The role of cuticular waxes in the prepenetration processes of *Blumeria graminis* f.sp. *hordei*

Der Einfluss kutikulärer Wachse auf die Präpenetrationsprozesse von *Blumeria graminis* f.sp. *hordei*

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades
der Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Klasse Integrative Biology

vorgelegt von

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aus

München

Würzburg 2012

Eingereicht am: ……

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Summary

The obligate biotrophic fungus *Blumeria graminis* f.sp. *hordei* is the causative agent of barley powdery mildew, a destructive foliar disease. The fungus infests barley (*Hordeum vulgare*), an important crop plant, which causes remarkable yield losses.

Leaf cuticular wax of barley consists mainly of primary alcohols (80%), alkyl esters (10%) and minor constituents such as fatty acids (2%), alkanes (2%) and aldehydes (1%). The asexual airborne conidia have an initial contact to the leaf surface, in an environment dominated by cuticular waxes, which trigger germination and differentiation. The conidia undergo a sequential morphogenesis during that phase, the so-called prepenetration processes. The conidium initially forms a short primary germ tube, followed by a secondary elongated germ tube, which swells and finally forms a septate appressorium. The fungal appressorium infests the epidermal cell of the host plant and establishes an initial haustorium, the feeding structure of the fungus.

In order to assess the effects of single host plant wax constituents on the prepenetration processes a novel *in vitro* assay based on Formvar® resin was established. This system permits the setting up of homogeneous surfaces as substrata, at which the adsorbed amounts and the surface hydrophobicity are highly reproducible, independently of the tested substance classes and chain lengths of the molecules. In this system, very-long-chain aldehydes promoted germination and differentiation of *B. graminis* f.sp. *hordei* conidia. The appressorium formation rates were decreasing in a concentration and chain-length dependent manner compared to *n*-hexacosanal (C_{26}), which was the most effective aldehyde (C_{22}<<C_{24}<C_{26}>C_{28}>>C_{30}). The tested alkanes with even and odd numbers (C_{24}-C_{33}), fatty acids (C_{20}-C_{28}), alkyl esters (C_{40}-C_{44}) and primary alcohols (C_{20}-C_{30}) did not induce germination and appressorium formation. The primary alcohol *n*-hexacosanol (C_{26}) was an exception, as it was capable of significantly stimulating conidial germination and appressorial germ tube formation.

To elucidate the impact of very-long-chain aldehydes on an intact plant surface *in vivo*, *B. graminis* f.sp. *hordei* conidia were inoculated on *glossy11* mutant leaves of the non-host plant maize (*Zea mays*), which are - unlike the wildtype - completely devoid of very-long-chain aldehydes. On *glossy11* leaves 60% of *B. graminis* f.sp. *hordei* conidia remained ungerminated and 10% developed a mature appressorium, which is three times less than on wildtype plants. Spraying of synthetic *n*-hexacosanal or wildtype leaf wax on *glossy11* leaves fully restored the fungal prepenetration processes. In contrast, spraying of non-inducing *n*-alkanes, primary alcohols or very-long-chain fatty acids on wildtype leaves of maize mimicked the aldehyde deficient phenotype of *glossy11*.

During the prepenetration processes an appressorium is formed, which is a newly formed specialized cell. Germination and subsequent morphogenesis are linked to the cell cycle in certain phytopathogenic fungi. It was investigated to what extent the prepenetration processes of *B. graminis* f.sp. *hordei* are synchronized with cell cycle progression. Hence, a distinct staining procedure of nuclei for fixed samples of *B. graminis* f.sp. *hordei* conidia based on DAPI (4,6-diamidino-2-phenylindole) was developed. In combination with a pharmacological approach it was possible to trace mitosis in dependency of conidial germination and differentiation *in vivo* and *in vitro.* The uninucleate conidium germinated and after formation of the appressorial germ tube, a single mitosis occurred in the primordial conidium six hours after inoculation. The inhibition of S-phase with hydroxyurea or M-phase with benomyl prevented appressorium formation, but not the development of the appressorial germ tube. These results indicate that mitosis and a successful cytokinesis are necessary prerequisites for the appressorium formation but not for conidial morphogenesis.

In order to identify genes that are expressed in response to certain host plant wax constituents, which may be critical for the prepenetration phase, cDNA clone libraries were constructed by suppression subtractive hybridization (SSH) after inoculation. The Formvar® resin based *in vitro* system provided a stable platform to enrich cDNA sequences that were expressed in *B.graminis* f.sp. *hordei* conidia incubated on *n*-hexacosanal coated surfaces for 22 minutes. Among various candidates, a cDNA sequence was identified, which was upregulated on barley leaves and on surfaces coated with *n*-hexacosanal or extracted barley leaf wax. The hexacosanal responsive transcript was cloned by 3’ and 5’ RACE. The cDNA sequence showed no homologies to genes of known function in fungal development and fungal pathogenicity in plants.

Zusammenfassung

Der obligat biotrophe Pilz *Blumeria graminis* f.sp. *hordei* gilt als Erreger des Gerstenmehltaus, einer destruktiven Erkrankung der Gerste (*Hordeum vulgare*). Als Folge des Befalls mit *B. graminis* f.sp. *hordei* drohen erhebliche Ernteeinbußen.

Das kutikuläre Wachs von Gerstenblättern besteht hauptsächlich aus primären Alkoholen (80%), Alkylestern (10%) sowie aus geringfügig vorkommenden Bestandteilen wie Fettsäuren (2%), Alkanen (2%) und Aldehyden (1%). Der initiale Kontakt der asexuellen und durch die Luft verbreiteten Konidien findet auf der Blattoberfläche in einer Umgebung statt, die von den kutikulären Wachsen bestimmt ist, welche Keimung und Differenzierung stimulieren. Während der Keimungs- und Differenzierungsphase durchlaufen die Konidien eine sequenzielle Morphogenese, die so genannten Präpenetrationsprozesse. Dabei bilden die Konidien auf der Pflanzenoberfläche zunächst einen primären, kurzen und im weiteren Verlauf einen sekundären, elongierten Keimschlauch aus. Im Anschluss daran schwillt dieser an und wird letztlich zu einem septierten Appressorium differenziert. Mit Hilfe des Appressoriums dringt der Pilz dann in die Epidermiszelle der Wirtspflanze ein und bildet ein initiales Haustorium, das die Ernährung des Pilzes sicherstellt.

Um den Einfluss von einzelnen Wachsbestandteilen der Wirtspflanze auf die Präpenetrationsprozesse systematisch zu untersuchen wurde ein neues *in vitro* System auf der Basis von Formvar®-Harz etabliert. Dieses System ermöglicht die Erzeugung homogener Oberflächen als Substrate für den Pilz, bei denen sowohl die aufgelagerten Mengen als auch die Oberflächenhydrophobizität unabhängig von den getesteten Substanzklassen und Kettenlängen der Moleküle hochgradig reproduzierbar sind. In diesem System haben langkettige Aldehyde die Keimung und die Differenzierung von *B. graminis* f.sp. *hordei* Konidien am wirksamsten induziert, wobei die Raten der Appressorienbildung in Abhängigkeit von der Konzentration und der Kettenlänge im Vergleich zu *n*-Hexacosanal (C_{26}), das sich als am effektivsten zeigte, abnahmen (C_{22}<<C_{24}<C_{26}>C_{28}>>C_{30}). Die getesteten gerad- und ungeradzahligen Alkane (C_{24}-C_{33}), Fettsäuren (C_{20}-C_{28}), Alkylester (C_{40}-C_{44}) und primären Alkohole (C_{20}-C_{30}) hatten keinen signifikanten Einfluss auf die Keimung und die Appressorienbildung des Pilzes. Der primäre Alkohol *n*-Hexacosanol (C_{26}) stellte hierbei eine Ausnahme dar, da er die Keimung und die Bildung des Appressorium-Keimschlauchs signifikant erhöhte. Um die Rolle von langkettigen Aldehyden auf einer intakten Pflanzenoberfläche *in vivo* genauer zu untersuchen wurden *B. graminis* f.sp. *hordei* Konidien auf Blätter von *glossy11* Mutanten der Nicht-Wirtspflanze Mais (*Zea mays*) inokuliert. Anders als der Wildtyp weisen *glossy11* Blätter keine langkettigen Aldehyde auf. Auf *glossy11* Blättern keimten 60% der *B. graminis* f.sp. *hordei* Konidien nicht und nur 10% der Konidien

entwickelten ein reifes Appressorium, was einer dreimal geringeren Rate als auf Wildtyp-Blättern entspricht. Durch das Besprühen von *glossy11* Blätter mit synthetischem *n*-Hexacosanal oder mit Wachs des Wildtyps wurden die pilzlichen Präpenetrationsprozesse wieder vollständig durchlaufen. Wurden im Gegensatz dazu Blätter des Mais-Wildtyps mit nicht induzierenden *n*-Alkanen, primären Alkoholen oder langkettigen Fettsäuren besprüht, konnte das den Aldehyd-defizienten Phänotyp von *glossy11* imitieren.

Während der Präpenetrationsprozesse wird ein Appressorium gebildet, wobei es sich hierbei um eine neu gebildete Zelle handelt. Die Keimung und die anschließende Morphogenese sind wichtige Schritte in der Etablierung der pilzlichen Infektionsstrukturen. Da diese Prozesse in einigen phytopathogenen Pilzen mit dem Zellzyklus gekoppelt sind wurde untersucht, inwieweit die Präpenetrationsprozesse von *B. graminis* f.sp. *hordei* mit dem Verlauf des Zellzykluses synchronisiert sind. Hierfür wurde eine Methode basierend auf DAPI (4,6-diamidino-2-phenylindole) zur Färbung der Zellkerne für fixierte Präparate von *B. graminis* f.sp. *hordei* Konidien entwickelt. Mittels eines pharmakologischen Ansatzes war es auf diese Weise erstmals möglich die Abhängigkeit der Präpenetrationsprozesse von der Mitose *in vivo* und *in vitro* zu verfolgen. Sechs Stunden nach der Inokulation trat nach Ausbildung des Appressorium-Keimschlauchs eine Mitose in der einkernigen Konidie auf. Die Hemmung der S-Phase mit Hydroxyharnstoff oder die Hemmung der M-Phase mit Benomyl verhinderten eine Bildung des Appressoriums, nicht aber die Entwicklung des Appressorium-Keimschlauchs. Diese Ergebnisse weisen darauf hin, dass die Mitose und eine abgeschlossene Zytokinese notwendige Voraussetzungen für die Appressoriumsbildung, jedoch nicht für die Morphogenese der Konidie, sind.

Als Reaktion auf bestimmte Wachsbestandteile der Wirtspflanze werden pilzliche Gene, die während der Präpenetrationsprozesse eine wichtige Rolle spielen können, differenziell exprimiert. Um solche Gene zu identifizieren wurden cDNA Klonbibliotheken mittels der *suppression subtractive hybridization* (SSH) 22 Minuten nach der Inokulation erstellt. Das auf Formvar®-Harz basierende *in vitro* System ermöglichte die selektive Anreicherung von cDNA Sequenzen aus *B. graminis* f.sp. *hordei* Konidien, die auf *n*-Hexacosanal beschichteten Oberflächen inokuliert wurden. Aus einer Reihe von Kandidaten wurde eine cDNA-Sequenz identifiziert, die sowohl auf Gerstenblättern als auch auf mit *n*-Hexacosanal oder extrahiertem Gerstenwachs beschichteten Oberflächen hochreguliert war. Mittels 3’ und 5’ RACE wurde das *n*-Hexacosanal induzierte Transkript kloniert. Diese cDNA-Sequenz wies keine Homologien zu bekannten Genen, die Funktionen in der pilzlichen Entwicklung und der Ausbildung von Pathogenität in Pflanzen haben, auf.

