairs, which are most susceptible to the powdery mildew fungus, were in most cases the second leaf pairs from the top.

As at the beginning of this study fresh plant material was seasonally limited, gene expression studies were performed with *in vitro* plants. For this, middle parts of vigorously growing hop shoots where cut between the nodes into single nodal segments, surface sterilized in 4% NaOCl for five minutes and washed three times for 10 min in autoclaved water. To remove infiltrated NaOCl, nodal segments were trimmed and immediately transferred into culture vessels (Wächter, Leopoldshöhe, Germany) containing Murashige and Skoog (MS) media that consisted of 0.6% agar (w/v), 2% glucose (w/v), 1 mgl^{-1} 6-benzylaminopurine and 4.4 gl^{-1} MS media including vitamins (Duchefa, Haarlem, the Netherlands), with a pH of 5.8. The vessels were incubated at 23°C with a night setback of 3°C, a photoperiod of 16 h, and a light intensity of 40-45 µmol $m^{-2}s^{-1}$. After ten days, the first fully expanded leaf pairs from axillary shoots were used for the experiment. A thin 0.6% water agar layer on top of the MS media avoided contamination of the MS media with microorganisms due to the non-sterile inoculation procedure with the powdery mildew fungus for the expression studies.

2.2 Microscopic investigation of the interaction of hop epidermal cells with *P. macularis* and *E. cruciferarum*

2.2.1 Selection of hop genotypes

Table 1: Investigated hop genotypes and their reaction to the *Pm* isolate BU10[a]

Genotype	Geographical origin	Isolate BU10 (v3, v4, v6, vB)
Cv. Northern Brewer	UK	10
Cv. Hall. Merkur	Germany	0
Cv. Hall. Herkules	Germany	0
BL 093 010 036	Germany	0
BL 2002 047 011	Germany	0
WH 2001 137 001	Germany	0
WH 018 097 008	Germany	0
WH 2002 186 740	Japan	0
WH 2002 186 047	Japan	0
WH 2001 139 003	Germany	0
WH 2002 182 001	Germany	0
WH 2002 185 002	Turkey	0
WH 2006 268 001	USA	0

[a] Data are based on a detached leaf assay performed in this study. Powdery mildew resistance phenotypes were scored on a scale of 0 to 10 (0 = no symptoms; 10 = strong sporulation, like susceptible reference variety). Cv. = cultivar; BL = advanced breeding line; WH = wild hop.

Together with A. Lutz, (LfL, Freising, Germany), I chose twelve powdery mildew resistant hop genotypes to investigate their interaction with the hop powdery mildew fungus *Pm* on the single-cell level. Among them were eight wild hops, two advanced breeding lines and two cultivars, the powdery mildew susceptible cv. Northern Brewer served as a control. These genotypes all stem from the German breeding program of the LfL (Freising, Germany). The eight wild hops were chosen from a collection of 54 broad-spectrum powdery mildew resistant genotypes according to their suitability for the breeding program. Here special attention was given on their aroma quality, the time point of flowering and in particular, on their geographical origin assuming that hop genotypes from Europe, Asia or the USA differ in their resistance mechanisms to the powdery mildew fungus. These 54

genotypes derived from a screening of 25,000 wild hops, which were collected from Europe, Asia, Australia and North America (Seigner et al., 2006). This screening was performed in the green house and subsequently with a detached leaf assay with pathogen isolates containing the virulence genes *v1-v6* and *vB* (Neve, 1991; Darby, 2001). The two breeding lines were selected because they represent a source for powdery mildew resistance in the breeding program, whereas the cultivars were interesting because they were recently released on the market. Table 1 lists the wild hops, cultivars and breeding lines investigated in this study and their reaction to the *Pm* isolate BU10. BU10 contains the virulence genes *v3*, *v4*, *v6*, *vB* and was used for all inoculation procedures in this study.
Cv. Northern Brewer was selected to investigate the non-host interaction of hops with a powdery mildew fungus usually adapted to *Arabidopsis thaliana*, *Erysiphe cruciferarum*.

2.2.2 Maintenance of the pathogens, inoculation procedures and evaluation of interaction sites

Pm isolate Bu10, kindly provided by EpiLogic GmbH (Freising, Germany), was maintained on detached leaves of cv. Northern Brewer. To inoculate fresh plant material, leaves from first fully expanded leaf pairs were detached and placed in petri dishes containing 0.75% water agar. An open acrylic glass cylinder with a diameter of 11 cm and a height of 35 cm served as a settling tower and was placed vertically over the petri dishes. Leaves were inoculated by blowing conidia carefully from infected leaves with a spray bottle or an air brush to give an inoculation density of 10 to 30 conidia mm^{-2}. This inoculation procedure and the following incubation of the petri dishes for spore production were performed according to Seigner et al. (2006).
E. cruciferarum was maintained on plants of the *A. thaliana* ecotype Columbia 0 (Col-0) that were grown in a growth chamber at 22°C, with 70% relative humidity, a photoperiod of 10 h and a light intensity of 120 µmol $m^{-2}s^{-1}$. Leaves of the resistant hop genotypes were inoculated as described above to give an inoculation density of 10 to 30 conidia mm^{-2} and incubated in a growth chamber at 19°C with

70% relative humidity, a photoperiod of 14 h and a light intensity of 40-45 µmol $m^{-2}s^{-1}$.

To investigate defence reactions of normal hop epidermal cells to *Pm* and *E. cruciferarum*, for each of the twelve macroscopically resistant genotypes three leaves from three individual plants were inoculated together with one leaf of the susceptible control cv. Northern Brewer. Altogether three independent experiments were performed for each resistant genotype. At 1 day after inoculation (dai) leaf samples were taken to study the host interactions of the twelve powdery mildew resistant hop genotypes with *Pm* and at 2 dai to study the non-host interaction of cv. Northern Brewer with *E. cruciferarum*. At least 79 interaction sites per leaf were analysed. The 7 dai time point was only sampled for the wild hop 018 097 008 because this genotype showed a late defence reaction. Here, at least 17 germinated spores that formed elongated secondary hyphae were investigated on each leaf. All experiments were evaluated by calculating the mean of the technical repetitions and then the mean of the three independent repetitions.

To investigate the cell type-specific susceptibility of hop leaf hairs to *Pm* and *E. cruciferarum*, the experimental setup was the same as described above, except for the following modifications: Leaf samples were taken at 2 dai and instead of calculating the mean of the technical repetitions means of all interaction sites from technical repetitions were calculated because single fungal-hair cell interactions could only be found infrequently. In this way, at least 55 fungal-hair cell interaction sites per genotype and independent experiment were obtained. For assessment of sporulation events at 7 dai for each resistant genotype, one to three leaves from three individual plants were screened under the microscope for sporulating colonies. Here, also three independent experiments were performed. Single-cell interactions of *E. cruciferarum* with hair cells of cv. Northern Brewer were examined in the same way. Fungal fitness of *E. cruciferarum* was controlled in each independent experiment by determining the percentage of germinated spores that formed elongated secondary hyphae on one leaf of the powdery mildew susceptible *Arabidopsis* accession Col-0.

As an overall control of fungal fitness sporulation of *Pm* and *E. cruciferarum* on susceptible control leaves was checked visually in all experiments at 7 dai.

2.2.3 Histochemical staining methods

Fungal structures and defence reactions were detected through histochemical stainings, autofluorescence or were visible in the transmitted light channel. First, leaves were cleared for at least two hours in a 3:1 (v/v) mixture of ethanol:chloroform containing 0.15% trichloroacetic acid (w/v). Destained leaves were mounted in 50% glycerol until histochemical stainings were performed. Fungal structures were stained with the red fluorescing wheat germ agglutinin tetramethylrhodamine conjugate (WGA-TMR, Molecular Probes, Life Technologies GmbH, Darmstadt, Germany). This method was modified from Deshmukh et al. (2006). Leaves were rinsed with water to remove remaining glycerol and incubated in 1x PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4) for 20 min before they were transferred to 1x PBS containing 10 $\mu g ml^{-1}$ WGA-TMR und 10 μg ml^{-1} bovine serum albumin. After five to seven days, fungal haustoria were visible and detection of defence reactions took place. HRs and cell wall appositions were identified by staining of callose depositions with methylene blue (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). For this purpose, remaining WGA-TMR staining solution was removed, leaves were rinsed with water and left for five minutes in 0.1 M K_2HPO4 (pH 9.2). Subsequently, 0.067 M K_2HPO4 containing 0.05% (w/v) methylene blue was vacuum infiltrated at 25 inch Hg. Leaves were incubated over night in this solution, washed with water and directly subjected to microscopy. In addition to staining of callose depositions and autofluorescence, cell reactions of attacked epidermal cells that were visible in the transmitted light channel as discolouration and cytoplasm granulation were also taken as a reliable signs for cell death (Koga et al., 1990; Görg et al., 1993; Heath, 2000).

All experiments were analyzed with an Axiostar Plus Fluorescence Microscope (Zeiss, Jena, Germany). WGA-TMR was detected with filter set 43 (excitation at 545/25 nm, emission at 605/70 nm) and methylene blue with filter set 49 (excitation at 365 nm, emission at 445/50 nm). Autofluorescence was detected with filter set 38 (excitation at 470/40 nm, emission at 525/50 nm) and filter set 43. Pictures were taken with a confocal laser-scanning microscope (Leica SP5, Mannheim, Germany). Here, the fluorescence dye WGA-TMR was excited by a 561 nm laser

line and detected at 570 to 616 nm. Methylene blue was excited by a 405 nm laser line and detected at 420 to 490 nm. Autofluorescence was excited by a 488 nm laser line and detected at 500 to 550 nm or detected with the same settings as for WGA-TMR. The number of optical sections and the increment size is specified in the description of each figure in the results section.

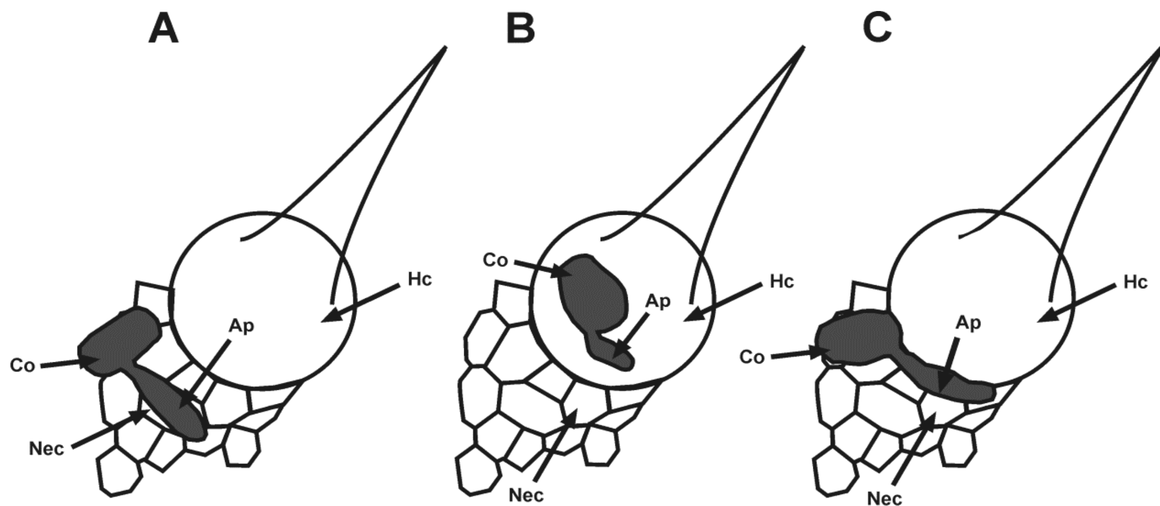

Figure 3: Categories of single fungal-epidermal cell interactions. A-C, On hop leaves, appressoria of germinated conidia (Ap) were either found lying directly on normal epidermal cells (Nec) (A), on hair cells (Hc) (B) or lying simultaneously on a normal epidermal cell and lateral of a hair cell (C).

The evaluation methodology for single fungal-epidermal cell interactions was as follows: Three different categories of fungal penetration attempts could be found. Fungal appressoria were i) lying directly on normal epidermal cells, ii) lying directly on hair cells or iii) lying simultaneously laterally of hair cells and on adjacent normal epidermal cells (Figure 3). A lateral penetration of hair cells, which was often observed, is possible because hair cells rise from the leaf surface. In the first two cases it was clear, which cell was attacked, in the latter case only if a haustorium was visible in one of the two cell types. Hence, it was decided to count interaction sites of type iii) as interactions with normal epidermal cells, if only defence reactions of normal cells were observed or normal epidermal cells contained haustoria. In all other cases, interaction sites of type iii) were counted as interactions with hair cells.

2.3 DAPI nucleic acid stain of hop epidermal cells

To compare the DNA content of hair cells to the DNA content of normal epidermal cells, DNA in nuclei of cv. Northern Brewer was stained with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Life Technologies GmbH, Darmstadt, Germany) according to Walker et al. (2000). For this purpose, leaves were fixed over night, incubated in the staining solution for two hours and transferred to 50% glycerol in McIlvaine’s buffer for microscopy. Pictures from at least 20 hair cell nuclei were taken with a confocal laser-scanning microscope (see chapter 2.2.3). DAPI was excited by a 405 laser line and detected at 431 to 551 nm.

2.4 Cell area measurements

To compare the area of normal epidermal cells to the area of hair cells at least 15 cells from the first fully expanded leaf pair from a Northern Brewer plant were measured for each cell type. Therefore, leaves were destained (see chapter 2.2.3) and pictures were taken with an Axiostar Plus Microscope equipped with Axio-CamICc 1 (Zeiss, Jena, Germany). Then the outline of the epidermal cells was traced using the ImageJ software (http://rsbweb.nih.gov/ij/index.html) and the area was calculated.

To obtain information about the composition of the leaf surface, the leaf area covered by hair cells was determined for Northern Brewer. Three evenly distributed pictures from a leaf of a first fully expanded leaf pair as a well as from a leaf from the following node were made with a binocular (Stemi SV 11, Zeiss, Jena, Germany). Hair cell bases were measured as described above with the ImageJ software. In addition to the cell areas, the area of the analyzed image sections was determined.

2.5 Establishment of a transient transformation assay

2.5.1 Maintenance of the pathogen

Because the transient transformation assay in hops is limited in terms of the number of single-cell interactions between transformed cells and the powdery mildew fungus, a high inoculation density is extremely important. Therefore, a method for maintaining BU10 on whole plants of cv. Northern Brewer was established. For this purpose, plants were grown in a growth chamber at 19°C with 70% relative humidity, a photoperiod of 14 h and a light intensity of 40-45 µmol $m^{-2}s^{-1}$. Fully expanded leaf pairs were inoculated by detaching an infected leaf and pressing it on the surface of a healthy leaf. After seven to ten days, when the fungus showed heavy sporulation, leaves were used for the inoculation procedure.

2.5.2 Phylogenetic analysis of the putative hop *MLO* gene

A phylogenetic classification of the deduced partial amino acid sequence of the hop *MLO* EST (GenBank accession number: ES653367) within *Arabidopsis* and grapevine MLO family members was performed by carrying out a multiple sequence alignment with T-coffee (http://www.ebi.ac.uk/Tools/msa/tcoffee/) followed by a bootstrap analysis with 100 replicates with RAxML (Stamatakis et al., 2008). Default settings of programs were used and the best scoring maximum likelihood tree was visualized with dendroscope (Huson et al., 2007).

To investigate the sequence conservation in detail, the partial putative hop MLO amino acid sequence was aligned to the amino acid sequences of *Arabidopsis* and grapevine MLO clade V family members with T-coffee. Sequences were taken from GenBank and accession numbers are (Devoto et al., 2003; Feechan et al., 2008): AtMLO1 (CAB08605), AtMLO2-15 (AAK53795-AAK53808), VvMLO1 (CAO41068, superceded by XP_002266377), VvMLO2 (CAO66267, superceded by XP_002273026), VvMLO3 (CAO18135, superceded by XP_002275390), VvMLO4 (CAO21819, superceded by XP_002282216), VvMLO5 (CAO22254, superceded by XP_002266927), VvMLO6 (CAO66388, superceded by XP_002273434), VvMLO7 (CAO46388, superceded by XP_002280697), VvMLO8 (CAO71699, superceded by XP_002276608), VvMLO9 (CAN84002, superceded

by XP_002275487), VvMLO10 (CAO18134, superceded by XP_002275360), VvMLO11 (CAO21818, superceded by XP_002282198), VvMLO12 (CAO39251, superceded by XP_002282190), VvMLO13 (CAO68971, superceded by XP_002274608), VvMLO14 (CAO66265, superceded by XP_002273002), VvMLO15 (CAO47031, superceded by XP_002266144), VvMLO16 (CAO48195, superceded by XP_002265520), VvMLO17 (CAO68972, superceded by XP_002274642).

2.5.3 Expression analysis of the putative hop *MLO* gene

Before transient-induced gene silencing studies were performed, expression of the putative hop *MLO* gene was analyzed in leaves of the powdery mildew resistant cv. Wye Target and the susceptible cv. Northern Brewer after powdery mildew infection. Therefore, first fully expanded leaf pairs of *in vitro* plants were evenly inoculated by pressing gently an infected leaf, obtained with the method described in chapter 2.5.1, on healthy leaves. Subsequently, spores were dispensed on the leaf surface with a brush. At 0, 4, 6, 8, 10, 24, 48 and 168 hai, leaves were harvested and immediately frozen in liquid nitrogen. Leaf tissue was ground into fine powder, and RNA was extracted according to the manufacturer's instructions with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), including a DNase digestion with the RNase-Free DNase Set (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA quality was checked by examining rRNA bands after electrophoresis on a denaturing agarose gel. cDNA synthesis was primed with the iScript cDNA Synthesis Kit (Bio-Rad, München, Germany) in a 20 µl reaction containing 1 µg RNA, oligo (dT) and random hexamer primers. Subsequently, 2 µl aliquots were used for the PCR amplification of a 252 bp fragment of the putative hop *MLO* cDNA with gene specific primers. As a constitutive control, a 186 bp *polyubiquitin* cDNA fragment (GenBank accession number: EU700059) was amplified (Castro et al., 2008). Annealing temperatures were 55°C for both the putative hop *MLO* cDNA fragment (32 cycles) and the *polyubiquitin* fragment (26 cycles). Primer sequences are 5’-ACCATCACTTTGGAGGTGGA-3’ (forward primer) and 5’-GAGACGGAGGACAAGGTGAA-3’ (reverse primer) for *polyubiquitin* and

5'-AGGGACACGTCATTTGGAAG-3' (forward primer) and 5'-GAACCAAATCGGA-GGACTGA-3' (reverse primer) for the putative hop *MLO* cDNA fragment.

2.5.4 Construction of the transient-induced gene silencing vector pIPKTA30N-MLO

The transient-induced gene silencing (TIGS) vector pIPKTA30N-MLO was generated by the Gateway cloning strategy published by Douchkov et al. (2005). It contained inverted repeats of the 252 bp fragment of the partial putative hop *MLO* cDNA (see chapter 2.5.3) separated by an intron deriving from the wheat RGA2 gene under the control of the CaMV 35S promoter. For the construction of pIPKTA30N-MLO, the 252 bp fragment of the putative hop *MLO* EST was amplified from cv. Northern Brewer by PCR. The *MLO* cDNA fragment was ligated in inverse orientation into the pIPKTA38 entry vector following Douchkov et al. 2005. Integration and inverse orientation of the *MLO* fragment were checked by colony PCR with vector-specific primer 5'-AGCAGGCTTTAAAGGAACC-3' (forward primer) lying in the aTTL1 site of pIPKTA38 and the forward primer of the putative hop *MLO* cDNA (see chapter 2.5.3). After sequence confirmation, a standard Gateway LR reaction with the Gateway LR Clonase Enzyme Mix (Invitrogen, Carlsbad, CA, USA) was carried out according to manufacturer's instructions with 300 ng of the pIPKTA38-MLO entry clone and 300 ng of the pIPKTA30N destination vector, resulting in pIPKTA30N-MLO.

2.5.5 Coating of the gold particles and particle bombardment

Coating of gold particles was adapted from Knudsen and Müller, (1991). First, 30 mg of gold particles were dissolved in 1 ml of 100% EtOH by vortexing for 3 min to deagglomerate them. Then, particles were spun down for 1 minute at 9,500 rcf and the supernatant was removed. To wash the particles, 1 ml of sterile water was added, particles were resuspended, and spun down again. This washing step was repeated once. Particles were finally dissolved in 1 ml of sterile water and stored in aliquots at -20°C. Gold particles were coated by a $CaCl_2$ precipitation. For this purpose, 50 µl of 2.5 M $CaCl_2$, 20 µl of 0.1 M spermidine and vector DNA were added to a reaction tube. The combination of expression plasmids was de-

pendent on the experiment performed. To determine transformation rates of hair cells and normal epidermal cells at different helium gas acceleration pressures, 6µg of pGY-1-GFP (0.75 µg per shot) and 9 µg (1.13 µg per shot) of empty pGY-1 vector (GFP under control of CaMV 35S promoter, Schweizer et al., 1999) were added to the coating solution. To determine co-expression rates in hair cells, 6 µg of pGY-1-GFP and 9 µg of pe35AscIoptRed (DsRed-C1 under control of CaMV 35S promoter, Dietrich and Maiss, 2002) were added to the coating solution. For TIGS experiments, 6 µg of the reporter gene vector pUbiGUS (β-glucuronidase gene from *Escherichia coli* under control of the maize *UBIQUITIN* promoter, Schweizer et al., 1999) and 9 µg of the RNAi vector pIPKTA30N-MLO (see chapter 2.5.4) or 9 µg or the empty vector control pIPKTA30N were added to the coating solution.

The coating solution was mixed by pipetting and transferred to 50 µl of the gold suspension. The vector DNA was precipitated onto the particles by vortexing the mixture for 3 min. Subsequently, particles were spun down at 9,500 rcf for 10 s, and the supernatant was removed. Then, particles were resuspended in 250 µl of 100% EtOH upon vortexing for 1 min. After this step, particles could be stored on ice for up to 3 h. Finally, particles were spun down at 9,500 rcf for 10 s, the supernatant was removed and particles were resuspended in 56 µl of 100% EtOH. Coated particles (1.875 µg per shot) were pipetted in 7 µl aliquots onto the macrocarriers, a maximum of 6 macrocarriers was prepared at one time.

For particle bombardment, detached leaves from cv. Northern Brewer were placed in 0.75% agar plates. Leaves were bombarded depending on their size separately or in twos with the PDS-1000/He-gun (Bio-Rad, Munich, Germany). The bombardment was carried out under the following conditions. The distance between the leaves and the macrocarrier was 10 cm, and the distance between the macrocarrier and the rupture disk was 2.5 cm. During bombardment, a vacuum of 27 inch Hg was applied. Particles were accelerated with 650, 900 or 1100 psi to determine the transformation rates dependent on the acceleration pressure, and with 650 psi to determine co-expression rates and to perform the TIGS experiments.

2.5.6 Determination of co-expression rates and determination of transformation rates at different acceleration pressures

Incubation of detached leaves in a growth chamber (see chapter 2.1) followed particle bombardment to allow the expression of the reporter genes. Microscopic evaluation of transformation rates was performed after one day, microscopic evaluation of co-expression rates after three days.

To assess transformation rates at different acceleration pressures, the total number of GFP-expressing hair cells and normal epidermal cells was counted for whole leaves. Subsequently, photographs of the leaves were analysed with the ImageJ software to measure the leaf area (see chapter 2.4) and to calculate the number of transformed cells per mm^2. For each acceleration pressure, the mean of 13 leaves was calculated. To assess co-expression rates, GFP-expressing hair cells were investigated for DsRed fluorescence. Transformed hair cells from three leaves with each leaf being from an individual plant were counted. At least 158 GFP-expressing hair cells were examined in one experiment. The mean of 3 independent experiments was calculated.

Microscopy was done with a Zeiss Axiostar Plus Fluorescence Microscope equipped with filter set 38 for the detection of GFP fluorescence and filter set 43 for the detection of DsRed fluorescence (see chapter 2.2.3).

2.5.7 Inoculation procedure and evaluation of TIGS experiments

For one independent experiment, four to seven leaf pairs of cv. Northern Brewer were detached, one leaf of the pair was always bombarded with the *MLO* TIGS vector as described in chapter 2.5.5, the other always with the empty vector. Subsequently, leaves were incubated for three days in a climate chamber (see chapter 2.1) to allow progression of the RNAi-mediated knockdown of *MLO* expression. Heavily infected leaves obtained with the method described in chapter 2.5.1 were detached and used to inoculate transiently transformed leaves with a settling tower as described in chapter 2.2.2 to give an inoculation density of 250 conidia mm^{-2}. The fungus was allowed to develop for another three days in a climate chamber (see chapter 2.2.2) and leaves were stained over night for GUS-activity. The GUS staining solution was prepared according to Schweizer et al. (1999) with the ex-

ception that concentrations of potassium hexacyanoferrat (II) and potassium hexa-cyanoferrat (III) were adapted to 8 mM to avoid too intensive staining of the trans-formed cells. After removal of the chlorophyll with destaining solution (see chapter 2.2.3), leaves were mounted in 50% glycerol until WGA-TMR staining for fungal structures was performed (see chapter 2.2.3). Microscopy was done with a Zeiss Axiostar Plus Fluorescence Microscope equipped with AxioCamICc 1 and filter set 43 for the detection of WGA-TMR (see chapter 2.2.3).

For the functional analysis of the putative hop *MLO* gene, a modified susceptibility index (Douchkov et al., 2005) was calculated as a measure of powdery mildew susceptibility of hair cells. It represents the percentage of transformed hair cells containing at least one mature haustorium, whereby only hair cells with appres-soria nearby were evaluated.

$$Susceptibility\ index = \frac{100\ \times (transformed\ hair\ cells\ containing\ at\ least\ one\ mature\ haustorium)}{(transformed\ hair\ cells\ with\ an\ appresorium\ on\ or\ adjacent\ to\ the\ hair\ cell)}$$

Because the fungus grew for three days, secondary and tertiary haustoria may also be included in the susceptibility index. With this method, 50 to 260 fungus-transformed hair cell interactions were obtained for each variant in one experi-ment. Altogether, seven independent experiments of the transient knockdown of the putative hop *MLO* gene were evaluated. Values of the empty vector control variant were set to 100% and the mean susceptibility index for the *MLO* knock-down variant was calculated. After testing for normal distribution with a qq-plot, a *t*-Test was conducted. Statistical analysis was supported by Thomas Eckl (LfL, Freising, Germany).

3 Results

3.1 Investigations on the cellular basis of resistance in the hop-powdery mildew interaction

3.1.1 Defence reactions of normal epidermal cells to *P. macularis*

I characterized types and frequencies of phenotype-related defence reactions to *Pm* in twelve broad-spectrum powdery mildew resistant hop genotypes from the German breeding program, among them eight wild hops, two advanced breeding lines and two cultivars. For this, single-cell interactions of *Pm* with normal epidermal cells were investigated microscopically at 24 hai. The susceptible cv. Northern Brewer served as a control. Table 2 lists mean frequencies of the two observed defence reactions, the HR, and papillae formation that is often associated with pre-penetration resistance (Thordal-Christensen, 2003; Prats et al., 2007; Niks and Marcel, 2009). Furthermore, I determined the haustorial index, i.e. the percentage of investigated fungal germlings that had produced a mature or immature haustorium, and the formation of elongated secondary hyphae as an indicator of susceptibility or late defence reactions. Single-cell interactions of *Pm* spores that formed only one short germ tube with epidermal cells that showed no visible defence reactions are also listed. Results of breeding line 093 010 036 are in the supplement (Supplementary Table 1) because only two independent experiments were obtained from this genotype.

Conidia of *Pm* formed elongated secondary hyphae on the susceptible cv. Northern Brewer at 35.8% of the interaction sites and on the resistant wild hop 018 097 008 at 15.0% of the interaction sites (Table 2, Figure 4A, B). On the other resistant genotypes, conidia produced only single, short germ tubes.

Consistent with the formation of elongated secondary hyphae, mature haustoria represented the major fraction of the haustorial indexes determined for the susceptible cv. Northern Brewer and the resistant wild hop 018 097 008 (Table 2, Figure 4A, B), whereas haustorial indexes determined for the other resistant genotypes consisted of rudimentary haustoria (Table 2, Figure 4C). In comparison to normal epidermal cells of the susceptible cv. Northern Brewer with an haustorial

index of 41.3%, the resistant wild hop 2002 186 740 showed with 71.6% the highest haustorial index and the resistant wild hop 2001 137 001 with 1.7% the lowest (Table 2). Haustorial indexes determined for the other resistant genotypes ranged in-between.

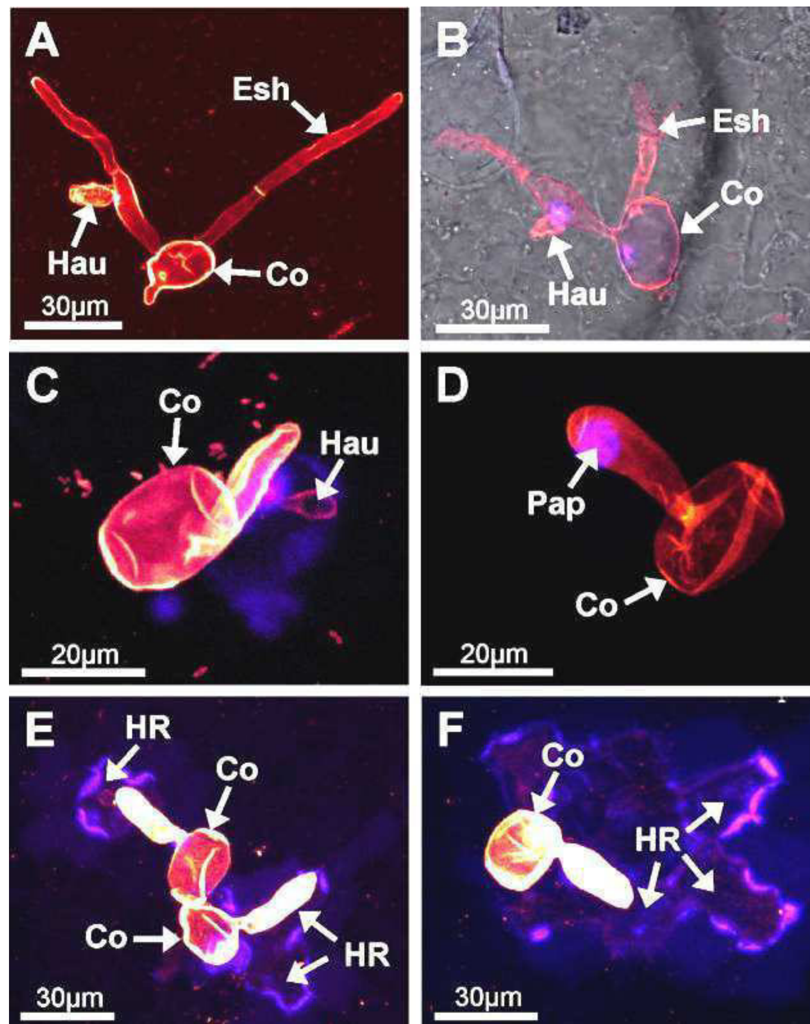

Figure 4: Single-cell interaction phenotypes between *Pm* and the resistant hop genotypes as well as between *Pm* and the susceptible control cv. Northern Brewer. Pictures were taken with a confocal laser-scanning microscope and show maximum projections of z-stacks, picture B includes an overlay with a single image of the transmission channel. Fungal structures were stained with the red fluorescing wheat germ agglutinin tetramethylrhodamine (WGA-TMR). Co = conidium, Hau = haustorium, HR = hypersensitive reaction, Esh = elongated secondary hyphae, Pap = papilla. **A,** a germinated conidium of *Pm* penetrated the cell wall of a normal epidermal cell of the susceptible cv. Northern Brewer and established a mature haustorium, elongated secondary hyphae are visible. The picture is a maximum projection of 13 optical sections at 2 µm increments. **B,** a germinated conidium of *Pm* penetrated the cell wall of a normal epidermal cell of the resistant

wild hop 018 097 008 and established a mature haustorium, elongated secondary hyphae are visible. The picture is a maximum projection of 11 optical sections at 2 µm increments. **C,** a normal epidermal cell of cv. Merkur contains a rudimentary haustorium of *Pm*, the conidium produced only a single, short germ tube. Staining of callose depositions with aniline blue indicate an HR. The picture is a maximum projection of 12 optical sections at 2 µm increments. **D,** a penetration attempt of *Pm* resulted in the formation of a papilla by a normal epidermal cell of wild hop 2002 182 001. The picture is a maximum projection of 6 optical sections at 2 µm increments. **E,** conidia of *Pm* produced single, short germ tubes and attacked normal epidermal cells of the resistant wild hop 2002 182 001. Staining of callose depositions with aniline blue indicate that one (top left) and two (bottom right) normal epidermal cells per interaction site underwent an HR. The picture is a maximum projection of 11 optical sections at 2 µm increments. **F,** single-cell interaction between *Pm* and a normal epidermal cell as described in (E), staining of callose depositions with aniline blue indicate a multicellular HR of more than five cells at one interaction site. The picture is a maximum projection of 15 optical sections at 2 µm increments.

Early HR arrested fungal growth in ten out of the twelve resistant genotypes. In nine of these ten genotypes, more than 90% of the normal epidermal cells lying beneath fungal appressoria underwent an HR (Table 2, Figure 4E, F). HR of normal epidermal cells of the resistant wild hop 018 097 008 were less frequently detected and accounted for 67.0% (Table 2). However, this wild hop additionally exhibited a late HR to arrest fungal growth (see below). In contrast to these resistant genotypes, normal epidermal cells of the susceptible cv. Northern Brewer underwent an HR only at 57.4% of the interaction sites (Table 2).
Fungal penetration attempts led to the formation of papillae only rarely. In all investigated genotypes mean frequencies of papillae ranged from 0.0% to 1.2% (Table 2, Figure 4D).
A certain percentage of germinated conidia neither penetrated normal epidermal cells nor triggered visible defence reactions. This observation did not seem to be dependent on the genotype and ranged from 3.3% to 19.4% (Table 2).

Table 2: Microscopic assessment of defence reactions of normal epidermal cells to *Pm* at 24 hai in susceptible and resistant hop genotypes[a]

Genotype	Phenotype	% (±SD) HI	% (±SD) Esh	% (±SD) HR	% (±SD) Pap	% (±SD) no def
Cv. Northern Brewer	sus	41.3 ± 12.8	35.8 ± 12.7	57.4 ± 12.1	0.2 ± 0.2	6.6 ± 3.4
BL 2002 047 011	res	19.0 ± 0.5	0 ± 0	94.4 ± 0.1	0.4 ± 0.7	5.2 ± 0.7
Cv. Hall. Merkur	res	23.7 ± 14.9	0 ± 0	93.6 ± 1.9	0.3 ± 0.5	6.1 ± 1.6
WH 2001 137 001	res	1.7 ± 1.7	0 ± 0	96.7 ± 0.2	0.0 ± 0.0	3.3 ± 0.2
Cv. Hall. Herkules	res	13.9 ± 8.7	0 ± 0	92.0 ± 2.7	0.5 ± 0.5	7.5 ± 2.4
WH 2002 186 740	res	71.6 ± 6.1	0 ± 0	91.9 ± 1.9	1.1 ± 2.0	7.0 ± 3.0
WH 2002 186 047	res	31.8 ± 17.0	0 ± 0	80.3 ± 30.8	0.3 ± 0.3	19.4 ± 30.8
WH 2001 139 003	res	10.4 ± 7.1	0 ± 0	94.0 ± 1.1	0.8 ± 1.2	5.1 ± 2.0
WH 2002 182 001	res	38.9 ± 16.4	0 ± 0	95.4 ± 2.2	1.2 ± 1.4	3.4 ± 1.4
WH 2002 185 002	res	21.6 ± 10.9	0 ± 0	93.3 ± 6.9	0.5 ± 0.6	6.3 ± 7.2
WH 2006 268 001	res	61.2 ± 16.3	0 ± 0	92.0 ± 5.4	0.5 ± 0.2	7.4 ± 5.7

[a] Data represent mean percentages of three independent experiments and corresponding standard deviations. In each experiment at least three leaves with 79 fungal germlings per leaf were evaluated. The cv. Northern Brewer served as the susceptible control. The haustorial index (HI) represents the percentage of germlings that formed a rudimentary or mature haustorium. Fungal Germlings that formed elongated secondary hyphae (Esh) after establishment of a mature haustorium were assessed. Defense reactions of normal epidermal cells to *Pm* attacks were categorized into hypersensitive reactions (HR) and the formation of effective papillae (Pap). At some interaction sites, germlings neither penetrated normal epidermal cells nor triggered defense reactions (no def). Mean frequencies of Esh, HR, Pap and non-penetrated cells with no defense reaction add up to 100%. BL breeding line, res = resistant (no macroscopic powdery mildew symptoms), sus = susceptible (macroscopic powdery mildew symptoms), WH = wild hop.

Single as well as multicellular reactions occurred at the interaction sites. To reveal differences between the investigated genotypes, I set HRs that are listed in Table 2 to 100%, and categorized each interaction site into one of three categories. The categories depended on the number of normal epidermal cells that underwent an HR at one interaction site, i) 1, ii) ≤ 5 and iii) > 5 (Table 3, Figure 4E, F). In the susceptible cv. Northern Brewer at 17.3% of the interaction sites 1, at 74.4% ≤ 5 and at 8.3% > 5 cells underwent an HR. The resistant wild hop 2001 137 001 showed the highest differences to cv. Northern Brewer, here at 0.2% of the interaction sites 1, at 16.0% ≤ 5 and at 83.8% > 5 cells performed an HR. Also the resistant wild hop 2002 186 740 differed clearly from cv. Northern Brewer, here at 41.3% of the interaction sites 1, at 56.3% ≤ 5 and at 2.5% > 5 cells underwent an HR. All other genotypes behave similar to cv. Northern Brewer (Table 3).