Acknowledgements

Die vorliegende Arbeit wurde am Julius-von-Sachs Institut, am Lehrstuhl für Botanik II durchgeführt. Ganz besonders möchte ich mich bei Prof. Dr. Markus Riederer für die Bereitstellung des interessanten Themas, die Betreuung sowie das entgegengebrachte Vertrauen bedanken. In gleicher Weise danke ich Dr. Ulrich Hildebrandt für die engagierte Begleitung der Arbeit, für die anregenden und hilfreichen Diskussionen aber auch für die Freiheit, eigene Ideen verfolgen zu können. Ebenso danke ich Prof. Dr. Roy Gross für die Übernahme der Drittbetreuung und für den regelmäßigen Gedankenaustausch, der zum Projektfortschritt beigetragen hat. Herrn Prof. Dr. Martin Müller danke ich für die Übernahme des Prüfungsvorsitzes.

Ich danke der Deutschen Forschungsgemeinschaft, die im Rahmen des SFB 567 die Finanzierung des Projektes ermöglichte. Dem GK1342 und der Graduate School of Life Sciences in Würzburg danke ich für die Strukturierung des Promotionsvorhabens sowie für das umfangreiche wissenschaftliche Ausbildungs- und Qualifizierungsprogramm.

Ich danke Prof. Dr. Gerhard Bringmann vom Institut für Organische Chemie für die Bereitstellung seines Labors zur Aldehyd-Synthese. Sebastian Bischof danke ich für die Unterstützung bei der Umsetzung der Synthesen. Für die Beantwortung aller meiner Fragen bezüglich der Elektronenmikroskopie, danke ich Prof. Dr. Georg Krohne und Daniela Bunsen. Bei Prof. Dr. Ralph Hückelhoven der TU-München möchte ich mich für das Überlassen von *B. graminis* f.sp. *tritici* bedanken.

Ich danke auch allen gegenwärtigen und ehemaligen Kollegen des Lehrstuhls für Botanik II. Ganz besonders möchte ich jenen danken, die mich auf fachlicher und persönlicher Ebene unterstützt haben: Dr. Katja Arand, Dr. Elham Atteran, Ilaria Forieri, Dr. Thomas Griebel, Dr. Franziska Kuhlmann, Dr. Jana Leide, Silvia Niemann, Eva Reisberg, Dr. Anna Smirnova, Manja Wendt und Theresa Wollenberg. Ganz besonderer Dank gilt Andrea Knorz für die Charakterisierung unzähliger Konidien unter dem Mikroskop und für die Unterstützung bei der alltäglichen Laborarbeit. Ich möchte mich auch bei Olga Frank für die Wartung der Massenspektrometer und Gaschromatographen und bei Natascha Sieling für ihr Organisationstalent bedanken. Ich danke auch Jutta Winkler-Steinbeck für die Pflege der Versuchspflanzen und des Mehltaus. Wilma Kressmann danke ich für die Literaturbeschaffung. Monika Noak, Michaela Jäger und Bianca Putz danke ich für die engagierte Organisation des Lehrstuhls.

Statement of author contributions

"Dissertation Based on Several Published Manuscripts"
Statement on individual author contributions and on legal second publication rights

Publication (complete reference): Anton Hansjakob, Sebastian Bischof, Gerhard Bringmann, Markus Riederer and Ulrich Hildebrandt. (2010) Very-long-chain aldehydes promote *in vitro* prepenetration processes of *Blumeria graminis* in a dose- and chain length-dependent manner. *New Phytologist* **188:** 1039-1054

Participated in	**Author-Initials,** Responsibility decreasing from left to right				
Study Design	AH	UH	MR	SB	GB
Data Collection	AH	SB	UH	MR	GB
Data-Analysis and Interpretation	AH	SB	UH	MR	GB
Manuscript Writing	UH	AH	MR	GB	SB

Explanations (if applicable):

Publication (complete reference): Anton Hansjakob, Markus Riederer, Ulrich Hildebrandt. (2011) Wax matters: absence of very-long-chain aldehydes from the leaf cuticular wax of the *glossy11* mutant of maize compromises the prepenetration processes of *Blumeria graminis*. *Plant Pathology* **60:** 1151-1161

Participated in	**Author-Initials,** Responsibility decreasing from left to right				
Study Design	AH	UH	MR		
Data Collection	AH	UH	MR		
Data-Analysis and Interpretation	AH	UH	MR		
Manuscript Writing	UH	AH	MR		

Explanations (if applicable):

Publication (complete reference): Anton Hansjakob, Markus Riederer, Ulrich Hildebrandt Appressorium morphogenesis and cell cycle progression are linked in the grass powdery mildew fungus *Blumeria graminis.* (*Fungal Biology*, in revision)

Participated in	**Author-Initials,** Responsibility decreasing from left to right				
Study Design	AH	UH	MR		
Data Collection	AH	UH	MR		
Data-Analysis and Interpretation	AH	UH	MR		
Manuscript Writing	AH	UH	MR		

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Anton Hansjakob 22.05.2012 Würzburg

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1 Introduction

1.1 The plant cuticle

Plants are exposed to various abiotic and biotic stress factors. The most meaningful biotic stress for plants is a consequence of the interaction with organisms, such as fungi, bacteria or viruses. The initial contact between airborne pathogenic fungi and plants is the cuticle. Aerial organs of higher plants are covered by the cuticle, which is an extracellular membrane between the plant and its environment. The cuticle is a passive protection barrier against herbivores, pathogenic fungi and microorganisms. On the contrary the cuticle itself influences the behaviour of herbivores or phytopathogenic fungi, as the physical and chemical properties may guide and induce the interaction or the infection process, respectively (Müller, 2006; Carver & Gurr, 2006). The most important physiological function of the cuticle is to provide an effective barrier against water loss by transpiration, which is an essential prerequisite for the survival of terrestrial plants (Kerstiens, 1996; Riederer & Schreiber, 2001; Burghardt & Riederer, 2006). Other physiological functions of the cuticle are controlling uptake and loss of solutes, gases and lipophilic organic compounds. The cuticle is also a protection barrier against abiotic influences like shielding of UV radiation or shelter against wind. In some cases the cuticle provides a mechanical support to maintain structural integrity. During developmental processes the cuticle has a critical role in preventing the fusion of distinct tissues in organo- and embryogenesis and the cuticle has a function in the communication process between pollen and pistil (Tanaka & Machida, 2006).

Structurally, the cuticle consists of a core polymer matrix (cutin) anchored with a pectin lamella to the cell wall layer of the epidermal cells and the embedded cuticular waxes. The cell wall layer itself is composed mainly of cellulose and proteins (Carpita & Gibeaut, 1993). Cutin is an amorphous biopolymer of mainly esterified ω-epoxy and hydroxy C_{16} and C_{18} fatty acids (Stark & Tian, 2006). The cuticular waxes can be divided in two spatially distinct layers. Outermost are the epicuticular waxes (wax crystals and/or wax film) and the intracuticular waxes, which are embedded in the cutin matrix (Jeffree, 1996). Cuticular waxes are a mixture of aliphatic very-long-chain fatty acid derivatives, e.g. primary and secondary alcohols, aldehydes, esters, alkanes or ketones and cyclic compounds like triterpenoids (Holloway, 1982; Walton, 1990; Jetter *et al.*, 2006; Nawrath, 2006). The current biochemical and genetic understanding of wax biosynthesis is based on work on the model plant *Arabidopsis thaliana*. The precursors of cutin and aliphatic wax constituents are fatty acids of C_{16} and C_{18} acyl chains and are synthesized *de novo* by the soluble fatty acid synthase complex (FAS) in the plastid stroma (Slabas & Fawcett, 1992; Ohlrogge & Browse, 1995). In the first step a β-ketoacyl synthase

(KASIII) initiates the fatty acid biosynthesis with acetyl-CoA bound to acylcarrier proteins, KASII and KASI elongate the chain by condensation of a C_2 moiety from a malonyl-acyl carrier protein (ACP). A reduction of β-ketoacyl-ACP followed by the dehydration of β-hydroxyacyl-ACP and the reduction of trans-enoyl-ACP are necessary to complete the elongation until a chain length of C_{16} and C_{18} is achieved (Kunst *et al.*, 2006; Figure 1-1).

The further elongation of the fatty acids proceeds in the endoplasmatic reticulum by repeated cycles of attaching a C_2 moiety, in a similar way as in the plastid by a fatty acid elongase (FAE) complex (Kunst & Samuels, 2003; Kunst *et al*., 2006). The C_{16} and C_{18} acyl precursors are hydrolysed by an acyl-ACP thioesterase and are exported from the plastid. Subsequently the precursors are esterified to free coenzyme A (CoASH) by a long-chain acyl-CoA synthetase (LACS). The β-ketoacyl-CoA synthase adds malonyl-CoA by condensation. The reaction product is further processed by β-ketoacyl-CoA-reductase (KCR), β-hydroxyacyl-CoA-dehydratase (HCD) and enoyl-CoA-reductase (ECR) to a fully saturated acyl-CoA, which serves as substrate for the next elongation cycle (Kunst & Samuels, 2009) (Figure 1-1). The final products of the FAS are very-long-chain fatty acids (VLCFA) that are converted into the aliphatic compounds of cuticular waxes.

In *A. thaliana* primary alcohols are products of the acyl reduction pathway (Kunst & Samuels, 2009; Kunst et al., 2006). Fatty acyl-CoA is reduced by two fatty acyl-CoA-reductases, with a released aldehyde intermediate. Mutants, defective in fatty acid reductase (FAR) activity show a decreased level of primary alcohols and wax esters, whereas the levels of aldehydes, alkanes and secondary alcohols are increased. Wax esters are synthesized by a wax synthase that is an acyltransferase, which transfers the acyl chain from fatty acyl-CoA to a fatty alcohol. The initial step of the decarbonylation pathway is the production of aldehydes from fatty acyl-CoA precursors (Kunst & Samuels, 2003). These aldehydes are then decarbonylated to odd-chain alkanes by release of carbon monoxide. The alkanes are hydroxylated to secondary alcohols that can be converted into ketones by oxidation.