Table 3: Single and multicellular HRs[a]

	Mean frequencies of HR cell categories in% ±SD		
Genotype	category i	category ii	category iii
WH 2001 137 001	0.2 ± 0.3	16.0 ± 0.5	83.8 ± 0.5
WH 2002 185 002	1.1 ± 0.2	62.3 ± 15.2	36.7 ± 15.1
WH 2002 182 001	6.8 ± 0.6	82.5 ± 8.0	10.6 ± 8.5
WH 2001 139 003	9.1 ± 3.6	78.5 ± 8.4	12.5 ± 5.1
Cv. Hall. Herkules	13.3 ± 14.7	63.7 ± 7.1	23.0 ± 8.5
WH 018 097 008	13.7 ± 10.1	71.4 ± 9.0	14.9 ± 6.2
Cv. Hall. Merkur	15.1 ± 17.6	78.0 ± 14.3	6.9 ± 3.6
Cv. Northern Brewer	17.3 ± 8.0	74.4 ± 9.8	8.3 ± 3.6
BL 2002 047 011	19.9 ± 13.4	69.9 ± 13.5	10.2 ± 4.8
WH 2006 268 001	27.0 ± 22.4	64.8 ± 26.1	8.1 ± 11.6
WH 2002 186 047	29.8 ± 9.9	66.6 ± 5.3	3.6 ± 5.2
WH 2002 186 740	41.3 ± 9.5	56.3 ± 6.0	2.5 ± 3.7

[a] Mean frequencies of normal epidermal cells that underwent an HR as a response to *Pm* attack (see Table 2) were set to 100%. Each interaction site was grouped into one of three categories depending on the number of HR cells at one interaction site. The three categories were i: 1, ii: <5 iii: ≥5 HR cells per interaction site.

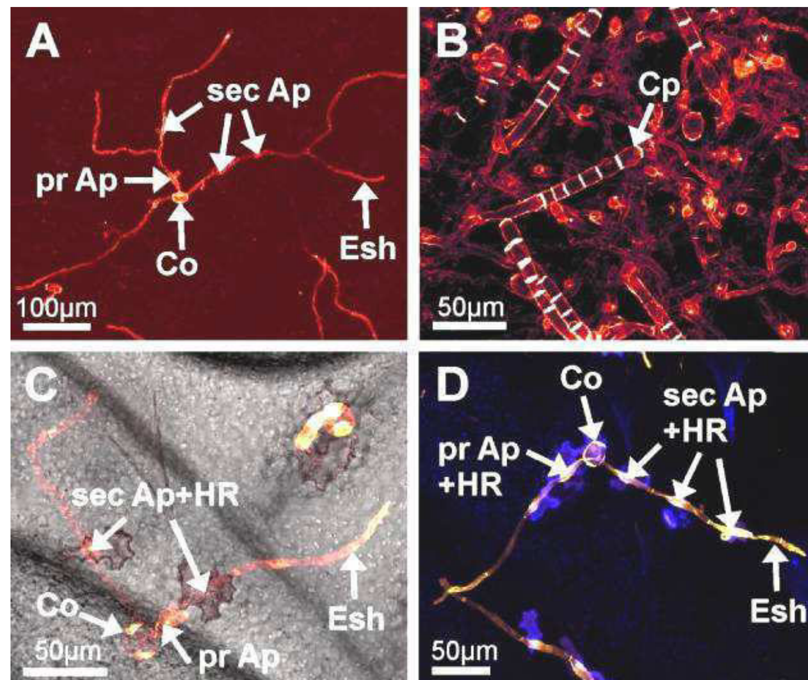

Figure 5: Development of *Pm* on the susceptible cv. Northern Brewer and on the resistant wild hop 018 097 008. Pictures were taken with a confocal laser-scanning microscope and show maximum projections of z-stacks. Picture C shows an overlay with single projections of the transmission channel. Fungal structures were stained with red fluorescing wheat germ agglutinin tetramethylrhodamine (WGA-TMR). Cp = conidiophores, Co = conidium, Esh = elongated secondary hyphae, HR = hypersensitive reaction, pr Ap = primary appressorium, sec Ap = secondary appressorium. **A,** a conidium of *Pm* formed elongated secondary hyphae on the susceptible cv. Northern Brewer at 48 hai. Hyphae are branching, and in addition to the primary appressorium secondary appressoria are visible. The picture is a maximum projection of 10 optical sections at 3 µm increments. **B,** *Pm* covers the leaf surface of the susceptible cv. Northern Brewer with a dense mycelium, and conidiophores arise at 7 dai. The picture is a maximum projection of 19 optical sections at 4 µm increments. **C,** a conidium of *Pm* formed elongated secondary hyphae on the resistant wild hop 018 097 008 at 48 hai. Hyphae are not branched, and whole cell autofluorescence of epidermal cells beneath secondary appressoria indicates HRs. The epidermal cell beneath the primary appressorium containing a mature haustorium stayed alive. The picture is a maximum projection of 8 optical sections at 2 µm increments. **D,** at 7 dai, elongated secondary hyphae of *Pm* grew only marginally on the resistant wild hop 018 097 008 compared to the formation of elongated secondary hyphae at the 48 hai time point (C). Staining of callose depositions (blue) with aniline blue indicates an HR of normal epidermal cells beneath the primary and secondary appressoria. The picture is a maximum projection of 7 optical sections at 3 µm increments.

To further examine the late defence reaction of the resistant wild hop 018 097 008, I investigated HRs beneath primary and secondary appressoria at 7 days after in-

oculation (dai) (Figure 5). At this time point, 97.3% of the normal epidermal cells beneath primary appressoria and 87.1% of the normal epidermal cells beneath secondary appressoria had died. Furthermore, the colony size of the fungus on leaves of the wild hop 018 097 008 (Figure 5D) remained clearly behind the colony size of the susceptible cv. Northern Brewer, on which a dense, sporulating mycelium was visible (Figure 5B). Preliminary data obtained from 48 hai (only two independent experiments) revealed that 16.6% of normal epidermal cells beneath primary appressoria and about 35.9% of normal epidermal cells beneath secondary appressoria exhibited cell death. This incomplete defence reaction is consistent with the colony size at 48 hai, which was slightly delayed in comparison to cv. Northern Brewer (Figure 5A, C).

3.1.2 Defence reactions of normal epidermal cells to non-adapted *E. cruciferarum*

Microscopic assessment of defence reactions in different powdery mildew resistant genotypes showed that the penetration attempts of *Pm* led to the formation of papillae only at a maximum of 1.2% of the interaction sites (see chapter 3.1.1). To generally investigate the role of papillae in the hop-powdery mildew interaction, I examined the non-host interaction of cv. Northern Brewer with *E. cruciferarum* because in non-host interactions pre-penetration resistance is often achieved or accompanied by the formation of papillae (Thordal-Christensen, 2003; Niks and Marcel, 2009). Categories of single-cell interactions between *E. cruciferarum* and epidermal cells listed in Table 4 are the same as described in 3.1.1. As expected, conidia of *E. cruciferarum* did not establish mature haustoria and did not form elongated secondary hyphae on normal epidermal cells of the non-host cv. Northern Brewer at 48 hai. Only at 4.0% of the interaction sites normal epidermal cells contained rudimentary haustoria, therefore haustoria were found less frequently in this non-host interaction than in most of the host interactions (Table 4, see chapter 3.1.1). At 68.5% of the interaction sites, HR arrested fungal growth, whereas fungal penetration attempts led to the formation of papilla at 6.9% of the interaction sites (Table 4, Figure 6). Germinated conidia that neither penetrated normal epidermal cells nor triggered visible defence reactions accounted for 24.5% (Table 4).

In the control variant, 74.1% of conidia formed elongated secondary hyphae on the susceptible *Arabidopsis* host accession Col 0 (Table 4).
These investigations on defence reactions of normal hop epidermal cells show that the HR is the basis for the resistance in hop genotypes to adapted or non-adapted powdery mildew fungi.

Table 4: Microscopic assessment of defence reactions of normal epidermal cells to *E. cruciferarum* at 48 hai[a]

	mean frequencies in% (± SD)				
Genotype	HI	Esh	HR	Pap	no def
Cv. Northern Brewer	4.0 ± 3.6	0.1 ± 0.1	68.5 ± 3.1	6.9 ± 2.0	24.5 ± 1.7
Col-0	n.d.	74.1 ± 11.0	n.d.	n.d.	n.d.

[a]Data represent mean frequencies of three independent experiments and corresponding standard deviations. In one experiment, three leaves with at least 79 fungal germlings per leaf were evaluated. The susceptible *Arabidopsis* host accession Col-0 served as a control. The haustorial index (HI) represents the mean frequency of germlings that formed a (rudimentary) haustorium. Fungal germlings that formed elongated secondary hyphae (Esh) after establishment of a mature haustorium were assessed. Defence reactions of normal epidermal cells to *E. cruciferarum* conidia were categorized into HR and the formation of effective papilla (Pap). At some interaction sites, fungal germlings neither penetrated normal epidermal cells nor triggered visible defence reactions (no def). Mean frequencies of HR cells, papilla formation and not penetrated cells with no defence reaction add up to 100%. n.d. = not determined.

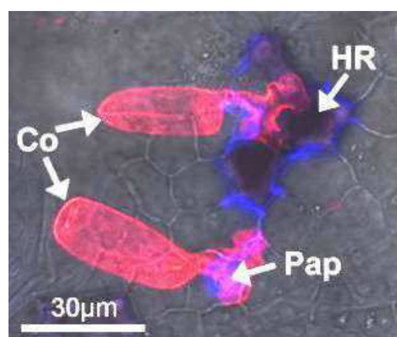

Figure 6: Observed defence reactions of normal epidermal cells of cv. Northern Brewer to *E. cruciferarum*. The picture was taken with a confocal laser-scanning microscope and shows a maximum projection of a z-stack in an overlay with a single projection of the transmission channel. Fungal structures were stained with red fluorescing wheat germ agglutinin tetramethylrhodamine (WGA-TMR). Co= conidium, HR = hypersensitive reaction, Pap = papilla. A normal epidermal cell underwent an HR (top), another formed a papilla (bottom) in response to attack of germinated *E. cruciferarum* conidia. Blue fluorescence indicates callose accumulation as visualized by aniline blue staining. The picture is a maximum projection of 12 optical sections at 2 µm increments.

3.2 Investigations on the cell type-specific susceptibility of hop leaf hairs to *P. macularis* and non-adapted *E. cruciferarum*

3.2.1 Single-cell interaction phenotypes between hop leaf hairs and the powdery mildew fungi *P. macularis* or non-adapted *E. cruciferarum*

During the microscopic assessment of defence reactions of normal epidermal cells to *E. cruciferarum* and *Pm*, I noticed cell type-specific susceptibility of hop hair cells. Therefore, I evaluated single fungal-hair cell interactions between *Pm* and twelve phenotypically resistant host genotypes, *Pm* and the susceptible host cv. Northern Brewer as well as between *E. cruciferarum* and the non-host cv. Northern Brewer. Table 5 lists mean frequencies of the observed single fungal-hair cell interaction phenotypes as described in 3.1. As an additional defence reaction haustoria packed in callose and as an additional sign of susceptibility sporulation events at 7 dai were investigated. The results of wild hop 018 097 008 are listed in the supplement because from the 7 dai time point no results were obtained (Supplementary Table 2).

Conidia of *Pm* penetrated hair cells, established mature haustoria and formed elongated secondary hyphae in both, the macroscopically resistant genotypes and in the susceptible cv. Northern Brewer. The mean frequency of conidia that formed elongated secondary hyphae ranged from 13.0% in the wild hop 2006 268 001 to 78.8% in the cv. Northern Brewer (Table 5). It was striking that on leaves of the cultivars and breeding lines (cv. Merkur, cv. Herkules, breeding line 093 010 036, breeding line 2002 047 011) conidia formed elongated secondary hyphae more frequently than on leaves of the wild hops. At 7 dai, on 9 of the 12 investigated resistant hop genotypes sporulating colonies spreading from a susceptible hair cell in the centre were observed (Figure 7A, Table 5). Simultaneously, attacked normal epidermal cells beneath secondary hyphae of these colonies underwent HRs (Figure 7C, see chapter 3.1.1). However, not every colony was sporulating and due to the limited number of fungal-hair cell interactions per leaf, only single sporulation events were observed. Also in the non-host interaction, *E. cruciferarum* established mature haustoria in hair cells of cv. Northern Brewer at 13.1% of the interaction sites, and formed elongated secondary hyphae (Table 5). In the control variant, 74.1% of the conidia formed elongated secondary hyphae on normal epi-

dermal cells of the susceptible *Arabidopsis* host accession Col-0 (Table 5). Although *E. cruciferarum* formed elongated secondary hyphae on cv. Northern Brewer, no sporulation events could be found at 7 dai (Figure 7B, D, Table 5).

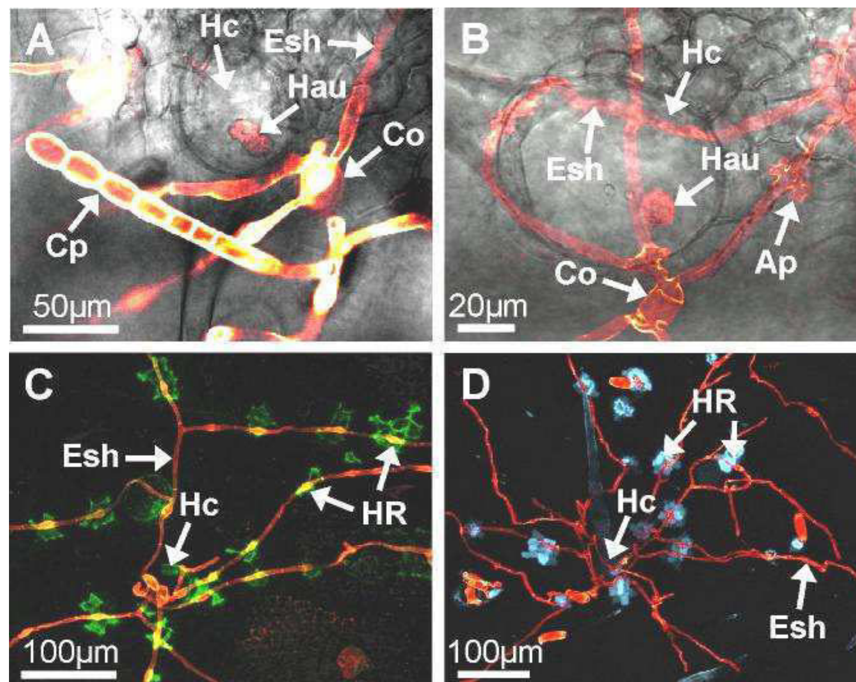

Figure 7: Cell type-specific susceptibility of hop leaf hairs in host and non-host interactions with *Pm* and *E. cruciferarum*. Pictures were taken at 7 dai with a confocal laser-scanning microscope and show maximum projections of z-stacks. Pictures A and B show an overlay with single projections of the transmission channel. Fungal structures were stained with red fluorescing wheat germ agglutinin tetramethylrhodamine (WGA-TMR). Ap = appressorium, Cp = conidiophore, Co = conidium, Esh = elongated secondary hyphae, Hau = haustorium. Hc = hair cell, HR = hypersensitive reaction. **A,** *Pm* penetrated a hair cell of the resistant host cv. Herkules and established a mature haustorium. The conidium, from which the colony originated, lies next to the hair cell, elongated secondary hyphae and a conidiophore arising from epiphytic fungal structures are visible. The picture is a maximum projection of 20 optical sections at 2.3 µm increments. **B,** *E. cruciferarum* penetrated a hair cell of the non-host cv. Northern Brewer and established a mature haustorium, elongated secondary hyphae are visible. The conidium, from which the colony derived, lies next to the hair cell. Note the lobed appressorium of *E. cruciferarum*. The picture is a maximum projection of 20 optical sections at 3 µm increments. **C,** elongated secondary hyphae of *Pm* on the resistant host cv. Herkules spread from a susceptible hair cell in the centre. Whole cell autofluorescence (green) of normal epidermal cells beneath secondary appressoria of the colony indicates an HR. The picture is a maximum projection of 39 optical sections at 3 µm increments. **D,** elongated secondary hyphae of *E. cruciferarum* on the non-host cv. Northern Brewer spread from a susceptible hair cell in the centre. Staining of callose depositions with aniline blue indicates an HR of normal

epidermal cells beneath secondary appressoria of the colony. The picture is a maximum projection of 45 optical sections at 4 µm increments.

Haustorial indexes of *Pm,* which include mature haustoria, rudimentary haustoria and haustoria packed in callose, ranged from 47.9% in the wild hop 2006 268 001 to 82.9% in the cv. Northern Brewer (Table 5). Similar to the formation of elongated secondary hyphae, the highest haustorial indexes were determined for the two cultivars and breeding lines and the lowest for the wild hops. The mean frequency of rudimentary haustoria in normal epidermal cells was not associated with the mean frequency of haustoria in hair cells. Here great differences between these two epidermal cell types were observed within one genotype. For example, only 1.7% of normal epidermal cells of the wild hop 2001 137 001 contained rudimentary haustoria, whereas the haustorial index was 65.3% in hair cells (Table 5). In comparison to host interactions, the haustorial index of *E. cruciferarum* in the non-host hair cell interaction with cv. Northern Brewer (26.5%) was much lower (Table 5).

In the host genotypes, single fungal-hair cell interactions resulting in HR ranged from 1.2% in cv. Northern Brewer to 45.3% in the wild hop 2002 185 002 (Figure 8A-C, Table 5). Cultivars and breeding lines, which derived from the German breeding program, exhibited HR in less or equal than 6% of all single fungal-hair cell interactions. The more cell death reactions occurred in the investigated host genotypes, the less haustoria and elongated secondary hyphae were detected. HRs of hair cells were generally less frequently observed than HRs of normal epidermal cells, for which the HR was the predominant resistance mechanism. In the non-host interaction, hair cells underwent only in 5.1% of the interactions an HR (Figure 8D, Table 5). In all investigated interactions, early HR before as well as late HR after the formation of elongated secondary hyphae were detected

Penetration attempts of *Pm* on leaves of the host genotypes resulted in the formation of papillae in 0.3 to 6.1% of single fungal-hair cell interactions (Figure 8E, Table 5). Thus, papillae occurred more frequently in hair cells than in normal epidermal cells (see chapter 3.1.1). In the non-host interaction, 12.5% of the penetration attempts of *E. cruciferarum* into hair cells led to the formation of papillae (Figure

8F, Table 5). Hence, papillae were more frequently detected in this non-host interaction than in the host interactions.