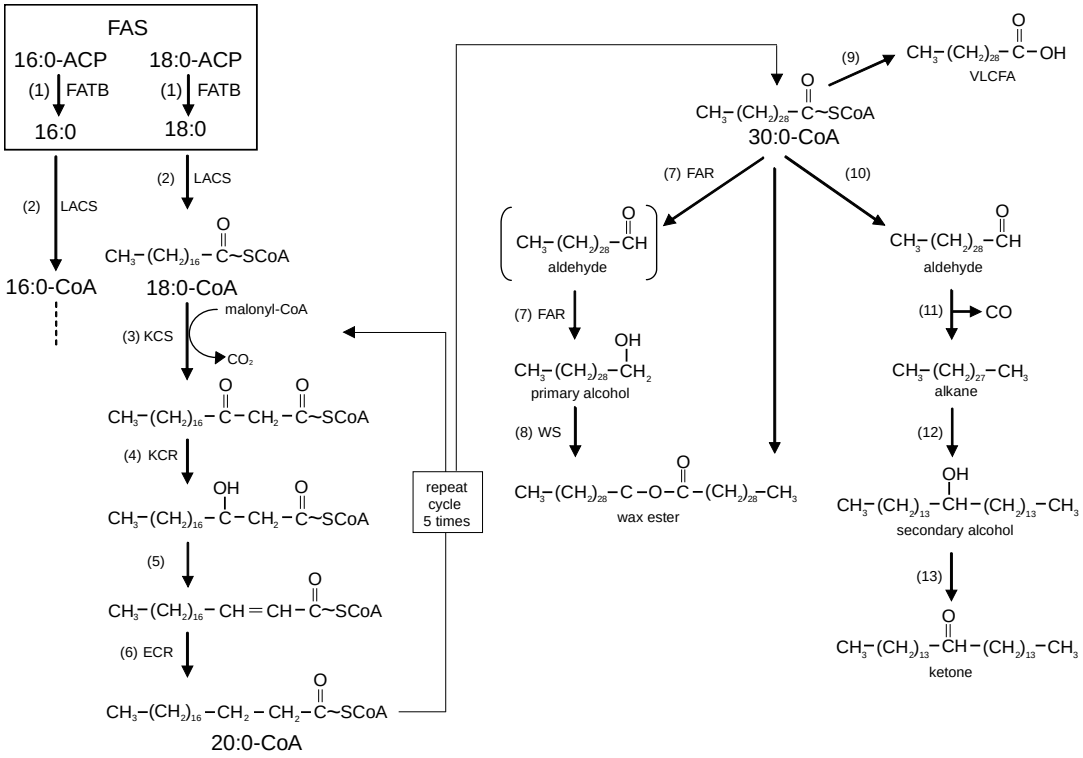

Figure 1-1 Fatty acid elongation pathway (>C_{20}) and conversion into aliphatic wax constituents by the acyl reduction and decarbonylation pathway. (FATB) acyl-ACP thioesterase (1), (LACS) long-chain acyl-CoA synthetase (2), (KCS) β-ketoacyl-CoA-synthase (2), (KCR) β-ketoacyl-CoA reductase (4), (HCD) β-hydroxyacyl-CoA-dehydratase (5), (ECR) enoyl-CoA-reductase (6), (FAR) fatty acyl-CoA-reductase (7), (WS) wax synthase (8). For the proposed enzymes thioesterase (9), aldehyde-forming fatty acyl reductase (10), decarbonylase (11), hydroxylase (12), oxidase (13) molecular biology data are lacking.

1.2 *Blumeria graminis*

Blumeria graminis is a plant pathogenic fungus in the order of the Erysiphales (family: Erysiphaceae), which belongs to the phylum of Ascomycota and is the causative agent of powdery mildew disease. Powdery mildew of cereals is one of the economically most important diseases of barley (*Hordeum vulgare*), which may lead to severe yield losses of up to one third (Johnson *et al.*, 1979; Murray & Brennan, 2010). The genus *Blumeria* is a phylogenetically distinct clade within the Erysiphales (Glawe, 2008). Members of the genus *Blumeria* only affect plants belonging to the family of Poaceae, whereas adapted special forms (*forma speciales,* f.sp.) infest particular genera of cereals. *B. graminis* f.sp. *avenae* infects oats (*Avena* spp.), f.sp. *secalis* rye (*Secalis* spp.), f.sp. *hordei* barley and f.sp. *tritici* wheat (Inuma *et al.*, 2007). Beside crop plants, also special forms are known to affect wild grasses, like f.sp. *poae* meadowgrasses of *Poae* spp., f.sp. *lolii* ryegrasses (*Lolium* spp.), f.sp. *agropyri* perennial grasses (*Agropyron* spp. and *Elymus* spp.) (Inuma *et al.*, 2007). *B. graminis* is a pleomorphic organism with a sexual

(teleomorph) and an asexual state (anamorph) during its life cycle and is an obligate biotrophic fungus. Obligate biotrophic fungi infect their host plants without causing cell death and establish a long term relationship (Perfect & Green, 2001). They cannot be cultivated on artificial media in axenic cultures. The obligate biotrophic lifestyle limits studying biological questions in vast comprehensiveness as it is possible with other common model fungi of Ascomycota, which have been used in past and present like *Neurospora crassa* (Raju, 2009) or *Aspergillus nidulans* (Etxebeste *et al.*, 2010). A targeted mutagenesis is not yet feasible, which is a prerequisite for studying the impact of single genes, though the transformation of a reporter gene by particle bombardment, which was stable over 24 asexual generations, was reported (Chaure *et al.*, 2000).

Nevertheless, *B. graminis* f.sp. *hordei* became an important model organism for understanding the genetics of host-pathogen interactions and resistance mechanisms towards fungicides (Brown, 2002). First genetic maps and DNA libraries of *B. graminis* f.sp. *hordei* were constructed during the 1990ies (Borbye & Giese, 1994) and linkage analyses for avirulence genes were performed (Christiansen & Giese, 1990). Recently, the genome of *B. graminis* f.sp. *hordei* was sequenced and annotated, providing a detailed insight into the genome organisation (Spanu *et al*., 2010). The estimated genome size is approximately 120 Mb, which is four times larger than in other ascomycetes, whereas the number of predicted genes is 5854, which is low in comparison to other fungal genomes. About 64% of the genome consists of transposable elements, which are evenly distributed in long stretches between clusters of 2-10 protein coding genes. A set of 99 genes that are assigned to primary metabolic pathways and its regulation are missing in *B. graminis,* though a subset of these genes (57% -77%) is also not present in other biotrophic phytopathogens. The number of key enzymes of secondary metabolic pathways is drastically reduced, similar as in other biotrophic fungi, such as *Ustilago maydis* or *Tuber melanosporum. B. graminis* encodes only one nonribosomal peptide synthase, one iron siderophore and a single polyketide synthase. Furthermore, the genome of *B. graminis* exhibits no plant cell wall degrading enzymes, such as cellulases, xylanases or pectinases. However, secreted enzymes were identified that might erode the plant cuticle during the infection process. Nicholson *et al*. (1988) described the activity of a non-specific esterase and Pascholati *et al*. (1992) specified cutinase activity. A secreted lipase that has the capabilities of liquefying cuticular wax was recently reported (Feng *et al*., 2009). A class of typical cell wall proteins of fungi, the hydrophobins are also missing in the genome. The genome provides 248 putatively secreted effector proteins that show high sequence diversity that presume specific functions in biotrophic lifestyle. The majority of these putative effectors are only found in the genome of *B. graminis* in comparison

to the mildews *Erysiphe pisi* and *Golovinomyces orontii* affecting the dicotyledones *Pisum sativum* and *Arabidopsis thaliana*, respectively.

Even though, the exploration of genomic information unravels features of biotrophic lifestyle, it does not give any information about gene expression and the proteomic inventory of the fungus during its development. Other studies, which circumvented these limitations, were analyses of transcript and proteomic profiles at different developmental stages during the asexual life cycle of *B. graminis* (Thomas *et al.*, 2002; Both *et al.*, 2005ab; Noir *et al.*, 2009; Bindschedler *et al.*, 2009, 2011). During the prepenetrative stage on the plant surface the *B. graminis* conidium forms a primary germ tube, followed by a secondary one. After 15 hours the secondary germ tube develops into an appressorium, which is a differentiated cell, in order to penetrate the host plant. (Carver & Ingerson, 1987; Both *et al*., 2005a). After successful plant cell wall penetration *B. graminis* develops a digitate haustorium, a specialized absorption structure (Green *et al*., 2002). The fungal mycelium spreads only epiphytically and forms secondary haustoria as the colony grows. The haustoria are restricted to the epidermal cell layer and are surrounded by the invaginated plasma membrane of the plant cell (Perfect & Green, 2001; Green *et al*., 2002). After three days, the now macroscopic colonies become visible and start to produce conidiophores, which release again a large number of asexual conidia. In ungerminated conidia most of the characterized proteins belong to metabolic pathways like in active fungal hyphae. This suggests that the molecular machinery for energy production and biogenesis of novel cellular material after conidia germination is preformed to guarantee a rapid growth on the plant surface (Noir *et al.*, 2009). About one fourth of the peptides are involved in carbohydrate metabolism, such as the citric acid cycle, the pentose phosphate pathway, glycogen degrading enzymes and the fatty acid metabolism. Glycogen and lipids are the most prevalent storage compounds of carbon and energy in *B. graminis* conidia. The second most abundant proteins in conidia are peptides functionally belonging to the category of protein metabolism and modification like peptidases, ribosomal proteins, translation- and elongation factors as well cellular stress related peptides like heat shock proteins (23%). Also components of the amino and nucleic acid metabolism are present in resting conidia (22%). Catalases and peroxidases that detoxify reactive oxygen species, released by the host as immune response, can be found preformed in the conidia and were demonstrated to be released during the infection process (Zhang *et al.*, 2004). About 6.5% of the proteome is of unknown function and could be effectors or proteins that are specific for the establishment of biotrophic lifestyle.

Detailed expression analyses of different asexual pre- and post penetrative stages of conidia and of growing or sporulating mycelium were performed by Both *et al.* (2005a) using high density cDNA microarrays. These expression analyses

revealed a dynamic regulation of genes involved in primary metabolism. During conidia germination, lipids are hydrolysed and glycogen is degraded that feeds into the upregulated glycolysis (1-4 hours post infection) to provide energy for the penetration of the cuticle and cell wall. After formation of the appressorium and establishment of an active haustorium, glucose is directly taken up from the host plant (3 days post infection). Protein and DNA synthesis are highly upregulated during the epiphytic growth phase. When the colonies start to produce conidiophores, lipid and glycogen biosynthesis are raised up, since these compounds are accumulating in the conidia, whereas the expression of degrading enzymes is repressed. A central aspect of these studies is the fact that *B. graminis* expresses and translates enzymes of the main metabolic pathways that all filamentous fungi have. But nevertheless, *B. graminis* is a biotrophic fungus that cannot grow on an artificial media. Therefore, it might be possible that *B. graminis* regulates its metabolic genes strongly in response to specific host plant signals, which still has to be elucidated (Both, *et al.*, 2005a).

1.3 The prepenetration processes of *Blumeria graminis*

Conidia of *B. graminis* are produced in chains, whereas the mature conidia can be found at the apical end of the chain and are separated by the formation of septa (Jarvis *et al.*, 2002). Under natural conditions, mature conidia are dispersed by wind and are preferentially released at high temperatures, at low relative humidity and when leaf surfaces are dry. In contrast, surface wetness, low temperatures and high relative humidity inhibit the release. Even circadian patterns of conidiation are characteristic for *B. graminis*. The infection process of the conidia is initiated immediately after landing on aerial parts of the plant and follows a complex sequential morphogenesis on the surface, the prepenetration processes (**Figure 1-2**). Approximately 30 min after contact with the plant surface the ungerminated conidium (not germinated, ng) forms a short (5-10 µm) aseptate primary germ tube (pgt). The pgt forms a short penetration hypha, which is a cuticular peg that does penetrate the cuticle, but not the cell wall and remains in the apoplast (Edwards, 2002). Through the pgt the conidium possibly can absorb water from the host plant (Carver & Bushnell, 1983) and molecules of low molecular weight (Nielsen, *et al.*, 2000). Later a second germ tube emerges that elongates up to approximately 40 µm and starts to swell (appressorial germ tube, agt). After nine to ten hours the apical part of the tube forms a hook and becomes septate, which marks the mature appressorium (app).