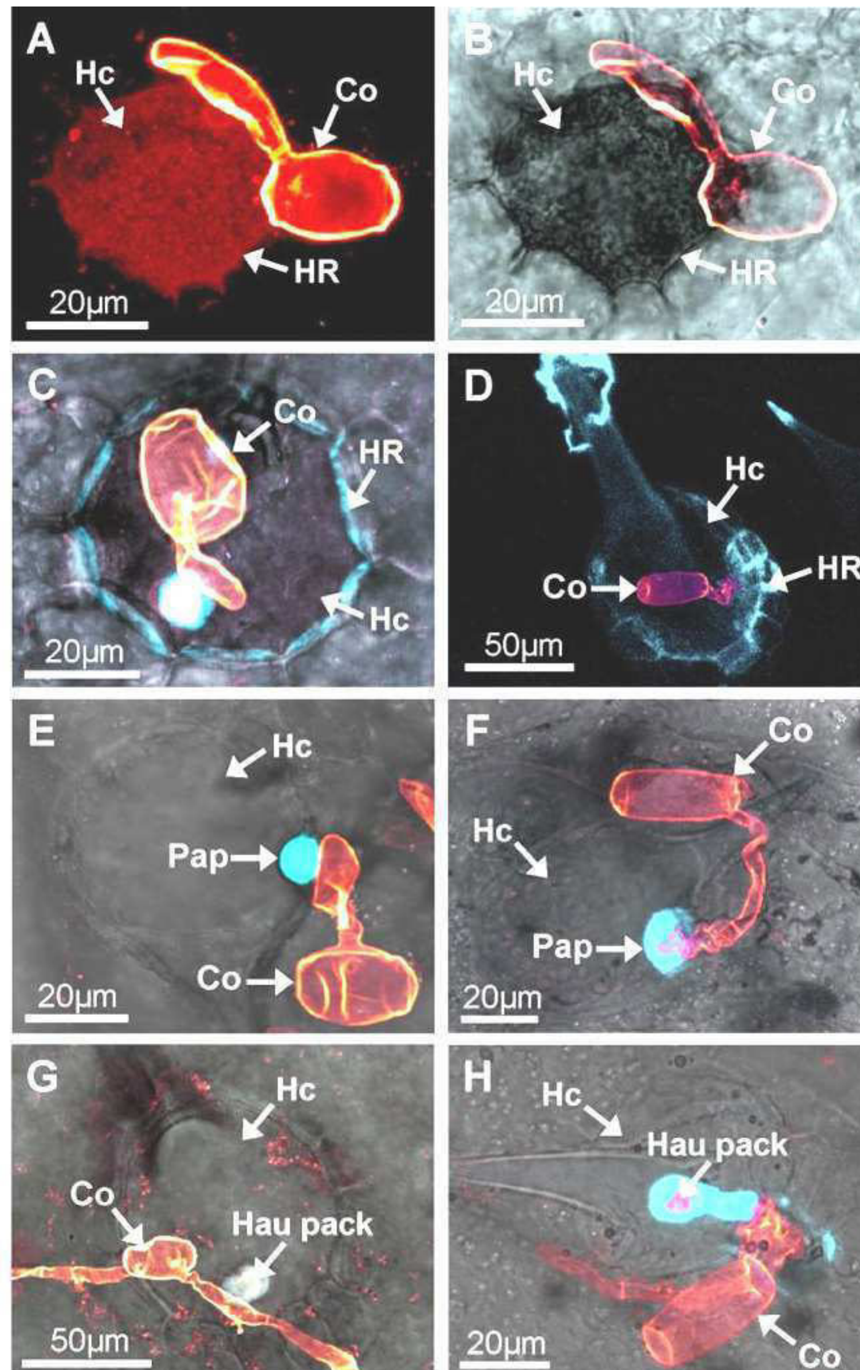

Figure 8: Observed defence reactions of hop leaf hairs in host and non-host interaction with *Pm* and *E. cruciferarum*. Pictures were taken at 48 hai with a confocal laser-scanning microscope and show maximum projections of z-stacks, pictures B, C, E, F, G and H show an overlay with single projections of the transmission channel. Fungal structures were stained with red fluorescing wheat germ agglutinin tetramethylrhodamine (WGA-TMR). Co = conidia, Hc = hair cells, Hau pack

= packed haustoria in callose, HR = hypersensitive reaction, Pap = papilla. **A-D,** germinated conidia of *Pm* (A-C) and *E. cruciferarum* (D) attacked hair cells of wild hop 2006 268 001 (A-C) and cv. Northern Brewer (D) that responded with an HR. Whole-cell autofluorescence (A), discoloration visible in the transmitted light channel (B) or staining of callose depositions with aniline blue (C, D) were taken as reliable signs for cell death. The pictures are maximum projections of 16 optical sections at 3 µm increments in A and B, 9 optical sections at 3 µm increments in C and 12 optical sections at 2 µm increments in D. **E-F,** penetration attempts of germinated conidia of *Pm* (E) and *E. cruciferarum* (F) on cv. Herkules (E) and cv. Northern Brewer (F) resulted in the development of a callose-containing papilla. The pictures are maximum projections of 34 optical sections at 0.63 µm increments in E and 47 optical sections at 0.63 µm increments in F. **G-H,** germinated conidia of *Pm* (G) and *E. cruciferarum* (H) penetrated hair cells of wild hop 2006 268 001 (G) and cv. Northern Brewer (H). The attacked hair cells packed haustoria in callose. The pictures are maximum projections of 21 optical sections at 3 µm increments in G and 13 optical sections at 2 µm increments in H.

In 1.1 to 10.4% of single fungal-hair cell interactions, haustoria of *Pm* were packed in callose (Figure 8G, Table 5). Normal epidermal cells did not show this defence reaction (see chapter 3.1.1). In 10.1% of non-host interaction sites, single hair cells packed haustoria of *E. cruciferarum* in callose (Figure 8H, Table 5).
In the host as well as in the non-host interaction, a considerable number of conidia neither penetrated hair cells nor triggered defence reactions. The frequency of this observation ranged from 17.4% in the host interaction of cv. Northern Brewer with *Pm* to 59.3% in the non-host interaction of this cultivar with *E. cruciferarum* (Table 5). The frequency of the occurrence of this type of interaction seemed to be related to reduced development of elongated secondary hyphae.

Table 5: Microscopic assessment of single-cell interaction phenotypes between *E. cruciferarum* or *Pm* and hop leaf hairs at 48 hai and sporulation events at 7 dai

		48 hai[a]						7 dai[b]
Genotype	Pathogen	% (±SD) HI	% (±SD) Esh	% (±SD) HR	% (±SD) Pap	% (±SD) Hau pack	% (±SD) no def	Spor
Cv. Northern Brewer	*Pm*	82.9 ± 3.9	78.8 ± 4.2	1.2 ± 0.9	1.5 ± 1.0	1.1 ± 0.8	17.4 ± 2.8	yes
BL 093 010 036	*Pm*	74.5 ± 15.6	68.5 ± 17.7	2.0 ± 0.4	5.2 ± 2.4	2.6 ± 0.8	21.7 ± 15.7	yes
BL 2002 047 011	*Pm*	75.6 ± 6.6	66.8 ± 10.8	4.9 ± 3.4	1.6 ± 0.9	1.3 ± 1.3	25.6 ± 7.7	yes
Cv. Hall. Merkur	*Pm*	72.2 ± 12.7	64.4 ± 15.9	2.3 ± 2.1	1.8 ± 2.0	1.3 ± 1.4	30.2 ± 15.5	yes
WH 2001 137 001	*Pm*	65.3 ± 7.1	60.0 ± 8.5	3.8 ± 2.6	2.7 ± 2.4	1.2 ± 0.7	32.4 ± 8.7	yes
Cv. Hall. Herkules	*Pm*	69.4 ± 3.8	57.7 ± 1.1	6.0 ± 3.1	6.1 ± 3.9	4.5 ± 1.9	25.6 ± 3.4	yes
WH 2002 186 740	*Pm*	71.5 ± 10.5	48.7 ± 6.8	11.9 ± 10.1	2.6 ± 2.0	7.9 ± 7.3	29.0 ± 12.1	yes
WH 2002 186 047	*Pm*	65.1 ± 9.7	48.2 ± 14.4	4.0 ± 1.1	3.3 ± 3.5	10.4 ± 2.5	34.2 ± 11.9	no
WH 2001 139 003	*Pm*	59.5 ± 16.7	43.8 ± 28.8	11.7 ± 16.2	5.1 ± 2.5	4.1 ± 1.8	35.2 ± 11.7	yes
WH 2002 182 001	*Pm*	50.3 ± 7.0	31.0 ± 9.6	23.0 ± 13.8	6.0 ± 1.5	3.5 ± 2.3	36.5 ± 6.5	yes
WH 2002 185 002	*Pm*	55.2 ± 6.2	19.4 ± 1.3	45.3 ± 20.7	2.2 ± 2.9	1.5 ± 2.6	31.6 ± 18.4	yes
WH 2006 268 001	*Pm*	47.9 ± 7.8	13.0 ± 3.2	41.4 ± 13.7	0.3 ± 0.5	1.2 ± 2.0	44.1 ± 13.6	no
Col-0	*Ec*	n.v.	74.1 ±11.0	n.v.	n.v.	n.v.	n.v.	yes
Cv. Northern Brewer	*Ec*	26.5 ± 12.3	13.1 ±12.1	5.1 ± 2.0	12.5 ± 6.2	10.1 ± 2.2	59.3 ±13.3	no

[a] Data represent mean frequencies of three independent experiments and corresponding standard deviations. In one experiment, at least 55 fungal-hair cell interactions were evaluated. The susceptible cv. Northern Brewer served as a control in the host interactions with *Pm* and the susceptible *Arabidopsis* accession Col-0 served as a control in the non-host interaction with *E. cruciferarum* (*Ec*). The haustorial index (HI) includes rudimentary and mature haustoria as well as haustoria packed in callose. Fungal germlings that penetrated hair cells, established mature haustoria and formed elongated secondary hyphae (Esh) were assessed. Defence reactions of normal epidermal cells to *Pm* conidia were categorized into HRs, the formation of effective papilla (Pap) and haustoria packed in callose (Hau pack). At some interaction sites, fungal germlings neither penetrated normal epidermal cells nor triggered defence reactions (no def). Mean frequencies of elongated secondary hyphae, HR cells, papilla formation, haustoria packed in callose and non-penetrated hair cells that showed no defence reaction add up to 100%.

[b] Occurrence of sporulating colonies (Spor) on whole leaves was evaluated at 7 dai. Three independent experiments, each experiment with one to three replicates were performed. A positive entry means, that in at least two of the three experiments sporulating colonies were found. A dense mycelium and numerous conidiophores covered the control leaves of cv. Northern Brewer and *Arabidopsis* wild-type Col-0 at this time point. BL = advanced breeding line, WH = wild hop, n.v. = no value.

3.2.2 Composition of the epidermal layer of hop leaves

To obtain a hint on the biological significance of the cell type-specific susceptibility of hop hair cells, I investigated the proportion of the leaf surface covered by hair cells for the second and the third leaf pair from the top (Figure 9A) of cv. Northern Brewer. The proportion of the leaf surface covered by hair cells strongly depends on the leaf age because normal epidermal cells enlarge during their development and was 17.6% on the second leaf pair from the top and decreased to 8.6% on the following leaf pair (Figure 9B).

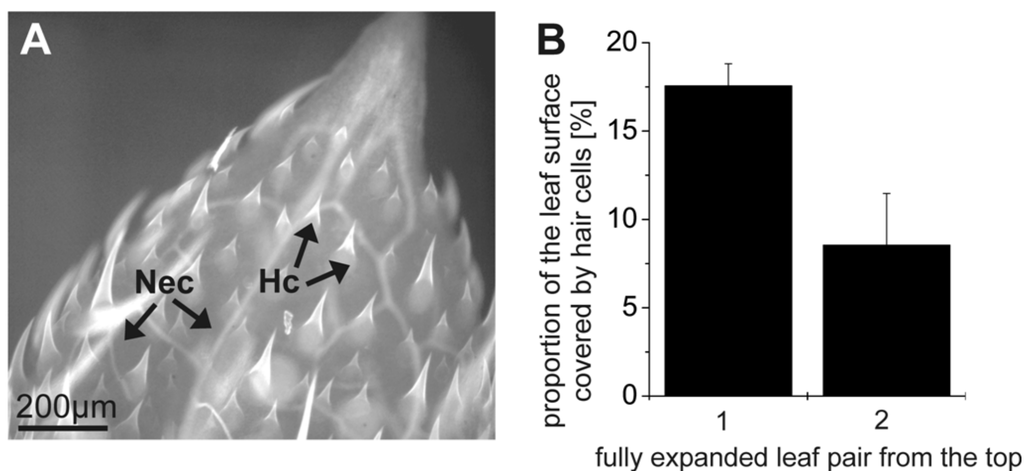

Figure 9: Epidermal layer of a young hop leaf. Hc = hair cells, Nec = normal epidermal cells. **A,** Epifluorescence picture of a young hop leaf with numerous hair cells exhibiting autofluorescence. Normal epidermal cells (not visible) are lying in-between the hair cells. **B,** Proportion of the leaf surface covered by hair cells. The second leaf pair from the top, which is most susceptible to the powdery mildew fungus, and the following leaf pair were examined. Columns represent mean values of three technical repetitions. Bars represent standard deviations. Area measurements were carried out with the ImageJ software.

3.2.3 Nuclear DNA contents in hair cells and normal epidermal cells

I compared the DNA content of hair cells to the DNA content of normal epidermal cells because simple trichomes from *Arabidopsis* show endopolyploidy (Melaragno et al., 1993) and endopolyploidy was reported to be associated with powdery mildew susceptibility (Wildermuth, 2010). The DNA of 20 hair cell nuclei was stained with DAPI, and size and DAPI fluorescence of each nucleus was visually compared to that of three nuclei of surrounding normal epidermal cells. Nuclei of hair cells were always slightly larger than nuclei of normal epidermal cells but showed considerably reduced DAPI fluorescence (Figure 10A). These results and the fact that hair cells have about 13 times the size of normal epidermal cells (Figure 10B,C) indicate a lower DNA content to cell size ratio of hair cells in comparison to normal epidermal cells.

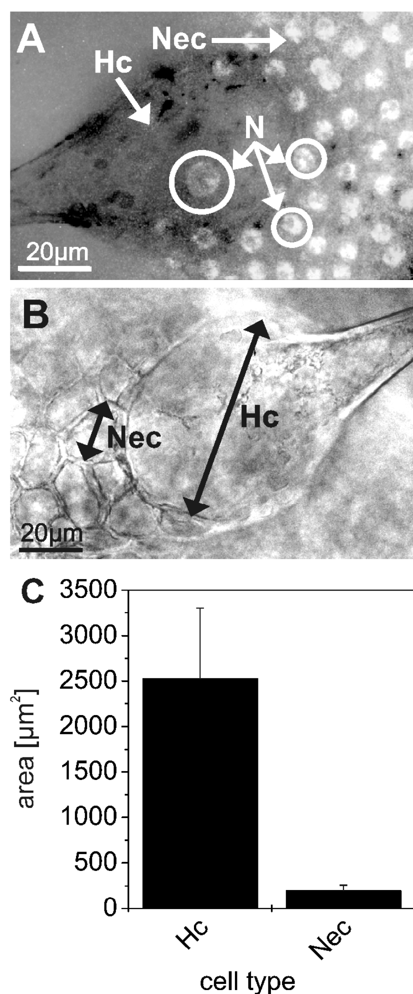

Figure 10: Relationship of DNA content and cell area of leaf hairs and normal epidermal cells. Hc = hair cell, Nec = normal epidermal cells, N = nucleus. **A,** DAPI stained nuclei of the two different epidermal cell types. The nucleus of the hair cell is bigger and DAPI staining is weaker compared to nuclei of the surrounding normal epidermal cells. The picture was taken with a confocal laser-scanning microscope and shows an overlay with single projections of the transmission channel to localize the nuclei in the different epidermal cell types. The picture is a maximum projection of a z-stack with 20 optical sections at an increment of 1 µm. **B,** Light microscope picture displaying the difference between the cell area of a hair cell and a normal epidermal cell. **C,** Mean area of hair cells in comparison to normal epidermal cells. Columns represent mean cell areas of 20 cells. Bars represent standard deviations. All experiments were performed with leaves from cv. Northern Brewer. Note that cell areas depend on the particular age of the leaves and probably also on the genotype taken for measurements and thus are within a certain range. Cell area measurements were carried out by analyzing light microscope pictures with the ImageJ software.

3.3 Establishment of the transient transformation assay for the assessment of gene function in hop-powdery mildew interactions

3.3.1 Comparison of plant epidermal and fungal cell areas

In barley and wheat, a transient transformation assay, based on the transformation of single epidermal cells by particle bombardment, followed by an inoculation with the powdery mildew fungus is routinely applied to identify resistance-associated genes. In this part of the work, I focused on the establishment of a transient transformation assay for hops, as for this crop a strong need for such a tool to support the breeding process exists.

The area of the epidermal cell is crucial for the feasibility and the validity of the transient transformation assay because an inoculation density of one conidium per

epidermal cell is aspired (Schweizer et al., 1999), and leaves composed of smaller epidermal cells consequently need a higher inoculation density. However, a too high inoculation density limits the evaluation because fungal spores and hyphae might lie on top of each other. To explore the suitability of hop epidermal cells for the transient transformation assay, I compared the areas of normal hop epidermal cells and hair cells, which both interact with the powdery mildew fungus, to the area of barley interstomatal epidermal cells (Koga, 1990). Barley epidermal cells were chosen for this comparison because a transient transformation assay is well established for the pathosystem barley-*B. graminis* f.sp. *hordei* (Nielsen et al., 1999; Shirasu et al., 1999). Cell areas were measured by analyzing pictures of the respective cells with the ImageJ software. The normal hop epidermal cells of cv. Northern Brewer had a mean area of 198 µm^2 and were in average 22 times smaller than barley epidermal cells that showed a mean cell area of 4386 µm^2. Hop hair cells were also smaller than barley epidermal cells, but with an area of 2529 µm^2 about 13 times larger than normal epidermal cells. Conidia of *Pm* had about 356 µm^2 and therefore had about the same area as normal hop epidermal cells (Figure 11). It is important to note that cell areas of normal hop epidermal cells strongly depend on the particular age of the leaves and presumably also on the genotype taken for measurements and thus vary within a certain range (data not shown).

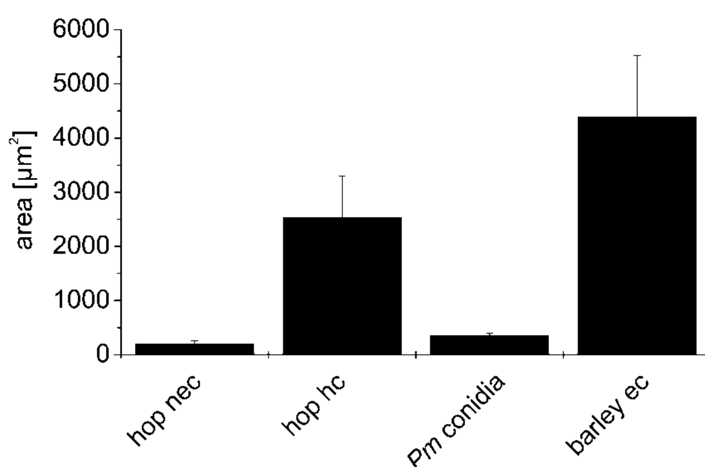

Figure 11: Areas of normal epidermal (nec) and hair cells (hc) of cv. Northern Brewer, *Pm* conidia and barley interstomatal epidermal cells (ec) of the cv. Golden Promise. Columns represent mean values of 15 to 20 cells measured by analyzing microscopic pictures of adaxial leaf surfaces with the ImageJ Software. Bars represent standard deviations.

3.3.2 Effect of different acceleration pressures on the transformation efficiency

For the successful transformation of epidermal cells, plasmid-coated gold particles have to penetrate the cuticle and the cell wall to reach the nucleus. Because the constitution of the leaf surface varies within different plant species and within different cell types of one species, the acceleration pressure for the coated gold particles has to be adapted in each experimental system. Therefore, I carried out particle bombardment of leaves of cv. Northern Brewer at acceleration pressures of 650, 900 or 1100 psi with the pGY-1-GFP reporter gene construct. After one day, I analyzed transformation rates of hair cells and normal epidermal cells separately.

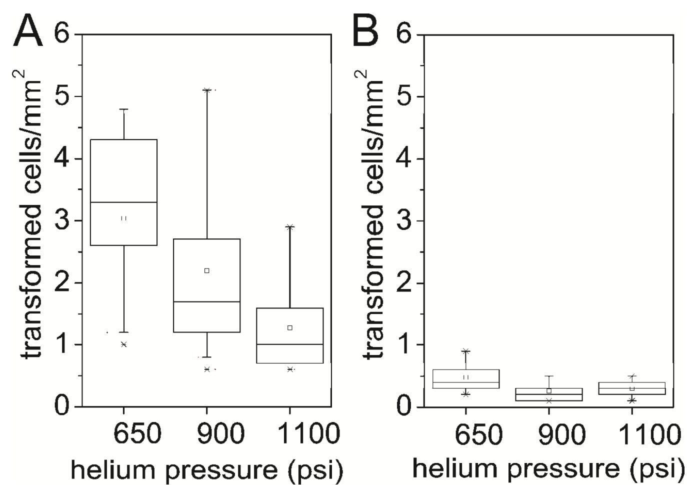

Figure 12: Transformation rates of normal epidermal cells and hair cells as a function of different helium pressures. Gold particles were coated with pGY-1-GFP and delivered into epidermal cells of cv. Northern Brewer with an acceleration pressure of 650, 900 and 1100 psi. GFP-expressing cells per leaf were determined one day after bombardment with an epifluorescence microscope and the leaf area was measured with the ImageJ software. **A,** box plots displaying transformation rates of normal epidermal cells. **B,** box plots displaying transformation rates of hair cells.

Figure 12 shows box plots displaying the transformation rates at different acceleration pressures of normal epidermal cells (Figure 12A) or hair cells (Figure 12B). The transformation rates of normal epidermal cells ranged between 0.1 and 5.1 cells per mm^2 and were higher than transformation rates of hair cells, which ranged from 0.1 to 0.9 cells per mm^2. In both cell types, an acceleration pressure of 650 psi seemed to result in the highest transformation rates.

3.3.3 Determination of co-expression rates

After identifying the most suitable cell type and the optimal acceleration pressure, in a last step the co-expression rates should be checked to establish a transient transformation assay. This is important because the transient transformation assay is based on the co-expression of a reporter gene and a knock- down or overexpression construct in single epidermal cells. To estimate co-expression, gold particles coated with pe35AscIoptRed and pGY-1-GFP plasmids were shot into hair cells. To evaluate the experiment, I investigated GFP-expressing hair cells three days after bombardment for DsRed fluorescence. Co-expression rates of GFP and DsRed were determined in three independent experiments resulting in a mean of 91.3% ± 1.5.

3.4 Proof of principle of the transient transformation assay by means of functional analysis of a putative hop *MLO* gene

3.4.1 Identification and phylogenetic classification of a putative hop *MLO* gene

I chose a putative hop *MLO* EST (GenBank accession number: ES653367) as a candidate gene for proof of principle of the transient transformation assay. *mlo* resistance reported from other plant species is a very promising approach in breeding for powdery mildew resistance because MLO proteins are susceptibility factors in plant-powdery mildew interactions and are obviously exploited by the fungus for penetration (Panstruga, 2005). Knockdown of *MLO* gene expression or naturally occurring loss-of-function mutations resulted in decreased powdery mildew susceptibility in barley, *Arabidopsis,* tomato and pea (Büschges et al., 1997; Consonni et al., 2006; Bai et al., 2008; Humphry et al., 2011; Pavan et al., 2011). Therefore, I expected an effect of the knockdown of hop *MLO* expression on the hop-powdery mildew interaction. I used the *Vitis vinifera* (*Vv*) protein sequence VvMLO4 (GenBank accession number CAO21819, Feechan et al. 2008, superceded by XP_002282216) for a tblastn search in the *H. lupulus* EST database (tissue type: trichome) of the NCBI GenBank and identified a partial putative hop *MLO* cDNA with a length of 799 bp (GenBank accession number: ES653367). RACE PCRs

should provide the full-length sequence of the 799 bp fragment but unfortunately they did not work (data not shown). Nevertheless, this study focused on *MLO* knockdown experiments to evaluate a possible implementation of hop *mlo* resistance in practice. A significant accord with another, shorter hop sequence indicates at least the presence of another hop *MLO* gene. I chose also five partial sequences of hop *R* gene analogues (Kozjak et al., 2009), 14 partial sequences of hop stress-related genes (Fortes et al., 2008) and a putative hop *BAX INHIBITOR-1* EST (GenBank accession number: EX521329) as possible candidate genes that are listened in the supplement (Supplementary Table 3, Supplementary Figure 1).

I performed a phylogenetic analysis with the deduced partial amino acid sequence of the putative hop MLO protein with all *Arabidopsis* (AtMLO1-AtMLO15) and grapevine MLO proteins (VvMLO1-VvMLO17). The classification of this putative hop MLO amino acid sequence is of interest because expression and functional analysis of MLO proteins from *Arabidopsis*, tomato and grapevine and pea indicate that in dicotyledonous plants MLO proteins that are important for powdery mildew susceptibility are assigned to clade V (Devoto et al., 2003; Chen et al., 2006; Consonni et al., 2006; Bai et al., 2008; Feechan et al., 2008; Humphry et al., 2011; Pavan et al., 2011). Figure 13 shows the phylogenetic relationship of the deduced, partial amino acid sequence of the putative hop MLO protein and of *Arabidopsis* and Grapevine MLO proteins. Clustering of the putative hop MLO amino acid sequence together with AtMLO2, AtMLO6, AtMLO12, VvMLO3, VvMLO4, VvMLO6, VvMLO9, VvMLO13 and VvMLO17 suggests a classification into clade V. Within clade V, VvMLO4 seems to be the closest relative of the putative hop MLO protein. MLO proteins for which an upregulation after powdery mildew attack was reported are marked with an asterisk.

Figure 14 shows a closer investigation of the sequence conservation between the clade V MLO proteins from *Arabidopsis* and grapevine and the putative hop MLO amino acid sequence. The putative hop MLO protein sequence spans the transmembrane domains 3 to 7. Lines above the sequences indicated these transmembrane domains, which are typical for MLO proteins (Devoto et al., 2003; Feechan et al., 2008).

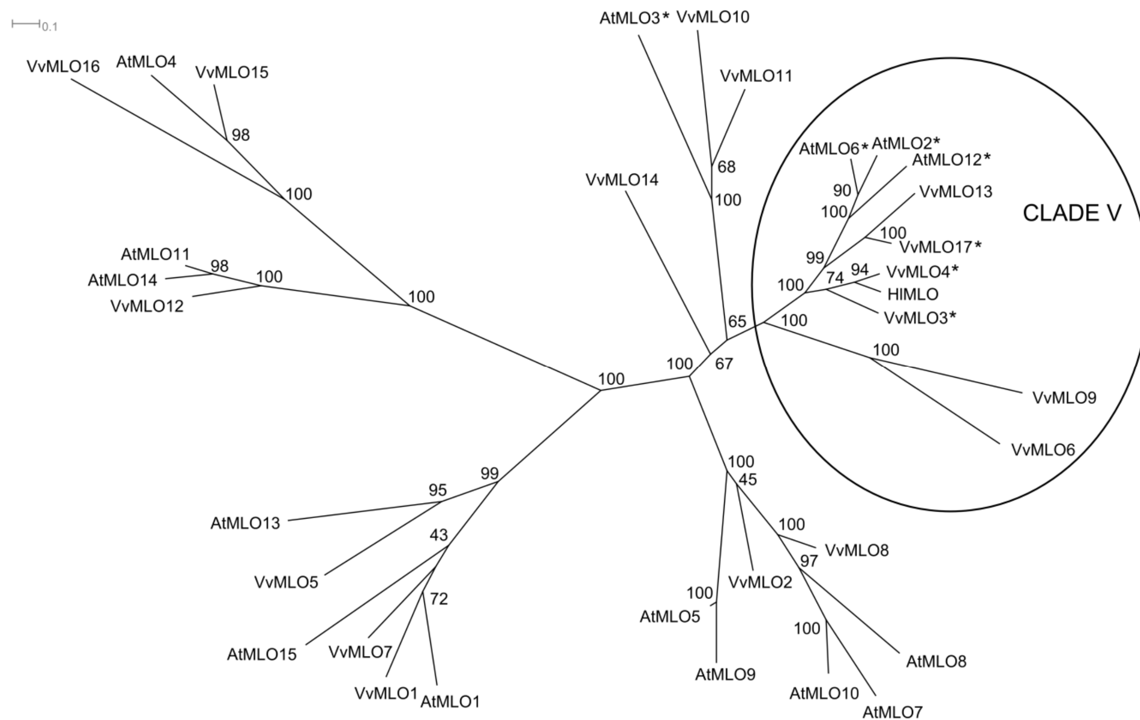

Figure 13: Best scoring maximum likelihood tree presenting the phylogenetic relationship of the putative hop MLO amino acid sequence to the *Arabidopsis* and grapevine MLO proteins. The alignment of the amino acid sequences was performed with T-coffee and maximum-likelihood phylogeny estimation was conducted using RAxML. Numbers show bootstrap values from 100 replicates. Asterisks indicate that the respective genes are significantly upregulated after inoculation with powdery mildew fungi (Chen et al., 2006; Feechan et al., 2008). Sequences were obtained from GenBank and accession numbers are: AtMLO1 (CAB08605), AtMLO2-15 (AAK53795-AAK53808), VvMLO1 (CAO41068, superceded by XP_002266377), VvMLO2 (CAO66267, superceded by XP_002273026), VvMLO3 (CAO18135, superceded by XP_002275390), VvMLO4 (CAO21819, superceded by XP_002282216), VvMLO5 (CAO22254, superceded by XP_002266927), VvMLO6 (CAO66388, superceded by XP_002273434), VvMLO7 (CAO46388, superceded by XP_002280697), VvMLO8 (CAO71699, superceded by XP_002276608), VvMLO9 (CAN84002, superceded by XP_002275487), VvMLO10 (CAO18134, superceded by XP_002275360), VvMLO11 (CAO21818, superceded by XP_002282198), VvMLO12 (CAO39251, superceded by XP_002282190), VvMLO13 (CAO68971, superceded by XP_002274608), VvMLO14 (CAO66265, superceded by XP_002273002), VvMLO15 (CAO47031, superceded by XP_002266144), VvMLO16 (CAO48195, superceded by XP_002265520), VvMLO17 (CAO68972, superceded by XP_002274642). *At* = *A. thaliana*, *Vv* = *V. vinifera*, *Hl* = *H. lupulus*.

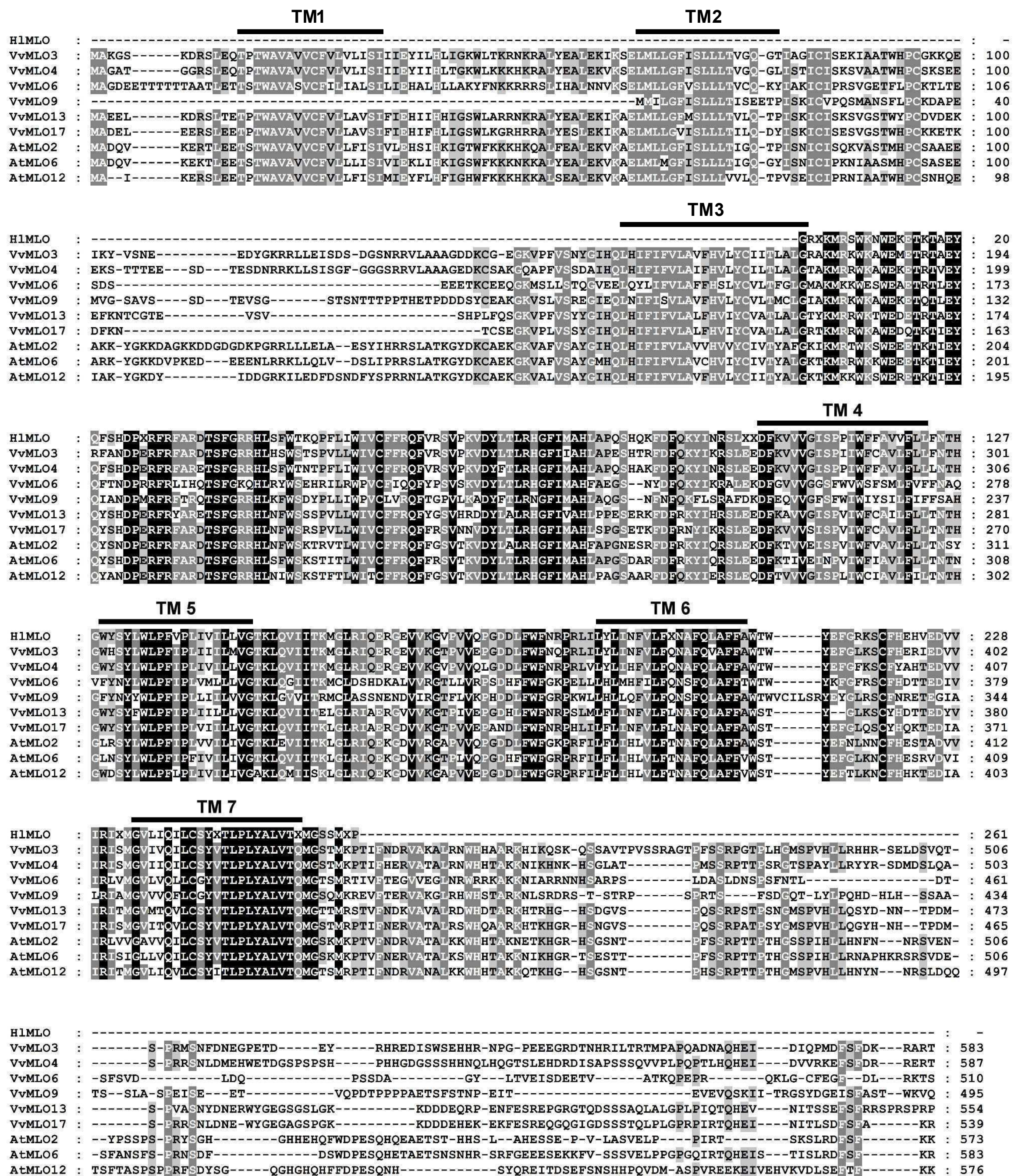

Figure 14: Alignment of the partial sequence of the putative hop MLO protein with the partial amino acid sequences of *A. thaliana* and *V. vinifera* MLO proteins of clade V. Transmembrane (TM) regions 1 to 7 are indicated by lines above the sequences (Devoto et al., 1999; Feechan et al., 2008). The alignment was performed with ClustalW and visualized with Gendoc. Sequences were obtained from GenBank (see Figure 13).

3.4.2 Expression analysis of the putative hop *MLO* gene in response to *P. macularis* infection

MLO genes from barley, *Arabidopsis* or grapevine associated with powdery mildew susceptibility are upregulated before or during fungal penetration (Piffanelli et al., 2002; Chen et al., 2006; Feechan et al., 2008). In order to elucidate the possible role of the putative hop *MLO* gene in the hop-powdery mildew interaction, I carried out expression analyses in the susceptible cv. Northern Brewer and in the resistant cv. Wye Target. After inoculation with the powdery mildew fungus, leaf samples were taken at 0, 4, 6, 8, 10, 24, 48 and 168 hai, and a semi-quantitative reverse transcription (RT) PCR was performed, whereby the expression of *UBIQUITIN* served as a control for equal amounts of PCR template. Transcript levels of the putative hop *MLO* gene increased between 4 to 6 hai in the susceptible cv. Northern Brewer. Increase in transcript levels was delayed in the resistant cv. Wye Target and peaked between 8 and 10 hai (Figure 15).

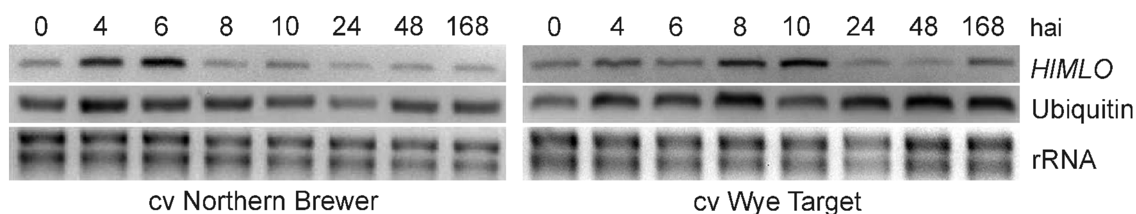

Figure 15: Temporal expression profile of the putative hop *MLO* gene in the susceptible cv. Northern Brewer and in the resistant cv. Wye Target after inoculation with *Pm*. RNA was isolated from leaves inoculated with conidia of *Pm*. Time points of sampling were 0, 4, 6, 8, 10, 24, 48 and 168 hai. RNA quality and equal loading was confirmed by ethidium bromide staining of rRNAs after electrophoresis. Amplification of a *UBIQUITIN* cDNA fragment (26 cycles) served as a constitutive control. The cDNA fragment of the putative hop *MLO* gene was amplified with 31 cycles. The figure shows inverted pictures of ethidium bromide stained gels.

Expression profiles of some of the other candidate genes are listed in the supplement as work was not continued with these sequences (Supplementary Figure 1).

3.4.3 Transient-induced gene silencing of the putative hop *MLO* gene

After establishing a protocol for the transient delivery of expression vectors into hop hair cells and identifying an appropriate candidate gene, suitability of the transient transformation assay for gene function analysis in hop should be estimated.

In order to confirm this, I performed a transient knockdown of the putative hop *MLO* gene in hair cells of the powdery mildew susceptible cv. Northern Brewer. For this purpose, hair cells were cotransformed by particle bombardment with the reporter gene construct pUbi-GUS and the TIGS construct pIPKTA30N-MLO or the empty pIPKTA30N vector as a control. After inoculation, the fungus was allowed to develop, leaves were stained for GUS activity, and the outcome of interactions of single fungus-transformed hair cell was evaluated by microscopy. A modified susceptibility index (Douchkov et al., 2005), which usually gives the percentage of GUS-stained transformed hair cells containing at least one mature haustorium, was calculated as a measurement for susceptibility. Here, only hop cells with an appressorium on or nearby were evaluated. In each variant 50 to 260 transformed hair cells were included into the evaluation. Figure 16A shows an example of a GUS-expressing transformed hair cell containing two haustoria of *Pm.* In seven experiments, the mean susceptibility index after TIGS of *MLO* was significantly reduced in comparison to the empty vector control by 9.8 % (Student's *t*-Test, $p<0.01$, Figure 16B).