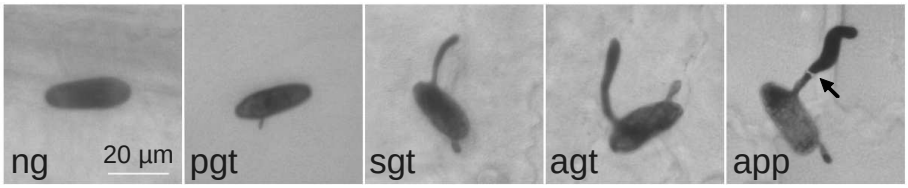

Figure 1-2 Prepenetration stages of *Blumeria graminis* conidia on leaf surfaces: non-germinated conidium (ng), conidium with only a primary germ tube (pgt), conidium with an additional secondary elongated germ tube (sgt), conidium with a swollen appressorial germ tube (agt) and a fully differentiated conidium with a lobed appressorium (app) and septum (arrow). Leaves were bleached and conidia were stained with trypan blue.

From beneath the appressorial germ tube, a so-called penetration peg emerges from the appressorium cell, which attempts to penetrate the host cuticle and cell wall. The forces that drive the penetration process are enzymatic activities and an increased turgor up to 2-4 MPa (Pryce-Jones *et al.*, 1999). The prepenetration phase of *B. graminis* is completed with the formation of a functional appressorium and the initial penetration peg. Within 24 hours past infection of an epidermal cell a digitate haustorium is established, the feeding structure. The fungus mainly takes up glucose (Sutton *et al.*, 1999), but can also metabolize sucrose and fructose (Bushnell *et al.*, 1987).

The leaf surface is the first barrier that has to be overcome by potential pathogens. Likewise, the outermost surface provides a multitude of physical and chemical cues that may promote the development of infection structures (Walters, 2006). Several studies corroborated a role of plant surface properties in triggering the germination and differentiation processes of pathogenic fungi. Beside *B. graminis* (Carver *et al.*, 1990; Francis *et al.*, 1996; Iwamoto *et al.*, 2002; Tsuba *et al.*, 2002; Zabka *et al*., 2008), the rice blast fungus *Magnaporthe grisea* (Gilbert *et al.*, 1996; Hegde & Kolattukudy, 1997), the anthracnose fungus *Colletotrichum gloeosporioides* (Podila *et al.*, 1993), the pea powdery mildew *Erysiphe pisi* (Gniwotta *et al.*, 2005), the cereal rust *Puccina graminis* (Reisige *et al.*, 2006), the grey mould *Botrytis cinerea* (Doehlemann *et al*., 2006) and the corn smut *Ustilago maydis* (Mendoza-Mendoza *et al.*, 2009) develop infection structures in response to cuticle derived constituents.

1.4 Prepenetration processes of *Blumeria graminis* on native and artificial surfaces

The whole prepenetration processes are epicuticular events and take place long before cell to cell interactions (fungus-plant) occur, in an environment dominated by cuticular waxes. The prepenetration processes during the early infection phase are essential parts of the fungal life cycle. The successful establishment of

appressoria and the overcoming of the cuticular barrier are needs for the subsequent host plant infestation.

The impact of cuticular wax components on the prepenetration processes of *B. graminis* were first studied with *eceriferum* (*cer*) barley mutants that had a glossy appearance and were altered in leaf wax composition and physical structure (Yang & Ellingboe, 1972). Altered germination and differentiation of *B. graminis* conidia on non-host plants and on artificial surfaces indicated that the prepenetration processes might be dependent on the substratum. *B. graminis* conidia germinated regularly and mature appressoria were formed on native host surfaces, epidermal strips and isolated cuticles but after removal of cuticular waxes mature appressoria were rarely observed (Yang & Ellingboe, 1972). On artificial surfaces such as water agar and cellulose, germination occurred but no mature appressoria could develop (Carver & Ingerson 1987). In other studies using cellulose film, the conidia developed infection structures at high levels, similar as on stripped barley epidermis (Kobayashi *et al.*, 1991). Other polymer films, like Formvar® films or nitrocellulose films, were capable of inducing infection structures at moderate levels, but on hydrophilic glass slides even germination was drastically reduced and no mature appressoria were observed.

A detailed study using different *cer*-mutants that showed a difference in quantity and a shift in the relative composition of cuticular waxes could demonstrate that chemical composition and surface hydrophobicity are of importance for triggering germination and differentiation of *B. graminis* conidia, whereas the micromorphology plays at best a minor role (Zabka *et al.*, 2008). Another study supporting the assumption that the chemical nature of cuticular waxes plays a major role in the prepenetration processes were performed on leaves of the perennial ryegrass *Lolium perenne* (Carver *et al*., 1990). The adaxial and abaxial sides of the leaves differ in chemical composition, surface hydrophobicity and in the physical microstructure (Ringelmann *et al.*, 2009). The most striking differences between the abaxial and the adaxial leaf surface were the absence of C_{26} aldehyde *n*-hexacosanal, decreased primary alcohol content and a reduced surface hydrophobicity. On the abaxial leaf surface the formation of differentiated conidia (agt+app) was reduced to 12% compared to the adaxial side (80% agt+app). After mechanical removal of epicuticular waxes from both sides the differentiation rates were similarly high (60% agt + app). Subsequent assays with mechanically removed wax sprayed on glass slides, revealed again significantly reduced differentiation rates on slides covered with the abaxial wax fraction (40% agt + app) compared to slides covered with the adaxial wax (70% agt + app).

Single chemical compounds of the cuticle that may act as a distinct signal were already identified previously. A minor wax constituent of barley, *n*-hexacosanal, is capable of strongly inducing the appressorium formation of *B.*

graminis conidia *in vitro*, whereas the C_{30} aldehyde *n*-triacontanal had less inducing capabilities (Tsuba *et al.*, 2002). The cutin monomers *cis*-9,10-epoxy-18-hydroxy-stearic acid and 8,16-dihydroxy-palmitic acid applied to glass as substratum were also able to enhance the rates of appressorium formation, (Francis *et al.*, 1996). The impact of the surface hydrophobicity was not considered in the experiments with synthetic cutin monomers. The cutin monomers may be released from the host cuticle by the activity of fungal cutinases and the breakdown products are presumably sensed via the primary germ tube, but experimental evidences are lacking (Kunoh *et al.*, 1990; Pascholati *et al.*, 1992). The release of a liquid extracellular matrix (ECM), which had an esterase activity three minutes after deposition upon contact with hydrophilic cellophane or hydrophobic glass surfaces were observed (Nicholson *et al.*, 1988; Nielsen *et al.*, 2000). When the esterase activity was inhibited chemically *in planta*, the number of powdery mildew colonies was significantly reduced (Francis *et al.*, 1996; Feng *et al.*, 2011). The ECM is also released after contact with the plant surface and goes along with a change of the conidial surface from a hydrophobic to a hydrophilic state (Nicholson *et al.*, 1993). This process is believed to decrease the hydrophobicity of the host leaf surface at the contact site, which is necessary to promote agt formation (Nicholson, *et al.*, 1988).

The release of the ECM was observed very rapidly within 20 seconds after contact with hydrophobic plastic surfaces. Therefore the ECM must be preformed in the conidium, since that process is insensitive towards the protein biosynthesis inhibitor cycloheximide (Carver *et al.*, 1999). The excretion of the ECM on barley leaves is much less in quantity and seems to be released from cell wall projections on the surface of the conidium (Carver *et al*., 1999; Green *et al.*, 2002). Another enzyme, the non-specific lipase LIP1 identified in the ECM had cuticular wax liquefying capabilities (Feng *et al.*, 2009). The enzyme hydrolyses a broad range of glycerides *in vitro* and presumably releases alkanes and primary alcohols from the epicuticular wax. Glass slides covered with the C_{24} alkane *n*-tetracosane or the C_{25} alkane *n*-pentacosane significantly enhanced the differentiation rates of *B. graminis* f.sp. *tritici* conidia. However, the substrate that is hydrolysed *in vivo* was not identified, but also a transesterification reaction between the conidium and the cuticular leaf wax was discussed by the authors.

1.5 Aims of this work

Influence of cuticular wax constituents on the prepenetration processes

Inoculation of *B. graminis* f.sp. *hordei* conidia on different fractions of barley cuticular waxes as substrata revealed the highest differentiation rates on surfaces covered with the aldehyde fraction (Tsuba *et al*., 2002). *B. graminis* conidia

inoculated on chemically synthesized *n*-hexacosanal (C_{26}) showed higher differentiation rates than on *n*-triacontanal (C_{30}). Physical properties of the surface, like the leaf surface microstructure and more important the surface hydrophobicity, additionally have an impact on the germination and differentiation (Zabka *et al*., 2008). Inoculation of *B. graminis* f.sp. hordei conidia on non-host waxes revealed significantly decreased germination and differentiation rates (Tsuba *et al*., 2002).

The central aim of this study was to systematically elucidate the impact of cuticular wax constituents using one *in vitro* experimental system. Existing *in vitro* systems, which were previously used, were quite diverse. The quantities and the homogeneity of the surface coatings proved not to be reproducible when the substances were sprayed with a glass chromatographic sprayer. Furthermore, the chemical nature of lipophilic substrata strongly influences the surface hydrophobicity, which made it difficult to distinguish between the impact of the physical properties and the substrata itself on *B. graminis* conidia during the prepenetration processes (Zabka *et al*., 2008). **Chapter 2** describes a novel highly reproducible system based on Formvar® resin, which provides a homogenous surface hydrophobicity and enables the deposition of equimolar amounts of lipophilic compounds on glass slides. This *in vitro* system offered the prerequisites to systematically address the following questions about the impact of cuticular waxes on *B. graminis* conidia differentiation:

• Which components, beside *n*-hexacosanal are capable of inducing *B. graminis* conidia differentiation and do certain cuticular wax constituents have an inhibitory effect on conidia differentiation?

• Do different chain lengths of molecules of the same chemical compound class have an effect on conidia differentiation?

• Do different blends of cuticular wax constituents influence conidia differentiation in a positive or negative way?

• Does a critical minimum concentration or proportion of a certain cuticular wax constituent exist, promoting or inhibiting conidia differentiation?