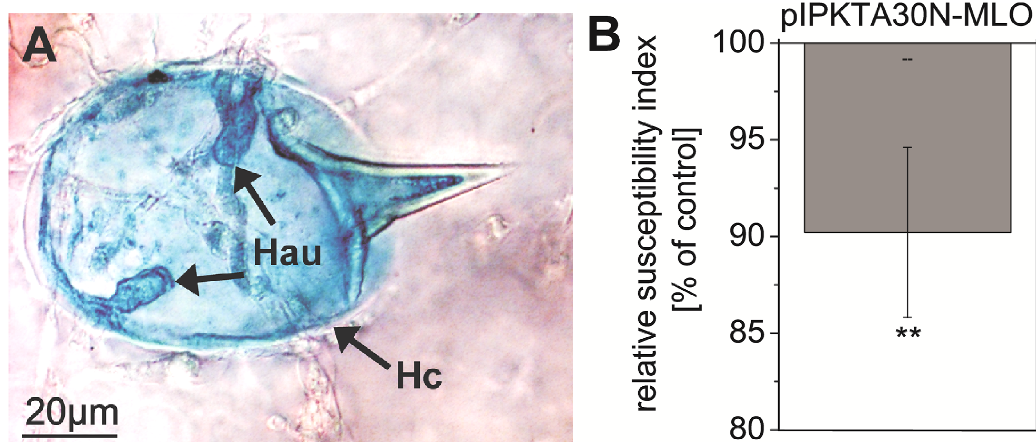

Figure 16: Transient-induced silencing of the putative hop *MLO* gene in hop leaf hairs. Gold particles were coated with a 2 to 3 ratio of the reporter gene construct pUbi-GUS to the pIPKTA30N-MLO or the pIPKTA30N empty vector control, respectively, and were transiently delivered into leaf hairs of cv. Northern Brewer. After three days, leaves were inoculated with conidia of *Pm* and another three days later stained for GUS activity. The outcome of the interaction of the single transformed hair cell with *Pm* was evaluated by microscopy. Hc = hair cell, Hau = haustorium. **A,** Single-cell interaction of *Pm* with a GUS-expressing hair cell. The hair cell contains two haustoria of *Pm*. To obtain a picture with an extended depth of field, a stack of light microscope

pictures was processed with the CombineZP software (http://www.hadleyweb.pwp.blueyonder.co.-uk/CZM/combinezm.htm). **B,** Transient-induced silencing of the putative hop *MLO* gene significantly reduces the susceptibility index (Student's *t*-Test, $p<0.01$). The susceptibility index is indicated relative to the empty vector control, which was set to 100%. The column represents the mean of seven independent experiments. In each independent experiment, 50 to 260 fungus-transformed hair cell interactions were evaluated for both the control and the TIGS variant. Altogether 908 single fungus-transformed hair cell interactions were examined in the control variant and a total of 976 in the TIGS variant. The bar represents the 95% confidence interval.

4 Discussion

The combination of different spatio-temporal defence reactions in one cultivar is proposed to mediate long-term powdery mildew resistance (Prats et al., 2007; Fernández-Aparicio et al., 2009). Therefore, detailed information about cellular defence reactions in plant-powdery mildew interactions as well as their timing could improve breeding. In the present study, resistance of twelve powdery mildew resistant hop genotypes was investigated. In these genotypes, resistance relies on early and late, single and multicellular HRs whereas pre-penetration resistance associated with papilla formation plays no obvious role. Also in the non-host interaction of hop with *E. cruciferarum,* pre-penetration resistance associated with papilla formation did not notably contribute to resistance.

Hop hair cells show a cell type-specific susceptibility to adapted *Pm* and non-adapted *E. cruciferarum.* The second part of this study focused on the characterization of this phenotype because the literature provides little information about this phenomenon for hop-, and for other plant-powdery mildew interactions. The hair type-specific susceptibility was visible as small, sometimes sporulating colonies, spreading from a susceptible leaf hair in the centre. The growth of these colonies was limited because leaf hairs accounted only for about 1/5 of the leaf surface. Compared to normal epidermal cells of the same genotype, hair cells underwent less frequently a hypersensitive reaction and contained more haustoria of *P. macularis* and *E. cruciferarum*. Defence reactions usually assigned to basal resistance like the encasement of haustoria in callose or papilla formation as a sign for pre-penetration resistance were more frequently present in hair cells. Furthermore, hair cells showed a reduced DNA content to cell size ratio when compared to normal epidermal cells.

Another part of this work focused on the adaption of a particle bombardment-based transient transformation assay for the functional assessment of resistance-associated genes in hop, as it is already available for barley and wheat (Nielsen et al., 1999; Schweizer et al., 1999; Shirasu et al., 1999). The results presented here indicate that hop leaves should be transformed with an acceleration pressure of 650 psi and that only hair cells should be subjected to microscopy. Proof of princi-

ple was successfully performed by TIGS of a putative hop homolog of the powdery mildew susceptibility factor *MLO* that resulted in reduced powdery mildew susceptibility.

4.1 Spatio-temporal aspects of defence reactions of normal epidermal cells to *P. macularis* and non-adapted *E. cruciferarum*

4.1.1 Defence reactions observed in the interaction of resistant hop genotypes with *P. macularis* and non-adapted *E. cruciferarum*

Pm formed elongated secondary hyphae on the susceptible cv. Northern Brewer and on the macroscopically resistant wild hop 018 097 008 at 24 hai, whereas on the other resistant genotypes *Pm* formed only a single, short germ tube (Figure 4, Table 2). This indicates that in wild hop 018 097 008 a late acting resistance mechanism might be active (see chapter 4.1.2).

Staining of callose depositions with aniline blue, autofluorescence and/or cell reactions revealed that HRs terminate fungal growth in all investigated resistant hop genotypes, whereas pre-penetration resistance associated with papilla formation seems not to contribute to the resistance phenotype (Figure 4, Table 2). In similar studies conducted with hops, Godwin (1985) and Godwin et al. (1987) reported that the *R2*, *R4* and *R5* major genes confer an HR-based resistance before *Pm* forms elongated secondary hyphae. A pre-penetration resistance of hops to *Pm* has not been reported yet. Godwin (1985) assumed a penetration resistance to *Pm* for one partial resistant hop genotype as he neither detected haustorial initials nor signs for cell death in most single-cell interactions. However, he chose no experimental setup to detect papillae. Prats et al. (2007) investigated the host interaction of ten resistant and moderately resistant *Medicago truncatula* accessions with *E. pisi*. They found failed penetration attempts of *E. pisi* associated with papilla formation in at least 10% of the investigated interaction sites, including the susceptible control. Fernández-Aparicio et al. (2009) examined defence reactions of 15 partially resistant beet genotypes to the adapted *E. betae* and reported papilla formation at more than 50% of the interaction sites and penetration resis-

tance. The study of Feechan et al. (2011) likewise investigated the host interaction of partially resistant *Vitaceae* genera with *E. necator.* These experiments revealed penetration resistance (i.e. appressorium formation without signs of cell death) in about 20% of single cell interactions of susceptible genotypes and in about 40-90% of single-cell interactions of partial resistant and resistant genotypes. Bai et al. (2005), who investigated *R* gene-mediated, race-specific resistance of tomato genotypes to *Oidium neolycopersici,* found no papilla indicating penetration resistance. In line with the above described examples, the fact that pre-penetration resistance associated with papilla formation does not contribute to resistance to *Pm* supports the assumption that resistance of the investigated hop genotypes relies on *R* gene-mediated resistance. *R* gene-mediated recognition of fungal effectors often restricts fungal growth at the post-penetration stage by triggering a fast HR conferring ETI (Heath, 2000; Jones and Dangl, 2006). On the other hand, pre-penetration resistance is often achieved or accompanied by papillae in MAMP triggered immunity or basal resistance (Hückelhoven, 2007; Lipka et al., 2008; Niks and Marcel, 2009).

Similar to the host interactions of hop with *Pm,* in the non-host interaction of the hop cv. Northern Brewer with *E. cruciferarum* resistance also relied mainly on an HR, and pre-penetration resistance associated with papilla formation was observed only at 7% of the interaction sites (Figure 6, Table 4). This finding was surprising because I expected to find papilla formation that is typical for non-host plant-powdery mildew interactions (Lipka et al., 2005; Niks and Marcel, 2009). However, it is difficult to detect haustoria of *E. cruciferarum* beneath the lobed appressoria and therefore the exact time point at which the HR is triggered in this non-host interaction could not be determined. Taken together, the lack of pre-penetration resistance associated with papilla formation in the interaction of hops with adapted and non-adapted powdery mildew fungi raises the question if this kind of resistance plays a role in defence of hop to powdery mildew fungi at all. This question would be of interest for further studies, especially as, in contrast to the well-characterized *R* gene-mediated HR, the molecular basis of papilla formation is less well understood (Hückelhoven, 2007; Underwood, 2012).

Interestingly, I detected genotype-dependent differences in single- or multicellular HRs at the site of primary *Pm* attack in the host interactions (Table 3, Figure 4). This may account for a different genetic basis of resistance in these genotypes. Similar to these findings, Godwin (1985) and Godwin et al. (1987) mentioned multicellular epidermal and mesophyll HRs in the race-specific interaction of hop genotypes (harbouring the R2, the R4 or the R5 gene) with *Pm*. Furthermore, Bushnell (1981) and Hückelhoven et al. (2000) noted multicellular epidermal and mesophyll HRs of barley *Mla* lines in response to *B. graminis* f.sp. *hordei*. Bai et al. (2005) reported multicellular HRs in *R* gene-mediated race-specific resistance of tomato to *O. neolycopersici*.

4.1.2 Timing of the defence reactions

Microscopic investigations at 7 dai confirmed the assumption that a late acting resistance mechanism confers resistance in the wild hop 018 087 008 (see chapter 4.1.1). At 24 hai, elongated secondary hyphae and no signs of cell death were visible at the interaction sites. Microscopic investigations at 7 dai revealed that at this time point more than 87% of attacked cells beneath primary and secondary appressoria had undergone an HR (Figure 5). Although the exact time point of this late HR was not determined in the present study, the fact that in many cases colonies remained relatively small indicated a time point before 7 dai. In hops, another late acting defence reaction is known, the so-called blister reaction conferred by the *RB* gene. Leaves showing this reaction are marked my raised blisters that are covered by thin mycelium and only a few sporulation events and sometimes by necrotic patches. This late acting defence mechanism probably relies on a delayed or incomplete HR (Liyanage, 1973; Royle 1978). Sedlavora et al. (2009) reported a similar blister reaction in the interaction of a resistant melon accession with *P. xanthii.* However, the wild hop 018 097 008 did not show macroscopically visible similarities to the blister reaction. Boyd et al. (1995) described *Mla3* and *Mla7* barley genotypes that perform a combined, extended mesophyll and epidermal HR after *B. graminis* f. sp. *hordei* has formed elongated secondary hyphae. Thus, the delayed HR of wild hop 018 097 008 might be similar to the *Mla3* and *Mla7* resistance in barley. Unfortunately, a newly emerged *Pm* race broke the resistance of

wild hop 018 097 008 in the green house in 2008 (A. Lutz, LfL, Freising, Germany, pers. comm.). However, this genotype might still be interesting for pyramiding different resistances in new cultivars because it is still resistant in the field to date.

Genotypes that stopped *Pm* development within 24 hai without elongated secondary hyphae being visible probably triggered the HRs at different time points, too. For example, HR cells of some genotypes like wild hop 2001 137 001 contained nearly no haustoria (1.7%) while in others up to 72% of HR cells contained rudimentary haustoria (Figure 4, Table 2). This indicates that *Pm* triggered the HR at the pre- and at the post-penetration stage, respectively. Here the study of Liyanage (1973), who investigated the early development of *Pm* on cv. Northern Brewer gives a hint for the exact time point. He found that *Pm* forms appressoria starting from 6 hai and mature haustoria starting from 12-15 hai. Although HR triggered at the appressorial germ tube stage seems to be rare in plant-powdery mildew interactions, this is also the basis of the race-specific *Mlg* resistance in barley (Görg, 1993). Here, the authors assumed that either fungal effectors are recognized earlier or alternatively the signalling leading to the HR is faster. However, to confirm the absence of rudimentary haustoria in the hop-powdery mildew interaction, confocal laser-scanning microscopy should be conducted. Probably, epifluorescence microscopy does not allow the identification of small haustoria lying under the swollen appressoria in every case.

4.2 Cell specificity in the hop-powdery mildew interaction

4.2.1 Characterization of the hair cell-specific powdery mildew susceptibility and postulated explanations

Despite the fact that host and non-host resistance of all hop genotypes investigated in this study relied on an HR of normal epidermal cells (see chapter 3.1.1 and 3.1.2), *Pm and E. cruciferarum* successfully penetrated hair cells, developed mature haustoria and formed elongated secondary hyphae at 48 hai (Table 5). In some genotypes like breeding line 093 010 036 or breeding line 2002 047 011 nearly all penetrations of hair cells led to the formation of elongated secondary hyphae. In others, fungal growth was partially stopped at an earlier post-penetra-

tion stage (Table 5). At 7 dai, single sporulating *Pm* colonies spreading from a susceptible leaf hair in the centre were observed in nine of the twelve investigated host interactions. In contrast, *E. cruciferarum* did not sporulate on cv. Northern Brewer (Figure 7, Table 5). In most cases, *Pm* formed only single conidiophores, whereas Shirasu et al. (1999) showed that *B. graminis* f.sp. *hordei* is capable of forming sporulating colonies with many conidiophores originating from a single penetrated cell. Although *Pm* established sporulating colonies on most of the leaves investigated, only a small proportion of the fungal spores that formed elongated secondary hyphae reached this stage. This indicates that the fungus not always gains enough nutrients from of a single hair cell to form a sporulating colony. In addition, late defence reactions stopping fungal growth between the investigated 48 hai and 7 dai time points could have prevented sporulation of these colonies. Unfortunately, no defence reactions were recorded at the 7 dai time point. Probably late defence reaction also prevented sporulation of *E. cruciferarum* on cv. Northern Brewer.

In some genotypes like the wild hop 2002 185 002 or the wild hop 2006 268 001, HRs limited fungal growth of *Pm* (Figure 8, Table 5) to a certain degree. However, in the same genotypes, hair cells underwent an HR less frequently than normal epidermal cells. Furthermore, I observed an inverse correlation between the formation of elongated secondary hyphae and the HR among the investigated genotypes. Therefore, a lacking HR might be the reason for the cell type-specific susceptibility of hair cells to adapted and non-adapted powdery mildew fungi.

Other kinds of defence reactions, like papilla formation indicating pre-penetration resistance or callose-encasements of haustoria were hardly detectable in normal epidermal cells but were more frequent in hair cells (Figure 8, Table 5). These two defence reactions are often assigned to basal or non-host resistance (Thordal-Christensen 2003; Lipka et al., 2008; Niks and Marcel 2009; Wen et al., 2011). Haustorial encasements in hop hair cells are probably a consequence of the lacking HR and the resulting higher penetration rates. The observation that hair cells form papillae more frequently than normal epidermal cells suggests that different defence pathways are active in these two cell types. These two defence reactions

are likely only of minor importance to limit the susceptibility of the hair cells because they always accounted for less than 11% of the interaction sites.
At some interaction sites, fungal development was stopped although no defence reactions were visible (Table 5). This observation occurred more frequently in hair cells than in normal epidermal cells and seemed to be inversely related to the formation of elongated secondary hyphae. This indicates an invisible defence reaction, e.g. antifungal metabolites, contributing to resistance. Another possibility might be that not every HR was detected, as for hop hair cells common HR markers like callose depositions were not reliable and therefore autofluorescence and cell reactions that were visible as discolouration and cytoplasm granulation had to be employed as signs for an HR in the investigations, too.
Levels of hair cell-specific susceptibility varied among the investigated genotypes and the relative percentages of the observed defence reactions (HR, papillae, haustoria packed in callose) differed, again indicating a different genetic basis of resistance in the investigated genotypes. This assumption is of special interest for this study because normal epidermal cells limited fungal growth solely through an HR. Thus, the characterization of the hair type-specific susceptibility might extend the knowledge about *Pm* resistance that I gained from normal epidermal cells (see chapter 3.1.1 and 3.1.2).
Interestingly, the original German germplasm (without the recently introduced wild hops) comprising cv. Merkur, cv. Herkules, breeding line 093 010 036 and breeding line 2002 047 011 clustered together because hair cells of these genotypes were more susceptible than those of the wild hops (Table 5). This might reflect a better adaptation of the pathogen, loss of genetic resistance due to the breeding process or less genetic variation among these genotypes compared to the wild hops. The wide geographical origin of the wild hops (Japan, USA, Europe) indicates that the cell type-specific susceptibility of hop hair cells is a general feature of hops and not only a genetic defect of certain genotypes.

The cell type-specific susceptibility of hop hair cells is contradictory to the prevailing view in literature that assigns a defence related role to simple trichomes (i.e. hairs). In this context, Jakoby et al. (2008) reported that signalling pathways and

genes involved in stress response and defence like the biosynthesis of glucosinolates and flavanoids are up-regulated in *Arabidopsis* simple trichomes. Ebert et al. (2010), who performed a GC-MS (gas chromatography-time of flight-mass spectrometry) based metabolite profiling of *Arabidopsis* simple trichomes, pavement and basal cells, found significantly higher levels of trehalose and citric acid in cell saps of simple trichomes. They suggested a stress related role of simple trichomes as citric acid can function as a metal chelator in detoxification processes and trehalose is not only a storage carbohydrate and a transport sugar but also associated with the stress protection machinery (Callahan et al., 2006; Fernandez et al., 2010). Gutiérrez-Alcalá et al. (2000) proposed a role of trichomes as sinks during detoxification processes. Moreover, roles of simple trichomes in reducing the leaf wetness and therefore limiting fungal infections as well as a structural role in defence were reported (Johnson, 1975; Wagner et al., 2004; Kivimäki 2007). These examples show that the proven or proposed defence-related role of simple trichomes in certain pathosystems or plant species cannot be generalized. The reason for this may be that trichomes are highly specialized cells that vary considerably in their form and function and probably exert diverse functions among different plant species (Uphof, 1962; Johnson, 1975; Wagner et al., 2004).

The literature provides only little information about cell type-specificity in plant-powdery mildew or even in plant-pathogen interactions and is rather descriptive than explanatory. In the interaction of barley with *B. graminis* f.sp. *hordei*, frequency of haustorium formation seems to be related to cell morphology (Lin and Edwards, 1973; Jørgensen and Mortensen, 1977; Koga et al., 1990). Furthermore, subsidiary cells of mlo-resistant barley seedlings show a cell type-specific susceptibility and support colony formation (Jørgensen, 1992). Rumbolz and Gubler (2005) mentioned a high frequency of haustoria of the grapevine powdery mildew fungus *E. necator* in hair cells of buds. In hops, Godwin (1985) reported that *Pm* establishes haustoria and forms elongated secondary hyphae in hair cells of the hop cv. Wye Target. Except for Skibbe et al. (2010) who showed that the corn smut fungus *Ustilago maydis* expresses effectors in a tissue-specific manner in the interaction with maize other authors have only suggestions to explain tissue- and

cell type-specificity. For example, Hermanns et al. (2003) linked the tissue-specific susceptibility of roots in the non-host interactions of *Arabidopsis* with *H. parasitica* with a lacking oxidative burst and interpreted their observations in an evolutionary context. According to them roots being capable of performing HRs would make no sense, as roots surrounded in a microorganism-rich soil would die away all the time.

My experiments indicated that hop hair cells show no endopolyploidy and have the same DNA content as normal epidermal cells that are 13 times smaller than the hair cells (Figure 10). This is consistent with the study of Tschermak-Woess and Hasitschka (1953) giving the hint that hop leaf hairs are diploid and show no endopolyploidy. Endopolyploidy occurs if single or repeated DNA replication without cell division leads to doubling of the DNA content and therefore to different ploidy levels within one organism. Ploidy levels may range from 4C to more than 64C (the nuclear DNA content of gametes is 1C) (Kondorosi et al., 2000; Barow, 2006; Lee et al., 2009). A role of endopolyploidy in powdery mildew susceptibility has been suggested, as *Golovinomyces orontii* seems to induce endopolyploidy accompanied by nuclear and cell enlargement in *Arabidopsis* mesophyll cells. Here, endopolyploidy might influence metabolic processes by increasing the nutrient exchange between the host and the haustorial complex or reducing effector influence on dosage-sensitive host targets (Chandran et al., 2010; Wildermuth, 2010). Furthermore, endopolyploidy often correlates with cell size and probably supports the physiological function of a given cell, for example by promoting a higher metabolic activity in tissues and organs like endosperms and fruits (Kondorosi et al., 2000; Sugimoto-Shirasu and Roberts, 2003; Barow, 2006; Lee et al., 2009; de Veylder et al., 2011). The fact that susceptibility of hop hair cells does not correlate with endopolyploidy must not be contradictory to the discussed connection of endopolyploidy and powdery mildew susceptibility of Wildermuth (2010). Endopolyploidy might rather be a result or a feature of susceptibility than causing susceptibility itself. Moreover, the reduced DNA content to cell size ratio of hop hair cells might lead to a less physiologically active state resulting in slowed down defence reactions as less DNA template per cell volume is available. However, Barow (2006) wrote that a simple relationship between the functional activity of a

cell and the DNA template per cell volume probably not exists. Godwin (1985) noted a cell type-specific powdery mildew susceptibility of hop hair cells, too, and suggested a physiologically less active state. He made this conclusion because fungal germlings formed shorter elongated secondary hyphae when infecting hair cells and longer elongated secondary hyphae when infecting normal epidermal cells. However, when talking about endopolyploidy one has to consider that a DNA content to cytoplasm ratio would be much more significant than the DNA content to cell size ratio as the size of the vacuole and the cystolith inside hop hair cells is not known (Barow, 2006).

In addition, cell type-specific differences in the transcriptome and a different biochemical status of epidermal cell types might lead to the cell type-specific susceptibility of hop hair cells because the expression of defence or susceptibility related genes could be altered, avoiding the proper recognition of the pathogen, impairing downstream defence signalling or promoting susceptibility. For example in *Arabidopsis*, microarray analysis gave a twofold stronger signal of the powdery mildew susceptibility factor *AtMLO6* in simple trichomes than in leaf notrichome tissue (http://www.planttrichome.org/trichomedb/microarray.jsp?species=Arabidopsis%20 thaliana&chip=ATH1&dowhat=queryprobesetID; probeset ID: 265008_at). Furthermore, transcriptional analysis of simple trichomes of *Arabidopsis* revealed a set of 3,231 genes with a higher or equal expression in trichomes than in notrichome leaf tissue and among them 1,115 trichome-specifically expressed genes (Jakoby et al., 2008). Ebert et al. (2010) performed metabolite profiling of *Arabidopsis* simple trichomes, pavement and basal cells by analyzing cell saps. Different metabolic contents of fatty alcohols, amino acids, N-compounds, (polyhydroxy) acids, phosphates, polyols and sugars indicated a different biochemical status of simple trichomes. Furthermore, a different chemical composition of the plant cell wall or a different composition of cuticular lipids might affect the HR in simple trichomes because the cuticle and the cell wall are both involved in defence signalling (Vorwerk et al., 2004; Reina-Pinto and Yephremov, 2009). For example, cell wall fragments like polysaccharides, released by cell wall degrading enzymes and acting as signalling molecules in the HR of normal epidermal cells might not be present in simple trichomes (Vorwerk et al., 2004).

4.2.2 Biological significance of the hair cell-specific powdery mildew susceptibility

The susceptibility of hop hair cells does probably not affect the overall resistance phenotype of hop leaves as hair cells account only for 17.6% of the leaf surface. They are therefore too far away from each other to support an extensive growth of the fungus (Figure 9). Indeed, on resistant genotypes, colonies were only visible under the microscope or the binocular. It is possible that the hair cell-specific susceptibility reduces the evolutionary pressure on the fungus to overcome resistance. However, there is also the possibility that *Pm* rapidly breaks resistance during the breeding process (A. Lutz, LfL, Freising, Germany, pers. comm.).

Another possible biological significance might be that hair cells facilitate overwintering of *Pm* as mycelium in buds. In this context, Rumbolz and Gubler (2005) mentioned that *E. necator* forms haustoria frequently in hair cells of grapevine buds. Here it is conceivable that powdery mildew fungi have an advantage in overwintering if they are able to infect, in addition to normal epidermal cells, also the preformed leaf hairs that lie close together on the developing leaves (Figure 1C). In this regard, it would be possible that *Pm* developed hair cell-specific effectors. For example, *Ustilago mayidis* expresses tissue specific effectors in the interaction with maize (Skibbe et al., 2010). However, overwintering of the powdery mildew fungus in buds is no serious problem in Germany, where root stocks are pruned in spring so that infected buds are removed (Neve, 1991).

4.3 A transient transformation assay is suitable to examine gene function in the hop-powdery mildew interaction

Cell area investigations revealed that hair cells are more suited for the transient transformation assay than normal epidermal cells because hair cells are bigger than normal epidermal cells and bigger than *Pm* conidia (Figure 11). Established transient transformation assays for the functional characterization of genes in barley or wheat base on the interactions of single transformed epidermal cells with spores of the powdery mildew fungus. The ability to distinguish between penetrated cells that contain a fungal haustorium and cells that could ward-off fungal penetration is a prerequisite for the evaluation. A high inoculation density is re-

quired to provide a theoretical interaction of each epidermal cell with one conidium. Epidermal cell sizes are therefore crucial for the feasibility and the validity of the transient transformation assay. Thus, the small normal epidermal cells of hops would require an area-wide inoculation and leaves inoculated with such a high inoculation density could not be subjected to microscopic evaluation because spores and hyphae would lie on top of each other. However, hop hair cells that are bigger than normal epidermal cells are still smaller than barley epidermal cells for which the transient transformation assay is well established. The described difficulties regarding the cell sizes may be the reason for the fact that transient transformation assays based on particle bombardment have been reported only for certain plant species yet. Indeed, Humphry et al. (2011) noted that small cell sizes of pea epidermal cells make the transformation assay less efficient than in barley. Although normal epidermal cells were considered unsuitable for the transient transformation assay due to their size, they might be used in a special experimental design. Experiments that would aim at breaking race-specific resistance, e.g. through TIGS of *R* genes, might need only a couple of interaction sites to obtain significant results. In the interaction of pea and *E. pisi,* Humphry et al. (2011) performed a single-cell complementation of a *mlo* resistance phenotype by biolistic overexpression of a pea *MLO* gene. Although the authors achieved only comparatively few interactions sites in single experiments due to small cell sizes and low inoculation densities, they obtained significant results.

The cell type-specific susceptibility of hair cells described in chapter 3.2 might limit the applicability of certain candidate genes. In hairs cells the HR as a part of ETI often lacks, whereas basal resistance including the formation of papillae or the encasement of haustoria in callose like material does not seem to be affected (see chapter 3.2.1). Therefore, genes affecting MTI responses or basal resistance like *MLO* genes may be more appropriate candidates to be tested in hair cells than genes involved in ETI.

Particle bombardment at 650 psi resulted in highest transformation rates of both cell types investigated and was therefore used in all further experiments (Figure 12). High transformation rates are important to get enough interactions between transformed cells and the powdery mildew fungus. Probably, less hair cells than

normal epidermal cells are transformed because hair cells cover only 17.6% of the area of the leaf surface (see chapter 3.2.2). Comparing transformation rates achieved in this study to that of other studies is difficult because other studies specify their values in transformed cells per leaf and not per mm^2. For example in a typical experiment with wheat, 7 transformed cells per 3 cm leaf section (*UBI-QUITIN* promoter, GUS reporter gene) were reported (Schweizer et al., 1999). Compared to these transformation rates, I obtained relatively high transformation rates of up to 5 transformed cells per mm^2. One explanation for this might be that hop leaves are composed of smaller and therefore more epidermal cells than barley leaves (Figure 11). One could estimate that higher transformation rates of normal hop epidermal cells should lead to more fungus-transformed cell interactions. However, this advantage does not make up for the disadvantage of very small cell sizes in microscopy of single fungus-transformed cell interactions.
Co-expression rates of more than 90% observed after transient transformation of hop hair cells with the reporter genes *GFP* and *DsRed* should be high enough to detect effects of candidate genes on the interaction with *Pm* (Schweizer et al., 1999).
The protocol may be improved by evaluating the interaction sites at 1 hai instead of 3 hai. This might also produce a sufficient number of interaction sites and would have the advantage that only primary appressoria and haustoria are evaluated. Single-cell interactions at sites of primary penetration attempts are more significant because the fungus has not established compatibility yet. Furthermore, using the GUS reporter gene under control of the dicot 35S promotor instead of the monocot *UBIQUITIN* promotor could increase the number of interaction sites.

To estimate the applicability of the transient transformation assay for the functional characterization of genes, a putative hop *MLO* gene was chosen as a candidate because in other plant species loss of *MLO* function strongly impairs powdery mildew susceptibility (Jørgensen, 1992; Bai et al., 2008; Humphry et al., 2011; Pavan et al., 2011). Phylogenetic classification of the isolated partial hop MLO amino acid sequence into clade V and an upregulation of its expression after powdery mildew attack indicated that this gene is an appropriate candidate for the transient trans-

formation assay (Figure 13, Figure 15). The *MLO* gene family comprises of up to 17 members and clusters into 6 clades. In dicots only clade V has been associated with powdery mildew susceptibility (Feechan et al., 2008). Simultaneous loss-of-function of *AtMLO2, 6* and *12* conferred powdery mildew resistance in *Arabidopsis* (Chen et al., 2006; Consonni et al., 2006) and loss-of-function of *SlMLO1* and *PsMLO1* conferred powdery mildew resistance in pea and tomato, respectively (Bai et al., 2008; Humphry et al., 2011; Pavan et al., 2011). Therefore clustering of the putative hop MLO amino acid sequence into this clade may be a hint for a function in hop powdery mildew susceptibility. Furthermore, *Arabidopsis AtMLO2, 6* and *12*, tomato *SlMLO1* as well as barley *HvMLO*, show increased expression after powdery mildew attack (Chen et al., 2006; Bai et al. 2008; Feechan et al., 2008; Piffanelli et al., 2002). For this reason, upregulation of the putative hop *MLO* gene after *Pm* attack also supports a role of this gene in powdery mildew susceptibility. In addition, the delayed upregulation in the resistant cv. Wye Target is consistent with the proposed role of the hop *MLO* gene as a powdery mildew susceptibility factor.

After identifying the hop *MLO* gene as an appropriate candidate gene for the transient transformation assay, TIGS of this gene in hair cells was performed and led to a significant reduction of the susceptibility index (Figure 16). This suggests a function of the putative hop *MLO* gene in powdery mildew susceptibility. Thus, proof of concept of the transient transformation assay was successful, providing a tool for the functional analysis of resistance-associated genes in hops. However, for breeding purposes, genes that confer full resistance would be needed and TIGS of the putative hop *MLO* gene did not strongly affect haustorium formation. One reason might be that, like in grapevine, further hop *MLO* genes exist that may function redundantly and replace the reduced expression of a single *MLO* gene as has been shown for *Arabidopsis* (Consonni et al., 2006; Feechan et al., 2008).

4.4 Conclusions

4.4.1 Cell specificity and the cellular basis of resistance in the hop-powdery mildew interaction

Based on the literature and the results obtained from my studies, Figure 17 summarizes single cell interaction phenotypes that can be observed between hop epidermal cell types and the powdery mildew fungus, and explains the cellular basis of resistance in the hop-powdery mildew interaction. The hop leaf surface is composed of normal epidermal cells and hair cells. The area covered by hair cells accounts for about 1/5 of the leaf surface (Figure 17A). Defence reactions of normal epidermal cells determine the outcome of race-specific hop-powdery mildew interactions whereas hop hair cells show a cell type-specific susceptibility to adapted *Pm* and non-adapted *E. cruciferarum*. This cell type-specific powdery mildew susceptibility is visible as small, sometimes sporulating powdery mildew colonies spreading from a susceptible leaf hair in the centre (Figure 17B). The cell type-specific susceptibility of hop hair cells does probably not affect the overall resistance phenotype of hops because hair cells are separated by normal epidermal cells in between, and the number of hair cells on the leaf surface is limited (Figure 17A). Compared to normal epidermal cells, a reduced DNA content to cell size ratio of hair cells might lead to a less physiologic active state and thus explain the hair cell-specific powdery mildew susceptibility (Figure 17B). The HR is the major resistance mechanism of normal epidermal cells, whereas the HR of hair cells partially lacks resulting in the hair cell-specific powdery mildew susceptibility. In host interactions, single and multicellular HRs of normal epidermal cells, the presence or absence of haustoria initials or elongated secondary hyphae indicate the exact time point of the HR (Figure 17C). Only hair cells stop fungal growth through haustorial encasements (Figure 17D). Hair cells form papillae in both, host and non-host interactions. Pre-penetration resistance associated with papillae formation of normal epidermal cells contributes not to race-specific host resistance and occurs only occasionally in the non-host interaction (Figure 17E).

Taken together, my experiments characterized resistance mechanisms of hop epidermal cells present in the German hop germplasm to adapted and non-adapted

powdery mildew fungi. Different timings of the defence mechanisms and diverse combinations of defence mechanisms point to different genetic bases of resistance in the investigated genotypes. Thus, these results provide new insights into hop powdery mildew resistance and may support breeding of resistant cultivars. For example, resistance mechanisms with a different genetic basis could be combined in the future. Because pre-penetration resistance associated with papilla formation did not notably contribute to resistance of hops to adapted and non-adapted powdery mildew fungi, its role in hop-powdery mildew interactions remains open. Whether this kind of resistance could be used for breeding resistant hop cultivars, should be addressed in further experiments. Such experiments might for example investigate interactions of *Pm* with partially resistant hop genotypes or the interaction of hops with non-adapted powdery mildew fungi other than *E. cruciferarum*. Furthermore, also in hops *mlo* alleles may exist that confer recessively inherited penetration resistance similar to the naturally occurring *mlo* resistance that was found in barley, tomato and pea (Jørgensen, 1992; Bai et al., 2008; Humphry et a., 2011).

The hop hair cell-specific powdery mildew susceptibility extends the sparse knowledge of cell and tissue-specificity in plant-pathogen interactions and adds new knowledge about the function of simple trichomes as the literature rather describes them as defence or stress-related. In future studies it would be interesting to examine the role of hop leaf hairs in overwintering of the powdery mildew fungus in buds. Perhaps breeding for powdery mildew resistant hair cells could help to control this disease in future cultivars. On the molecular level, it would be interesting to investigate the presence of certain resistance-associated genes, e.g. *R* genes in leaf hairs. However, in my study it was not possible to isolate the RNA of hop hair cells, as they could not be shaved-off like simple trichomes of *Arabidopsis*.

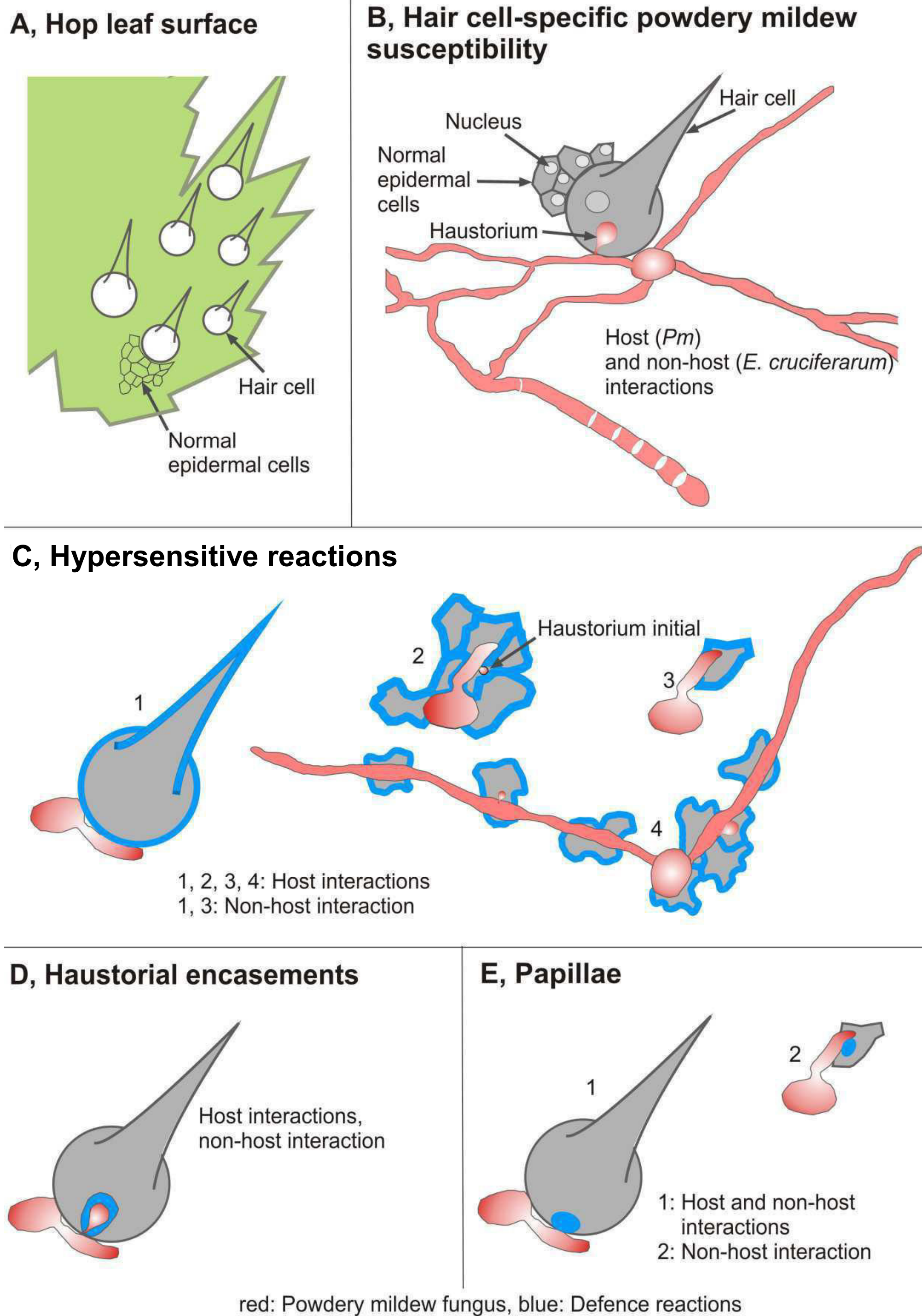

Figure 17: Cell specificity and the cellular basis of resistance in the hop-powdery mildew interaction. A, The hop leaf surface is composed of hair cells and normal epidermal cells (not drawn to scale). **B,** Leaf hairs show a cell type-specific susceptibility in incompatible interactions with *Pm* and in the non-host interaction with *E. cruciferarum*, visible as small colonies spreading from a susceptible leaf hair in the centre. On resistant host genotypes, these colonies sometimes sporulate. Although nuclei of hair cells are bigger than nuclei of normal epidermal cells, less intensive DAPI staining points to a reduced DNA content to cell size ratio. **C,** Upon powdery mildew

attack of adapted *Pm* and non-adapted *E. cruciferarum*, hypersensitive reactions of both, hair cells and normal epidermal cells occur. In host interactions, single and multicellular HRs of normal epidermal cells, the presence of haustoria initials or elongated secondary hyphae indicate the exact time point of the HR. **D,** Only hair cells form haustorial encasements (in both, the host and non-host interactions). **E,** Papilla formation of hair cells occurs in host and non-host interactions, papillae formation of normal epidermal cells only in the non-host interaction.