The *in vitro* studies presented in this work could clearly demonstrate that very-long-chain aldehydes promote germination and differentiation of *B. graminis* conidia in a chain length and concentration dependent manner. This finding raised up the question, which impact very-long-chain-aldehydes generally have on conidia differentiation during the prepenetration phase *in vivo* on the plant surface. In **Chapter 3**, the fungal development was studied on leaves of the non-host *Zea mays* wax mutant *glossy11* that is completely devoid of very-long-chain aldehydes. Additionally, the impact of elevated levels of very-long-chain aldehydes was studied on leaves of *Zea mays* wax mutant *glossy5*, which had a six times larger aldehyde content in its cuticular wax compared to wildtype plants. It was elucidated, whether increased aldehyde contents in leaf cuticular waxes lead to enhanced conidia

differentiation. The non-host Poaceae *Zea mays* was chosen for these experiments, since corresponding barley mutants are not available.

Timing of morphogenesis and cell cycle progression during the prepenetration processes

The germination of resting conidia is a crucial step in the life cycle of pathogenic fungi. The differentiation of infection structures is associated with a general reprogramming of the cell. The conidial morphogenesis on the plant surface is often tightly coupled to cell cycle progression. In the rice blast fungus *Magnaporthe grisea* a single round of mitosis in the previously formed appressorial germ tube is a prerequisite for appressorium formation (Saunders *et al*., 2010ab) and subsequent autophagic cell death is necessary for pathogenicity (Veneault-Fourrey *et al*., 2006). On the contrary, in the anthracnose fungus *Colletotrichum gloeosporioides* the emergence of the appressorial germ tube and the appressorium formation are independent of mitosis (Nesher *et al*., 2008).

So far, description of mitosis during the prepenetration phase for the important obligate biotrophic model organism *B. graminis* and a possible dependency on the morphogenesis was lacking. The work in **Chapter 4** describes in detail the mitotic processes during the prepenetration phase. In order to answer the question, whether development of the secondary germ tube and appressorium maturation are associated with the cell cycle, a pharmacological approach, using cell cycle inhibitors, was applied. Additionally, the observations of cell cycle progression and morphogenesis *in vitro* were compared with the situation on barley leaves *in vivo*. This part of the work focused mainly on two questions:

• Which impact has the inhibition of the cell cycle on the conidial morphogenesis and appressorium maturation?

• Are cuticular waxes sufficient to promote cell cycle progression during the prepenetration phase or are they even responsible?

To address these questions experimentally, the *in vitro* system based on Formvar® resin was improved, because the conidia are highly susceptible to free water and burst during the early stages of the prepenetration. Furthermore, the presence of free water impedes the germination of *B. graminis* conidia (Manners & Hossain, 1963; Sivapalan 1994). The system provides a Formvar® membrane with differentiation inducing barley leaf wax and simultaneously allows a treatment with water soluble inhibitors, without disturbing the development of the conidia. Using nuclei labelled with green fluorescent protein (GFP), a usual technical approach to trace nuclear division is currently impossible, since a stable transformation system for *B. graminis* is not yet available. Also well established staining methods for nuclei based on dyes, which are used for fungi were not applicable for *B. graminis*. Therefore, additionally a distinct procedure for staining nuclei of *B. graminis* based

on DAPI (4',6-diamidino-2-phenylindole), which made it possible to investigate morphogenesis and nuclear division during the prepenetration was developed in this work.

Surface dependent gene expression during the prepenetration processes

The dynamics of gene expression of *B. graminis* at different developmental stages during the whole asexual life cycle allowed detailed insights in the metabolic status of this obligate biotrophic fungus (Thomas *et al.*, 2002; Both *et al.*, 2005ab; Noir *et al*., 2009). So far, molecular biological work, which compares gene expression on substrata that induce appressoria formation and those that are non-inducing, is not available. This part of the work focuses on the question:

• Do genes exist, that are specifically transcribed in response to cuticular waxes or single wax constituents, which act as signals for conidial germination and differentiation?

Chapter 5 presents an approach for the identification of wax specific transcripts expressed by *B. graminis* conidia in response to *n*-hexacosanal, the most effective very-long-chain aldehyde present in the host plant cuticular waxes. The Formvar® resin based *in vitro* system, presented in Chapter 2, provided a stable platform for a suppression subtractive hybridization (SSH)-based experimental approach, because effects due to variations in the surface hydrophobicity can largely be excluded. The subtraction was performed with two pools of RNA originating from an inductive surface (*n*-hexacosanal) and a non-inductive surface (*n*-hexacosane). The non-inductive substratum was previously identified in the large scale screening of wax constituents (Chapter 2). After identification and cloning of the differentially expressed sequences it was investigated, to which extent these putative genes are induced *in vivo* on barley leaves and on artificial wax coated surfaces. The further characterization of transcripts, which are responding to cuticular waxes, could be of relevance for initiation and progression of the prepenetration processes of *B. graminis*.

2 Very-long-chain aldehydes promote *in vitro* prepenetration processes of *Blumeria graminis* in a dose- and chain length-dependent manner

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Published in *New Phytologist* (2010) **188:** 1039-1054

2.1 Abstract

- Surface properties of aerial plant organs have been shown to affect the interaction of fungal plant pathogens and their hosts. Conidial germination and differentiation – the so-called prepenetration processes – of the barley powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) are known to be triggered by *n*-hexacosanal (C_{26}-aldehyde), a minor constituent of barley leaf wax.

- In order to analyze the differentiation-inducing capabilities of typical aldehyde wax constituents on conidia of wheat and barley powdery mildew, synthetic even-numbered very-long-chain aldehydes (C_{22}–C_{30}) were assayed, applying an *in vitro* system based on Formvar®/*n*-hexacosane-coated glass slides.

- *n*-Hexacosanal was the most effective aldehyde tested. Germination and differentiation rates of powdery mildew conidia increased with increasing concentrations of very-long-chain aldehydes. Relative to *n*-hexacosanal, the other aldehyde compounds showed a gradual decrease in germination- and differentiation-inducing capabilities with both decreasing and increasing chain length.

- In addition to *n*-hexacosanal, several other ubiquitous very-long-chain aldehyde wax constituents were capable of effectively stimulating *B. graminis* prepenetration processes in a dose- and chain length-dependent manner. Other wax constituents, such as *n*-alkanes, primary alcohols (with the exception of *n*-hexacosanol), fatty acids and alkyl esters, did not affect fungal prepenetration.

Keywords: barley (*Hordeum vulgare*); *Blumeria graminis*; hexacosanal; mildew; octacosanal; very-long-chain aldehydes; wax; wheat

2.2 Introduction

Primary aerial plant surfaces are covered with the cuticle that forms the direct interface between a plant and its environment. The primary function of the cuticle is to form a permeability barrier against water vapour loss from tissues (Schönherr & Merida, 1981; Riederer, 1991; Riederer & Schreiber, 2001; Burghardt & Riederer, 2006). Other functions comprise protection against pathogens, herbivores, UV radiation and mechanical damage (Heredia, 2003). The plant cuticle, which represents the first site of contact with a variety of organisms, such as herbivores and fungal pathogens (Müller & Riederer, 2005; Müller, 2006), is composed of a polymer matrix (cutin) and associated solvent-soluble lipids (cuticular waxes), which can be divided into two spatially distinct layers: the intracuticular waxes embedded in the cutin matrix and the epicuticular waxes coating the surface (Jetter *et al.*, 2000). Cuticular waxes are typically composed of very-long-chain aliphatic compounds, such as, for example, primary and secondary alcohols, aldehydes, esters, ketones or alkanes, composed and arranged in a species-, organ- and tissue-specific manner (Baker *et al.*, 1982; Jeffree, 1996; Jetter *et al.*, 2000). Very-long-chain aldehydes (> C_{18}) are common, ubiquitous wax components that mostly consist of a homologous molecule series with chain lengths ranging from 20 to almost 40 carbons, and can be found in most of the cuticular plant waxes analyzed so far (Jetter *et al.*, 2006).

Plant cuticles have been found to contain substances that affect the germination and differentiation of several plant pathogenic fungi, including the grass powdery mildew *Blumeria graminis* (Carver *et al.*, 1990; Podila *et al.*, 1993; Gilbert *et al.*, 1996; Tsuba *et al.*, 2002; Reisige *et al.*, 2006; Zabka *et al.*, 2008). The obligate biotroph *B. graminis* attacks the cereals wheat and barley, two of the most important food crops, and causes one of the most destructive foliar diseases of cereals (Jørgensen, 1988). The asexual conidia of *B. graminis* germinate and continue their development following a highly ordered morphogenetic sequence. After initial contact with the host surface, *B. graminis* asexual conidia form a primary germ tube, which attaches to the leaf surface and forms a short peg that

penetrates the cuticle (Edwards, 2002; Yamaoka *et al.*, 2006). Subsequently, a secondary germ tube elongates that swells and differentiates into an appressorial germ tube, which matures and becomes a lobed, apical appressorium with a penetration peg formed to breach both host cuticle and cell wall. These prepenetration processes of fungal development are completed *c.* 12–15 h after inoculation.

To date, the chemical nature of only a few substances that promote *B. graminis* prepenetration processes on the leaf surface has been identified. Francis *et al.* (1996) reported that cutin monomers are involved in triggering appressorial germ tube development, and, more recently, it has been found that *n*-hexacosanal (C_{26}-aldehyde) and *n*-triacontanal (C_{30}-aldehyde), chemical constituents of the epicuticular wax layer of barley, are capable of inducing appressorium formation of *B. graminis* germlings in vitro (Tsuba *et al.*, 2002). Conidia inoculated onto *n*-triacontanal-coated polystyrene dishes, however, showed significantly lower differentiation rates than those on plates covered with *n*-hexacosanal, the main aldehyde component of barley leaf cuticular wax. These results suggest that aldehyde wax components of different chain length could affect the prepenetration processes of B. graminis in a substance- and/or chain length-specific manner. However, this remains to be demonstrated.

In addition to the chemical composition of the plant cuticle, physical parameters such as surface hydrophobicity can play an important role in the course of the prepenetration process. Indeed, a sufficiently high surface hydrophobicity was required for *B. graminis* conidia on wax-sprayed glass slides to effectively trigger germination and appressorium differentiation in the presence of *n*-hexacosanal (Zabka *et al.*, 2008). This important feature, however, was not under consideration in the study of Tsuba *et al.* (2002).

The central aim of the present study was to determine the germination- and differentiation-inducing capabilities of different very-long-chain aldehydes present in cereal leaf cuticular waxes in order to understand which chemical properties present in cuticular wax are required for the efficient promotion of appressorial differentiation of *B. graminis* conidia. For this purpose, a novel Formvar® resin-based in vitro system was developed, which provides highly homogeneous hydrophobic surfaces that exhibit essentially uniform contact angles. This permitted specific analysis of the effects of bioactive lipophilic extracts or single wax constituents on the prepenetration processes of *B. graminis* f. sp. *hordei* and *B. graminis* f. sp. *tritici*, excluding secondary effects caused by variations in surface hydrophobicity.

2.3 Material and Methods

Pathogen and plant material

Hordeum vulgare L. cv Bonus (obtained from the Nordic Gene Bank, Alnarp, Sweden) and *Triticum aestivum* cv Winnetou (Saatzucht Firlbeck, Atting-Rinkam, Germany) seeds were sown in plastic pots (diameter, 9 cm) filled with standard potting soil (Typ ED73; SteuderComp, Schermbeck, Germany). The plants were kept in growth chambers with 300 µmol photons m−2 s−1 light intensity in a 16 h : 8 h photoperiod (22°C : 18°C) and 70% relative humidity. For wax analysis, 14-d-old secondary leaves were harvested and analyzed with respect to surface hydrophobicity and epicuticular wax chemistry, and subjected to assays with B. graminis.