4.4.2 The transient transformation assay as a new tool to test gene function in hops

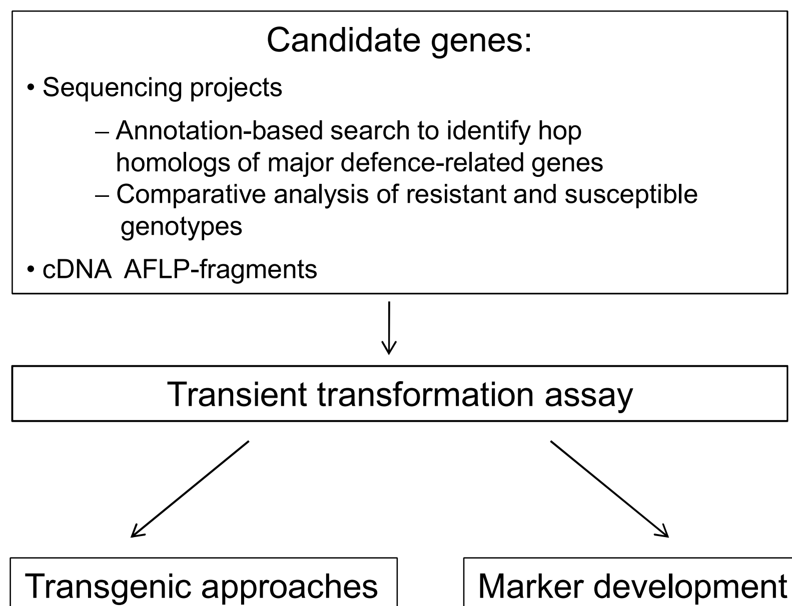

Figure 18: Possible application of the transient transformation assay in hops. Candidate genes may derive from sequencing projects or from cDNA-AFLP studies conducted at the LfL (Freising, Germany). The functional characterization of candidate genes in the hop-powdery mildew interaction might facilitate transgenic approaches, assist in the development of molecular marker or confirm the function of cDNA-AFLP derived sequences in the hop-powdery mildew interaction.

In the future, new resistance traits have to be incorporated into hop cultivars as *Pm* has already broken nearly all resistances in Germany. The transient transformation assay, which was adapted for hops in this study might support this breeding process through the characterization of resistance-associated genes (Figure 18). Candidate genes for this assay may derive from an annotation-based search in the soon available hop transcriptome that identifies homologues of major de-

fence-related genes known from other plant species. Alternatively, cDNA-AFLP studies or comparative transcriptome analysis of resistant with susceptible genotypes may provide candidates genes. Genes with a confirmed function in the hop-powdery mildew interaction can be used for genetic engineering or the development of molecular markers.

5 Summary/Zusammenfassung

Single-cell interactions between the hop powdery mildew fungus *Podosphaera macularis* (*Pm) and* twelve resistant hop genotypes were investigated to gain detailed knowledge about spatio-temporal aspects of resistance mechanisms in the German breeding program. Results indicated that resistance of all genotypes relies on early and late, single and multicellular hypersensitive reactions (HR). Neither in these interactions nor in the non-host interaction of hop with *Erysiphe cruciferarum* pre-penetration resistance associated with papillae formation did notably contribute to resistance and therefore it might play no role in hop defence to powdery mildew fungi at all. Taken together, these results provide valuable knowledge for resistance breeding, as for example resistance mechanisms with a different genetic basis might be combined in future cultivars.

All twelve genotypes showed a hair cell-specific susceptibility to adapted (*Pm*) and non-adapted (*E. cruciferarum*) powdery mildew fungi, resulting from a lacking HR. This was apparent as small, sometimes sporulating colonies spreading from susceptible leaf hairs. The cell type-specific susceptibility of hop hair cells might be explained by a less physiological active state due to a reduced DNA content to cell size ratio. However, the limited occurrence of hairs on the leaf surface leads only to small colony sizes and symptoms are therefore macroscopically not visible. These findings extend the knowledge about cell type-specificity in plant-pathogen interactions and the biological role of simple trichomes (leaf hairs).

In another part of this work, the transient transformation assay was adapted for the functional assessment of resistance associated-genes in the hop-powdery mildew interaction. Results obtained in this study indicated that in hops this assay should be performed with hair cells instead of normal epidermal cells and that particles should be accelerated with 650 psi. To perform proof of principle by transiently induced gene silencing (TIGS) of a hop homolog of the powdery mildew susceptibility factor *MLO* in hop hair cells, first suitability of this candidate gene was confirmed by phylogenetic and expression analysis. TIGS of this gene in hop hair cells led to a significant reduction of powdery mildew susceptibility and therefore demonstrates the applicability of this new tool to asses gene function in hop.

Diese Arbeit charakterisierte Resistenzmechanismen einzelner, gegen Hopfenmehltau, *Podosphaera macularis* (*Pm*), resistenter Hopfengenotypen des deutschen Zuchtprogramms. Die Resistenz aller untersuchten Genotypen beruht auf einem frühen, späten, einzel- oder multizellulären Zelltod. Penetrationsresistenz verbunden mit der Bildung von Papillen wurde kaum nachgewiesen und spielt auch in der Nicht-Wirts-Interaktion von Hopfen mit *Erysiphe cruciferarum* eine untergeordnete Rolle. Möglicherweise hat diese Abwehrreaktion in der Hopfen-Mehltau Interaktion lediglich eine geringe Bedeutung. In der Zukunft könnte das in dieser Arbeit erlangte, detaillierte Wissen über die Resistenzmechanismen einzelner Genotypen in der Mehltauresistenzzüchtung, z. B. für die Pyramidisierung von Resistenzmechanismen, eingesetzt werden.

Haarzellen aller zwölf untersuchten Genotypen zeigten gegenüber adaptierten (*Pm*) und nicht adaptierten Mehltaupilzen *(E. cruciferarum)* eine zelltypspezifische Anfälligkeit, der eine fehlende Hypersensitive Reaktion zugrunde liegt. Dies zeigt sich in der Form von kleinen, z.T. sporulierenden Kolonien. Die Auswirkung auf den Resistenzphänotyp ist allerdings gering, da Haarzellen auf der Blattoberfläche nur begrenzt vorkommen. Ein verringertes DNS-Menge/Zellgrößenverhältnis von Haarzellen deutete darauf hin, dass eine verminderte physiologische Aktivität Grund für die zellspezifische Mehltauanfälligkeit sein könnte. Diese Ergebnisse erweitern das Wissen über Zellspezifität in Pflanzen-Pathogen-Interaktionen und die biologische Funktion von einfachen Trichomen (Blatthaaren).

Ein anderer Teil dieser Arbeit beschäftigte sich mit der Adaptierung des transienten Transformationsassays zur Analyse der Genfunktion in der Hopfen-Mehltau Interaktion. Bei Hopfen ist für diesen Assay eine Transformation von Haarzellen mit einer Partikelbeschleunigung von 650 psi am besten geeignet. Um einen Machbarkeitsbeweis durchzuführen, wurde ein Hopfenhomolog des Mehltau-Anfälligkeitsfaktors *MLO* als Kandidatengen ausgewählt und dessen Eignung durch Expressionsanalysen und eine phylogenetische Klassifizierung bestätigt. Transient induziertes Gensilencing (TIGS) dieses Gens in Haarzellen führte zu einer signifikanten Reduzierung der Mehltauanfälligkeit. Damit wird ein neues Werkzeug für die Funktionsanalyse einzelner Gene in der Hopfen-Mehltau Interaktion bereitgestellt.

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7 Supplement

Supplementary Table 1: Microscopic assessment of defence reactions of normal epidermal cells to *Pm* at 24 hai in the susceptible cv. Northern Brewer and the resistant breeding line 093 010 36[a]

Genotype	Phenotype	% (±SD) HI	% (±SD) Esh	% (±SD) HR	% (±SD) Pap	% (±SD) no def
Cv. Northern Brewer	sus	41.3 ± 12.8	35.8 ± 12.7	57.4 ± 12.1	0.2 ± 0.2	6.6 ± 3.4
BL 093 010 36	res	22.7 ± 17.2	0.0 ± 0.0	91.0 ± 1.7	0.1 ± 0.2	8.9 ± 1.5

[a] Data represent mean percentages of three independent experiments with corresponding standard deviations. In each experiment, at least three leaves with 79 fungal interactions per leaf were evaluated. The cv. Northern Brewer served as the susceptible control. The haustorial index (HI) represents the percentage of fungal germlings that formed a rudimentary or mature haustorium. Fungal germlings that formed elongated secondary hyphae (Esh) after establishment of a mature haustorium were assessed. Defence reactions of normal epidermal cells to *Pm* attacks were categorized into HR and the formation of effective papillae (Pap). At some interaction sites, fungal germlings neither penetrated normal epidermal cells nor triggered defence reactions (no def). Mean frequencies of Esh, HR, Pap and non-penetrated cells with no defence reaction add up to 100%. BL advanced breeding line, res = resistant (no macroscopic powdery mildew symptoms), sus = susceptible (macroscopic powdery mildew symptoms).

Supplementary Table 2: Microscopic assessment of single-cell interaction phenotypes between *Pm* and hop leaf hairs of wild hop 018 097 008 at 48 hai

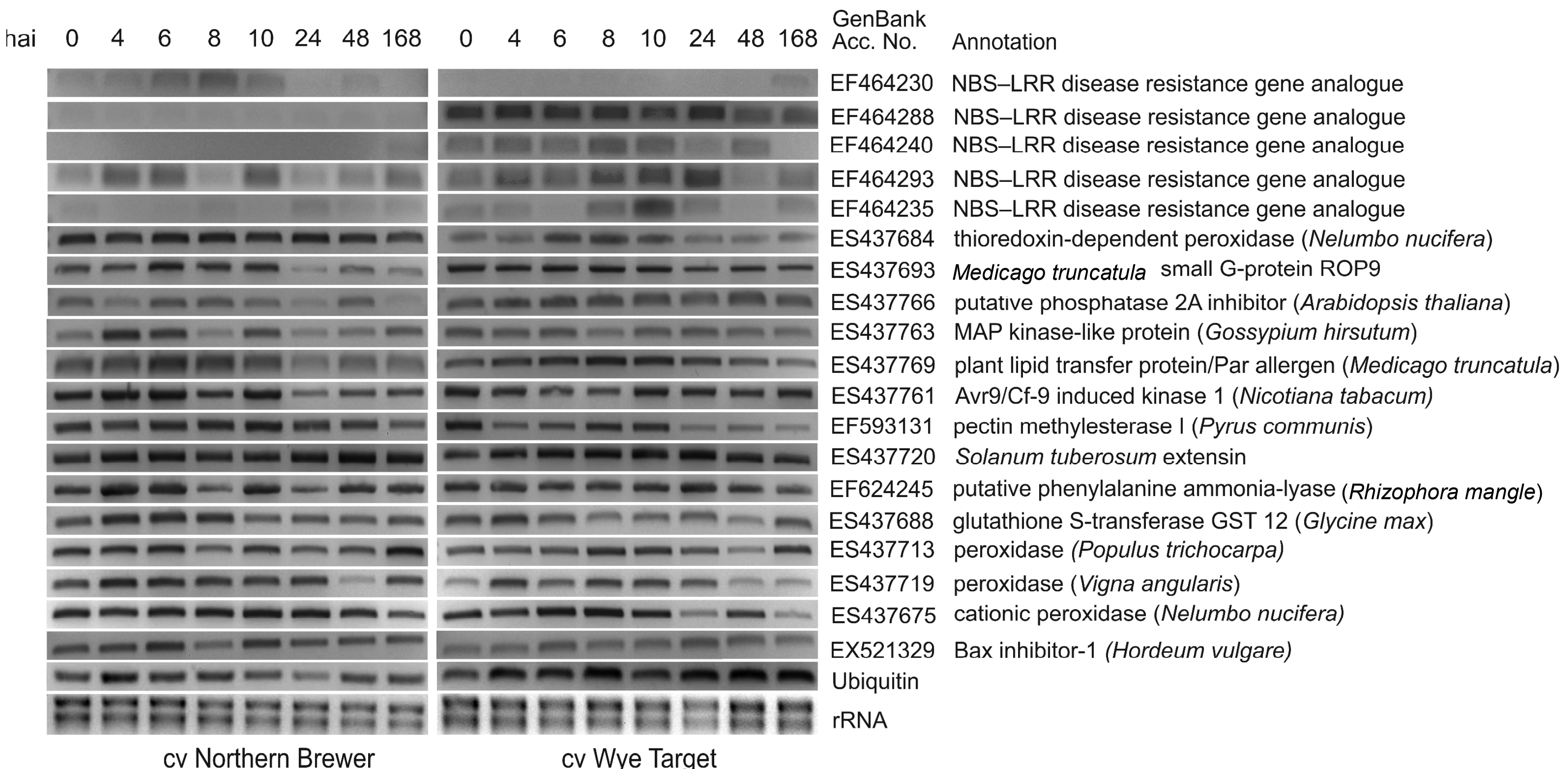
		48 hai[a]					
Genotype	Pathogen	% (±SD) HI	% (±SD) Esh	% (±SD) HR	% (±SD) Pap	% (±SD) Hau pack	% (±SD) no def
Cv. Northern Brewer	*Pm*	82.9 ± 3.9	78.8 ± 4.2	1.2 ± 0.9	1.5 ± 1.0	1.1 ± 0.8	17.4 ± 2.8
WH 018 097 008	*Pm*	77.2 ± 7.9	51.3 ± 10.7	22.0 ± 6.1	2.1 ± 2.3	1.6 ± 0.6	23.1 ± 7.7

[a] Data represent mean frequencies of three independent experiments with corresponding standard deviations. In one experiment, at least 55 fungal interactions with hair cells were evaluated. The susceptible cv. Northern Brewer served as a control. The haustorial index (HI) includes rudimentary and mature haustoria as well as haustoria packed in callose. Fungal germlings that penetrated hair cells established mature haustoria and formed elongated secondary hyphae (Esh) were assessed. Defence reactions of normal epidermal cells to *P. macularis* (*Pm)* conidia were categorized into HR, the formation of effective papilla (Pap) and haustoria packed in callose (Hau pack). At some interaction sites, fungal germlings neither penetrated normal epidermal cells nor triggered defence reactions (no def). Mean frequencies of elongated secondary hyphae, HR cells, papilla formation, haustoria packed in callose and non-penetrated hair cells that showed no defence reaction add up to 100%. WH = wild hop.

Supplementary Table 3: Primer sequences and PCR conditions for gene expression studies shown in supplementary Figure 1

GenBank Accession number	Primer sequences	Product size (bp)	Annealing temperature (°C)	Cycle number
EF464235	5’-AGACGACTCTAGCACATGCCC-3’ 5’-ACTCAGCATAAAGCTTTTCCC-3’	132	55	31
EF464293	5’-TCGTCATCAATTTGATCATGGGTGC-3’ 5’-AGCAACTTTTTATGACTTAGCATTC-3’	165	55	34
EF464240	5’-TTACCTGAAGTTGATTTAAGACGTTG-3’ 5’-ATGATGACAGAGTGAAGGTGTTTG-3’	150	50	35
EF464288	5’-AGAGATCTTTGAGGGGGTCAC-3’ 5’-AAGACTCGTTCCAAACATCATCAA-3’	131	50	34
EF464230	5’-CGACAAATGTGATACTGATAACTTG-3’ 5’-AATTTTACTTCCATGTGCTCCAG-3’	164	50	35
ES437675	5’-GAGGCCACATAGCACAGTCA-3’ 5’-CCGAAGCTTCTGTCCATCTC-3’	339	59	32
ES437719	5’-CCCTGTCATGGACAAAACCT-3’ 5’-TCTGAGGGTTCCCTAGCAGA-3’	332	59	32

ES437713	5’-GCTTGCCCTGCTACTGTTTC-3’ 5’-TCTATGGTCGGGTCAGGAAG-3’	321	59	32
ES437688	5’-TTTTGGGCAAGTCCTTATGC-3’ 5’-TGTAAGCATGCTCCCAAACA-3’	306	55	32
EF624245	5’-ACCACCCTGGTCAGATTGAG-3’ 5’-AGCTCAGAGAATTGGGCAAA-3’	361	55	34
ES437720	5’-AGACGATATGGGGTTTGACG-3’ 5’-TCCGCGTTGACTAAAAGGTT-3’	338	60	30
EF593131	5’-GCCCATGGACCTACAGAAAA-3’ 5’-AACTTGCTGGCTTCGACTGT-3’	363	59	31
ES437761	5’-ATGCCAAGAGGCAGCTTAGA-3’ 5’-TCGGAGTACAACCCTTCCAG-3’	494	60	40
ES437769	5’-CAAGACGATAATGGCGGTTC-3’ AGCTACGGCAGACTTGCATT-3’	301	59	32
ES437763	5’-GAAGTGGCATGGAACCAAGT-3’ 5’-CCTGGCCAATATTTCCATTG-3’	355	59	34
ES437766	5’-CGGAGCTTCAGAAGGTGAAC-3’ 5’-GCAGGGTGACTCAGAAAAGC-3’	312	59	31
ES437693	5’-GCGAAAGCTTATTGGAGCAC-3’ 5’-CAACCATGCAACTCGACAAT-3’	425	59	33
ES437684	5’-TTGCCGTTGGTGATGACTTA-3’ 5’-GCACCATCAGCAAGGAACTT-3’	304	59	32
EX521329	5’-TATTCTTGTCAGCGCGTTTG-3’ 5’-CGAACAAAGACAGCAACGAA-3’	327	59	27



Supplementary Figure 1: Temporal expression profile of candidate genes for the transient transformation assay in the susceptible cv. Northern Brewer and in the resistant cv. Wye Target after inoculation with *Pm*. RNA was isolated from leaves inoculated with conidia of *Pm*. Time points of sampling were 0, 4, 6, 8, 10, 24, 48 and 168 hai. RNA quality and equal loading was confirmed by ethidium bromide staining of rRNAs after electrophoresis. Amplification of a *UBIQUITIN* cDNA fragment (26 cycles) served as a constitutive control. Pictures show inverted pictures of ethidium bromide stained gels. The partial sequences of NBS-LRR disease *R* gene analogues were taken from Kozjak et al. (2009), the stress related ESTs from Fortes et al. (2008).

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