Blumeria graminis (D*C.*) E.O. Speer f. sp. *hordei* Em. Marchal [isolate CC1, originally obtained from Tim Carver, Institute of Grassland and Environmental Research (IGER), Aberystwyth, UK] was propagated on its host barley (*Hordeum vulgare* L. cv Bonus), and *Blumeria graminis* (D*C.*) E.O. Speer f. sp. *tritici* Em. Marchal (kindly provided by Professor R. Hückelhoven, Technical University of Munich, Germany) on its host wheat (*Triticum aestivum* L. cv Winnetou) under the same conditions as described above until distinct white powdery pustules appeared. One day before conidia were required for experimentation, spore-bearing leaves were shaken to remove older conidia, so that freshly emerged conidia were available for subsequent assays.

Sampling of cuticular waxes

Total leaf extracts of secondary leaves (n = 5) of 14-d-old barley or wheat plants were obtained by dipping entire leaves (apart from cut edges) for 2 min into 25 ml chloroform (> 99%; Roth, Karlsruhe, Germany). *n*-Tetracosane (Sigma-Aldrich, Steinheim, Germany) was added to all extracts as an internal standard. The solvent was removed under a gentle flow of nitrogen.

Chemical analysis

Before gas chromatography (GC) analysis, hydroxyl-containing compounds in all samples were transformed into the corresponding trimethylsilyl derivatives by reaction with bis-N,O-trimethylsilyltrifluoroacetamide (Macherey-Nagel, Düren, Germany) in pyridine (30 min at 70°C).

The quantitative compositions of the mixtures were studied using capillary GC (5890 Hewlett Packard Series II; Agilent Technologies, Santa Clara, CA, USA) and a flame ionization detector under the same conditions as a qualitative analysis (6890 N, Agilent Technologies), with mass spectrometric detection (m/z 50–750; MSD 5973, Agilent Technologies).

GC was carried out with on-column injection (30 m, 0.32 mm inner diameter, DB-1, df = 0.1 µm, J&W Scientific, Agilent Technologies). The oven temperature was programmed for 2 min at 50°C, 40°C min^{-1} to 200°C, 2 min at 200°C, 3°C min^{-1} to 320°C, 30 min at 320°C, and He carrier gas inlet pressures were programmed for 5 min at 50 kPa, 3 kPa min^{-1} to 150 kPa and 30 min at 150 kPa. Wax components were identified by comparison of their mass spectra with those of authentic standards and literature data. For the quantification of individual compounds, GC was used under the conditions described above, but with H_2 (5 min at 5 kPa, 3 kPa min^{-1} to 50 kPa and 30 min at 50 kPa) as the carrier gas and a flame ionization detector (HP ChemStation software package, Hewlett-Packard, Waldbronn, Germany).

Characterization of artificially coated surfaces and contact angle measurements

Small pieces of air-dried glass slides with different surface coatings were mounted on aluminum holders, sputter coated with gold–palladium (Bal-Tec SCD005 sputter coater; 25 mA, 300 s, Balzers, Oerlikon, Switzerland), and examined by scanning electron microscopy (SEM) (Zeiss DSM 962, 15 kV; Zeiss, Oberkochen, Germany). Sputtering conditions, depositing *c.* 20 nm of the alloy on the tissue samples, were optimized for the acceleration voltage used in SEM. To measure surface hydrophobicity, contact angles of 1 µl droplets of distilled water were determined (contact angle system OCA 15, software system SCA20; Dataphysics Instruments, Filderstadt, Germany). A total of 20 measurements on each of at least five independent surface samples was performed. Data are given as means ± SD.

Synthesis of very-long-chain aldehydes

Very-long-chain aldehydes were synthesized from their corresponding *n*-alkanols (*n*-docosanol, 0.61 mmol; *n*-tetracosanol, 1.8 mmol; *n*-hexacosanol, 0.91 mmol; n-octacosanol, 1.19 mmol; *n*-triacontanol, 0.26 mmol; all 99%, Sigma-Aldrich) according to the protocol developed by Corey & Suggs (1975). The respective *n*-alkanol was dissolved in CH_2Cl_2 (99.5%; Roth, Karlsruhe, Germany) and supplemented in a molar ratio of 1.2 : 1 to an ice-cold solution of pyridinium chlorochromate (PCC, 99%, Sigma) in CH_2Cl_2 containing 4 g Celite (Merck, Darmstadt, Germany).

After continuous stirring overnight at room temperature, the slurry formed was filtered over a silica gel column (Ø = 5 cm) and the aldehyde fractions were eluted with CH_2Cl_2. The purity of the aldehydes was checked by NMR spectroscopy and mass spectrometry (data not shown). Pure *n*-docosanal, *n*-tetracosanal, *n*-hexacosanal, *n*-octacosanal, and *n*-triacontanal were dissolved in chloroform and then applied to glass slides as described below.

Coating of glass slides

Histobond® glass slides (Marienfeld, Lauda-Königshofen, Germany) were carefully cleansed with a few drops of Deconex® 11 UNIVERSAL detergent (Applichem, Darmstadt, Germany), subsequently rinsed with distilled water, immersed in isopropanol for *c.* 10 s, again in distilled water for *c.* 1 min and finally dried at room temperature. A 0.5% solution of Formvar® resin (polyvinyl formal, CAS 63450-15-7, Applichem) in chloroform was supplemented with the C_{26} alkane *n*-hexacosane or other wax components to a final concentration of 7×10^{-4} mol l^{-1} (0.25 mg ml^{-1} *n*-hexacosane) only, or additionally with the respective very-long-chain aldehyde to final concentrations of 7×10^{-8}, 7×10^{-7}, 7×10^{-6} and 7×10^{-5} mol l^{-1} aldehyde. Isolated barley or wheat leaf wax was added to the 0.5% Formvar® solution to yield concentrations between 0.48 and 1 mg ml^{-1}. The dried glass slides were dipped into the respective coating solution for a few seconds. Subsequently, the slides were dried completely for 16 h at room temperature and then used for the experiments. All single wax components – except very-long-chain aldehydes – used for coating glass slides were purchased from Sigma-Aldrich.

Because of the similar solubility of the Formvar® resin and cuticular wax components in organic solvents, it was not possible to subsequently analyze the exact amounts of single wax components deposited onto the surface of the Formvar®-coated glass slides. In the following, we consequently refer to the concentrations of the single compounds present in the dipping solution. However, when the lipophilic dye Sudan III (Sigma-Aldrich) was employed as a 'model wax constituent' and supplemented to the Formvar solution in a defined concentration, increasing amounts of Sudan III resulted in similarly increasing amounts deposited onto the glass slide surfaces (data not shown). Hence, this dye might allow a rough estimate of the amount of wax compounds deposited onto the glass slide surfaces after dipping. Assuming that the deposition of Sudan III and of wax constituents was almost identical, Formvar®-coated glass slides had an average estimated total wax coverage of *c.* 0.5 µg cm^{-2}. Therefore, a concentration of 7×10^{-5} mol l^{-1} of a single wax compound in the dipping solution should correspond to a coverage of *c.* 50–60 ng cm^{-2}, depending on the molecular weight of the molecule.

For the production of glass slides coated with blends of wax constituents or extracted cuticular leaf waxes by means of spraying, the substances were dissolved in chloroform and sprayed onto Histobond® glass slides using a glass chromatography sprayer. In order to ensure full coverage of the glass surface, only those coated with ≥ 3 µg cm^{-2} of wax or wax constituents were used for data retrieval (Zabka *et al.*, 2008).

Studies of fungal prepenetration processes

Detached 14-d-old secondary leaves of *H. vulgare* or *T. aestivum*, with their adaxial surface up, and glass slides with different surface coatings were fixed at the base of a settling tower. Conidia from infected barley or wheat leaves, respectively, were blown into the tower using pressurized air to ensure their even distribution at a density of *c.* 2×10^3 conidia cm^{-2}. Artificial surfaces and leaves were kept moist with wet filter paper applied underneath to achieve a relative humidity of at least 90%. The samples were incubated for 16 h in the darkness at 20°*C.* To avoid displacement of ungerminated conidia, the leaves were placed, with their inoculated surface up, onto Whatman 3 MM paper moistened with ethanol : acetic acid (3 : 1, v/v) until bleached, and then transferred to filter paper moistened with lactoglycerol (lactic acid : glycerol : water, 1 : 1 : 1, v/v/v) for 3 h. Finally, fungal structures were stained for 30 min by carefully pipetting a few droplets of trypan blue (Merck) (0.05% w/v) acetic acid : glycerol : water (1 : 1 : 1, v/v/v) onto the inoculated surface (Lyngkjær & Carver, 1999).

Individual conidia were analyzed on each surface by light microscopy (Leica DMR with Leica IM1000 software package, Wetzlar, Germany) to determine whether they remained nongerminated (ng), had formed a primary germ tube only (pgt), a nonswollen secondary germ tube (sgt), a swollen appressorial germ tube (agt) or a hooked appressorium (app). In addition, the loss rate of conidia that were apparently damaged, burst or desiccated during the inoculation procedure was recorded (aberr). Only single, well-separated conidia were counted at each observation to eliminate the possibility of inhibition caused by crowding.

Statistical analysis

The basis for statistical analysis was n = 5 independent experiments in each case. For data concerning conidium development, n = 1 represents 500 examined conidia for in vitro experiments and 300 examined conidia for in vivo experiments. Significant differences ($P < 0.05$) between multiple datasets were tested by one-way ANOVA, followed by a Tukey HSD *post hoc* test; pairwise comparisons were tested by a Mann–Whitney U-test. In cases of significant differences ($P < 0.05$, Levene's test) of homogeneity between multiple datasets, the data were transformed.

2.4 Results

2.4.1 Composition of barley and wheat leaf cuticular waxes

The quantity and composition of the leaf cuticular waxes of the assayed barley and wheat cultivars were studied. In the wheat cv Winnetou, *n*-octacosanol – and not *n*-hexacosanol as in barley – was identified as the main wax constituent (Figure 1). The C_{28}-aldehyde *n*-octacosanal represented 1.1% of the cuticular wheat wax (0.12

µg cm^{-2}). In barley, the C_{26}-aldehyde *n*-hexacosanal amounted to only *c.* 0.8% of the total cuticular wax (0.06 µg cm^{-2}). The chain length distribution spectrum within the different substance classes (primary alkanols, aldehydes, alkyl esters and fatty acids) – with the exception of n-alkanes – was predominantly shifted by two carbon atoms towards a longer chain length in wheat. Approximately 3–4% of the leaf wax constituents of the two species remained unidentified. The absolute amount of cuticular wax of barley leaves (8 ± 1 µg cm^{-2}) did not largely differ from the amount that was extracted from the leaves of wheat (11 ± 1 µg cm^{-2}).

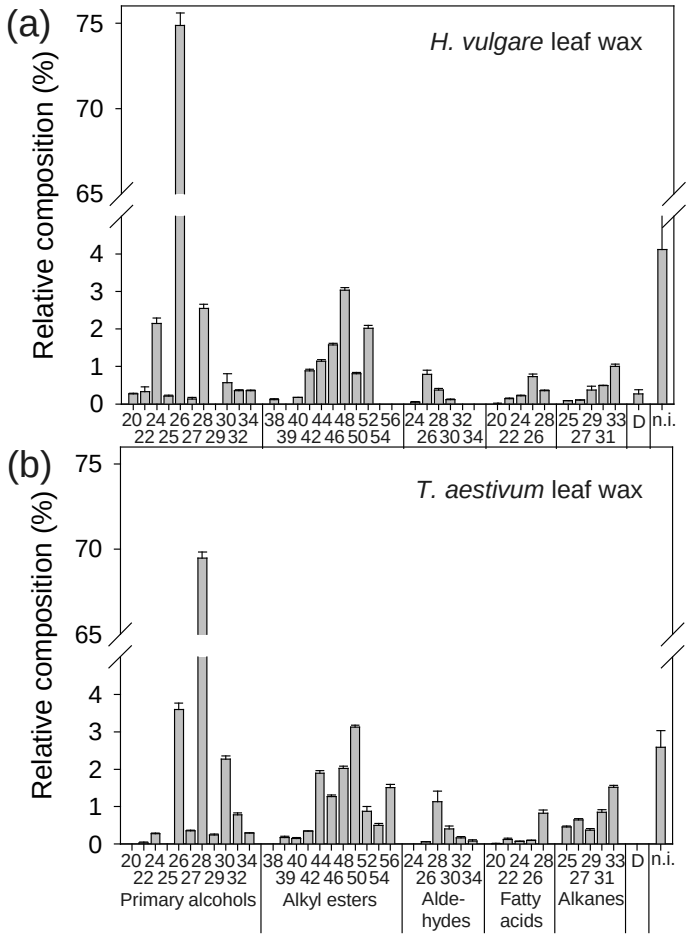

Figure 1 Relative composition (%) of total leaf wax extracts from *Hordeum vulgare* cv Bonus (a) and *Triticum aestivum* cv Winnetou (b). Carbon chain lengths are indicated for each chemical compound class, except for *n*-hexacosane-1,3-diol (D) and nonidentified (n.i.) compounds. Data are given as means from five replications ± SD.

2.4.2 Surface structures and contact angles

Adaxial barley and wheat leaf surfaces used for inoculation experiments were covered by the typical wax crystal platelets usually found on barley and wheat (data not shown), resulting in contact angles of 140 ± 5° for barley and 159 ± 7° for the even more hydrophobic wheat leaf surfaces. By contrast, SEM analysis of a glass slide surface coated with the Formvar® resin showed a largely homogeneous

smooth coverage, occasionally exhibiting fairly small irregularly scattered structures with contact angles of only 70 ± 3° (Figure 2a). When extracted barley leaf wax was added to the 0.5% Formvar® solution, yielding a final concentration of up to 1 mg l^{-1}, larger protrusions with a slab- to plate-like outer appearance and an average diameter of *c.* 2–5 µm appeared on the coated glass slide surface and exhibited contact angles of 108 ± 1° (Figure 2b). The addition of *n*-hexacosane (7×10^{-5} mol l^{-1}) to the 0.5% Formvar® solution resulted in smaller horizontal rod- or worm-like structures on the surface, having a length of *c.* 1 µm and similar contact angles of 114 ± 1° (Figure 2c). Supplementing the Formvar®/*n*-hexacosane mixture with different proportions of the assayed aldehydes did not result in further modifications of contact angle and surface topology – exemplified for 7×10^{-4} mol l^{-1} n-hexacosanal (Figure 2d).

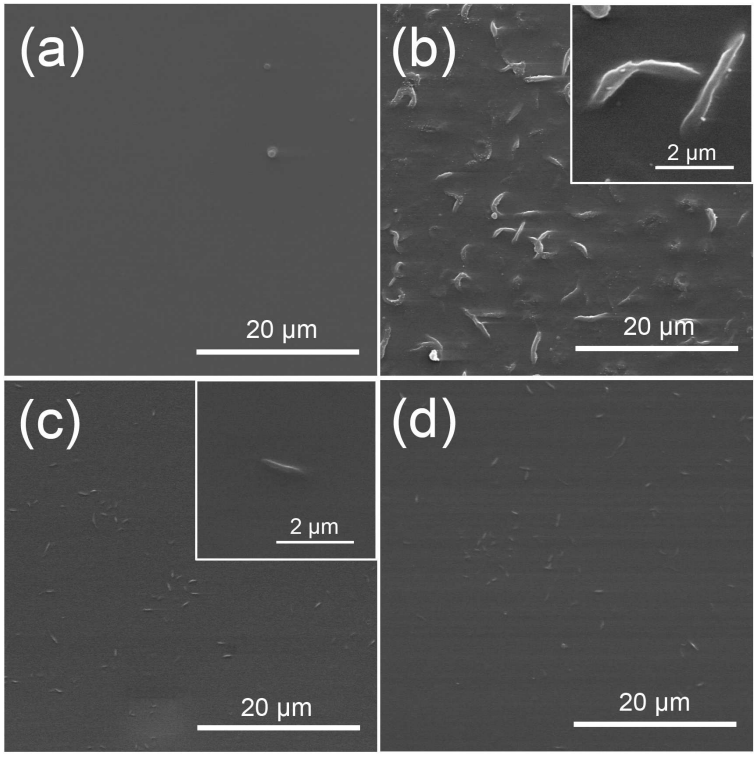

Figure 2 Scanning electron micrographs of glass slides with different surface coatings: (a) Formvar® only; (b) Formvar® resin supplemented with barley leaf wax (1 mg ml^{-1} in the dipping solution); (c) Formvar® with *n*-hexacosane; and (d) Formvar® with a mixture of *n*-hexacosane and *n*-hexacosanal. The insets in (b) and (c) show the respective surface at a higher magnification.

2.4.3 Prepenetration processes of *B. graminis* on native leaf surfaces and on wax-coated glass slides

After inoculation of host and nonhost adaxial leaf surfaces, *B. graminis* f. sp. *hordei* and *B. graminis* f. sp. *tritici* showed no significant differences with respect to conidial germination and differentiation (Figure 3a,c). More than 80% of the conidia formed a mature appressorium. Less than 4% of the barley powdery mildew conidia

remained nongerminated although apparently remaining intact (Figure 3a), whereas only < 1% of the wheat mildew conidia did not germinate (Figure 3c). This difference is also reflected by the slightly increased proportion of wheat mildew conidia with fully mature appressoria on wheat and barley leaves. The wheat and barley powdery mildew fungi showed very similar *in vitro* germination and appressorium formation rates on the Formvar®/*n*-hexacosane/isolated leaf wax-coated glass slides. However, comparable with the situation on adaxial leaf surfaces (Figure 3b), *B. graminis* f. sp. *tritici* exhibited slightly higher rates of germination and appressorium formation than did *B. graminis* f. sp. *hordei* on the respective wax glass slides (Figure 3d). Generally, formation rates of mature appressoria were distinctly reduced on Formvar®/*n*-hexacosane/isolated leaf wax-coated glass slides, whereas the majority of conidia (*c.* 60%) remained in the appressorial germ tube stage of fungal differentiation. On glass slides, the percentage of dead, burst conidia was, at most, 1%, whereas, on native surfaces, this proportion increased to 5–8% on average.

Among various single nonaldehyde wax constituents, only *n*-hexacosanol promotes germination and differentiation of *B. graminis* conidia

Control glass slides covered with Formvar® only (contact angle, 70 ± 3°) exhibited high germination rates (78 ± 8%), whereas only *c.* 7% formed an appressorial germ tube or an appressorium (Table 1). The majority of conidia, however, showed an aberrant development, resulting in mainly desiccated and burst conidia (56 ± 9%). Inoculation onto Formvar® membranes covering a block of 2% water agar (Kobayashi *et al.*, 1991) resulted in *c.* 21% differentiated conidia and only 5% showing an aberrant development.

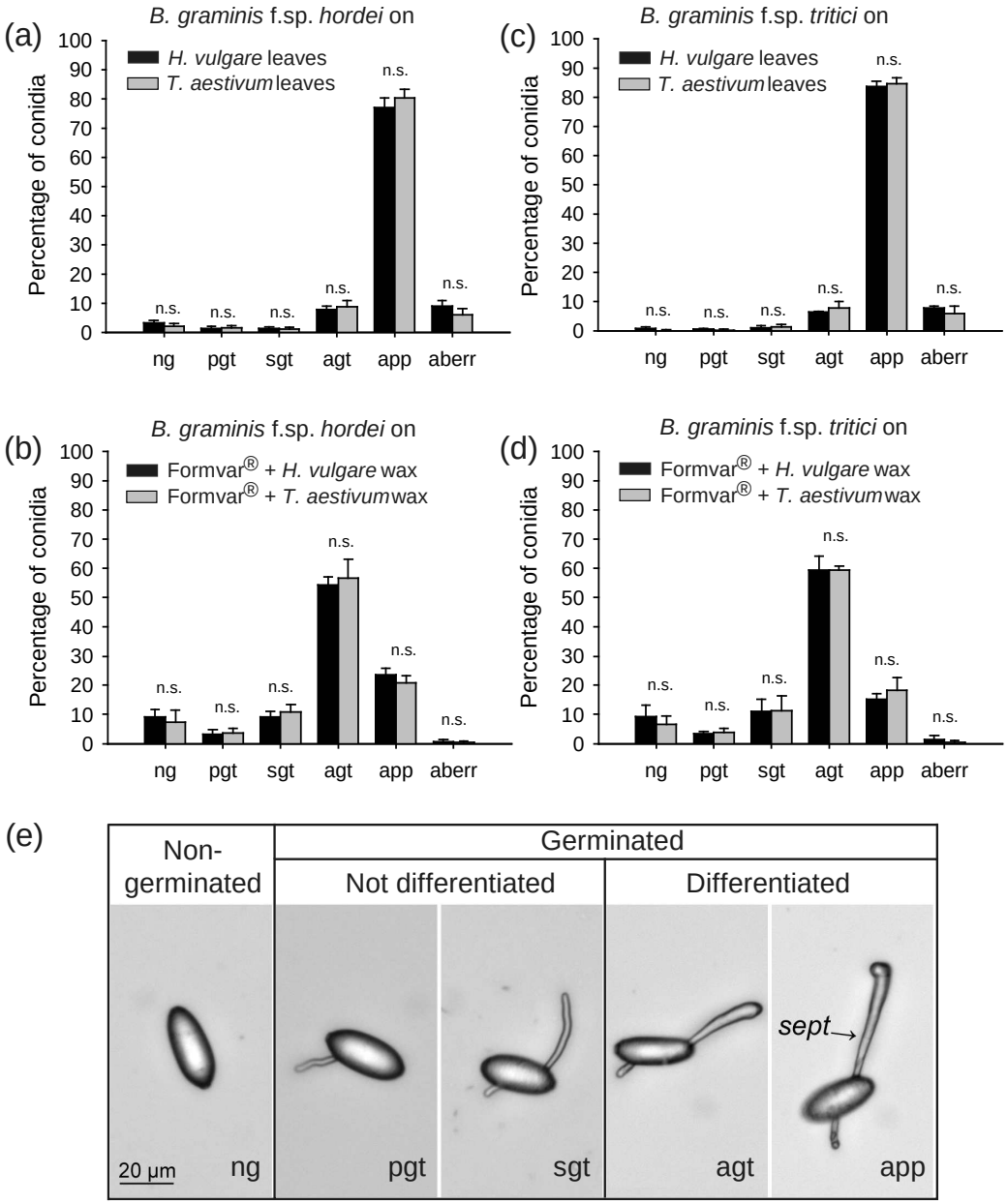

Figure 3 Development of *Blumeria graminis* f. sp. *hordei* (a) and *B. graminis* f. sp. *tritici* (c) conidia on adaxial leaf surfaces of *Hordeum vulgare* cv Bonus (black bars) and *Triticum aestivum* cv Winnetou (gray bars). Development of *B. graminis* f. sp. *hordei* (b) and *B. graminis* f. sp. *tritici* (d) conidia on glass slides covered with Formvar® resin supplemented with wax preparations of *H. vulgare* (black bars) and *T. aestivum* (gray bars). (e) Prepenetration developmental stages of *B. graminis* conidia: ng, nongerminated conidium without a germ tube; pgt, germinated conidium with a primary germ tube formed; sgt, conidium with an additionally formed and elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, fully differentiated conidium with a mature lobed and septated (sept) appressorium; aberr, burst, dead conidia. Values are given as mean ± SD of five independent experiments. ns, nonsignificant differences ($P \geq 0.05$, Mann–Whitney U-test) within a developmental category.

Coating glass slides with the Formvar®/*n*-hexacosane mixture resulted in distinctly reduced germination rates of *c.* 30%, whereas the differentiation rates were at best *c.* 4% with only 1% of the conidia forming appressoria, demonstrating the largely noninductive character of this Formvar®/*n*-alkane mixture (Table 1). In all Formvar®/*n*-alkane glass slide experiments, the percentage of dead, burst conidia was, at most, 1%.

In order to assay single wax constituents varying in chain length and compound class for their germination- and differentiation-promoting capabilities, Formvar®-only solutions and Formvar®/*n*-hexacosane mixtures were supplemented with the respective substance and subjected to inoculation bioassays with *B. graminis* conidia.

Even- and noneven-numbered *n*-alkanes as hydrophobic carrier substances in the Formvar® solution resulted in similar rates as with Formvar®/*n*-hexacosane, showing no specific induction of germination or differentiation (Table 1). The majority of conidia (*c.* 70%) remained in the nongerminated stage, whereas *c.* 20% of the conidia aborted their prepenetration development with the formation of a single primary germ tube.

Despite their low surface hydrophobicity, glass slides coated with Formvar®/*n*-hexacosanol (7×10^{-4} mol l^{-1}) showed significantly increased germination and differentiation rates compared with the other tested *n*-alkanols, which were not capable of distinctly promoting fungal differentiation. In addition to the *c.* 20% that were differentiated, the proportion of conidia with a formed secondary germ tube also increased, demonstrating that the presence of *n*-hexacosanol was capable of inducing the development of *c.* 40% of the conidia beyond the primary germ tube stage. Among the glass slides coated with a mixture of Formvar®/*n*-hexacosane/*n*-alkanol, only those supplemented with the C26-alkanol *n*-hexacosanol (7×10^{-5} mol l^{-1} *n*-hexacosanol) exhibited a significantly increased germination and appressorial germ tube formation, whereas appressorium formation remained unaffected.

The applied commercially available alkyl-esters (C_{40}–C_{44}) – with or without *n*-hexacosane in the dipping solution – did not result in a pronounced promotion of fungal germination and differentiation, although the proportions of conidia with an appressorial germ tube were distinctly higher when compared with the data from the *n*-alkane series. The assayed even-numbered fatty acids that are constituents of wheat and barley leaf wax resulted in differentiation rates of, at best, *c.* 6%, whereas *c.* 75% of the conidia remained nongerminated.

Table 1 *B. graminis* f.sp. *hordei* development on Formvar® coated glass slides supplemented with different non-aldehyde compounds occurring in barley and wheat leaf wax and corresponding surface contact angles.

Substratum		Percentage of conidia					Contact angle (deg)
		ng	pgt	sgt	agt	app	
0.5% Formvar®		22 ± 8^{A}	9 ± 4^{a}	$6 \pm 4^{\underline{A}}$	4 ± 3^{A}	$3 \pm 2^{\underline{A}}$	70 ± 3
0.5% Formvar® + 7 10^{-4} mol l^{-1} *n*-hexacosane		73 ± 3^{B}	18 ± 2^{b}	$2 \pm 1^{\underline{A}}$	3 ± 1^{B}	$1 \pm 1^{\underline{B}}$	114 ± 1
0.5% Formvar® membrane on 2% agarose		42 ± 3^{C}	28 ± 4^{c}	$7 \pm 2^{\underline{A}}$	12 ± 3^{B}	$9 \pm 3^{\underline{B}}$	nd
Concentration mol l^{-1}	Chain length						
Alkanes							
7×10^{-4}	24	71 ± 4^{a}	20 ± 2^{a}	2 ± 2^{a}	2 ± 1^{a}	1 ± 1^{a}	113 ± 1
	25	74 ± 3^{a}	16 ± 1^{a}	3 ± 1^{a}	3 ± 1^{a}	1 ± 1^{a}	113 ± 1
	26	73 ± 3^{a}	17 ± 1^{a}	2 ± 1^{a}	3 ± 1^{a}	1 ± 1^{a}	113 ± 1
	27	74 ± 4^{a}	17 ± 3^{a}	2 ± 1^{a}	3 ± 1^{a}	1 ± 1^{a}	112 ± 1
	28	72 ± 4^{a}	18 ± 3^{a}	2 ± 1^{a}	4 ± 1^{a}	1 ± 1^{a}	112 ± 1
	29	73 ± 3^{a}	17 ± 2^{a}	2 ± 1^{a}	3 ± 1^{a}	2 ± 1^{a}	112 ± 1
	30	75 ± 2^{a}	15 ± 2^{a}	2 ± 1^{a}	2 ± 1^{a}	2 ± 1^{a}	108 ± 1
	31	69 ± 4^{a}	19 ± 1^{a}	4 ± 1^{a}	4 ± 1^{a}	1 ± 1^{a}	110 ± 4
	33	76 ± 3^{a}	16 ± 3^{a}	2 ± 1^{a}	3 ± 1^{a}	1 ± 1^{a}	108 ± 1
Primary alcohols							
$7 \times 10^{-5(4)}$	20	76 ± 3^{ab}	14 ± 2^{a}	3 ± 2^{a}	3 ± 1^{a}	1 ± 1^{a}	115 ± 1
	22	73 ± 1^{abc}	17 ± 2^{ab}	2 ± 1^{a}	3 ± 1^{a}	1 ± 1^{a}	114 ± 1
	24	68 ± 4^{bc}	22 ± 3^{b}	3 ± 1^{a}	3 ± 2^{a}	1 ± 1^{a}	114 ± 1
	26	$^{67} \pm 5^{c}$	18 ± 4^{ab}	4 ± 3^{a}	9 ± 2^{b}	2 ± 1^{a}	114 ± 1
	27	80 ± 4^{a}	14 ± 3^{a}	2 ± 1^{a}	3 ± 1^{a}	1 ± 1^{a}	115 ± 1
	28	82 ± 4^{a}	13 ± 3^{a}	1 ± 1^{a}	3 ± 3^{a}	1 ± 1^{a}	115 ± 1
	30	77 ± 5^{a}	16 ± 6^{ab}	3 ± 1^{a}	3 ± 1^{a}	1 ± 1^{a}	115 ± 1
7×10^{-4}	20	80 ± 4^{a}	14 ± 3^{a}	1 ± 1^{a}	1 ± 1^{a}	1 ± 1^{a}	51 ± 8
	22	68 ± 3^{b}	25 ± 4^{b}	2 ± 1^{a}	1 ± 1^{a}	1 ± 1^{a}	33 ± 8
	24	68 ± 3^{b}	27 ± 3^{bc}	1 ± 1^{a}	1 ± 1^{a}	1 ± 1^{a}	37 ± 10
	26	27 ± 4^{c}	31 ± 4^{b}	17 ± 4^{b}	**17 ± 4^{b}**	**4 ± 3^{b}**	44 ± 3
	27	76 ± 3^{a}	21 ± 2^{ac}	1 ± 1^{a}	1 ± 1^{a}	1 ± 1^{a}	51 ± 2
	28	79 ± 3^{a}	17 ± 1^{a}	2 ± 2^{a}	2 ± 2^{a}	1 ± 1^{a}	62 ± 3
	30	77 ± 5^{a}	16 ± 5^{a}	3 ± 1^{a}	3 ± 2^{a}	1 ± 1^{ab}	89 ± 2
Alkyl esters							
$7 \times 10^{-5(4)}$	40[1]	71 ± 4^{ab}	20 ± 5^{ab}	2 ± 1^{a}	5 ± 3^{a}	1 ± 1^{a}	114 ± 1
	42[2]	67 ± 4^{b}	23 ± 5^{b}	2 ± 2^{a}	7 ± 1^{a}	1 ± 1^{a}	113 ± 1
	44[3]	75 ± 2^{ac}	14 ± 2^{ac}	1 ± 2^{a}	8 ± 3^{a}	1 ± 1^{a}	114 ± 1
7×10^{-4}	40[1]	76 ± 3^{a}	14 ± 4^{a}	2 ± 3^{a}	6 ± 1^{a}	1 ± 1^{a}	113 ± 1
	42[2]	69 ± 5^{a}	18 ± 3^{a}	4 ± 3^{a}	7 ± 1^{a}	1 ± 1^{a}	113 ± 1
	44[3]	70 ± 4^{a}	17 ± 2^{a}	3 ± 2^{a}	9 ± 5^{a}	1 ± 1^{a}	114 ± 1
Fatty acids							
$7 \times 10^{-5(4)}$	20	69 ± 5^{a}	21 ± 5^{ab}	4 ± 3^{ab}	5 ± 1^{a}	1 ± 1^{a}	114 ± 1
	22	68 ± 3^{a}	22 ± 2^{a}	5 ± 2^{a}	4 ± 2^{a}	1 ± 1^{a}	114 ±1
	24	69 ± 4^{a}	19 ± 2^{ab}	4 ± 1^{ab}	5 ± 2^{a}	1 ± 1^{a}	113 ± 1
	26	74 ± 2^{ab}	19 ± 2^{ab}	2 ± 1^{ab}	3 ± 1^{a}	1 ± 1^{a}	113 ± 1
	28	77 ± 4^{b}	15 ± 2^{b}	1 ± 2^{b}	4 ± 1^{a}	1 ± 1^{a}	113 ± 1
7×10^{-4}	20	75 ± 5^{a}	18 ± 3^{a}	3 ± 2^{a}	3 ± 2^{a}	1 ± 1^{a}	68 ± 2
	22	73 ± 2^{a}	20 ± 1^{a}	2 ± 1^{a}	4 ± 2^{a}	1 ± 1^{a}	72 ± 3
	24	79 ± 3^{a}	17 ± 3^{ab}	1 ± 1^{a}	2 ± 1^{a}	1 ± 1^{a}	73 ± 3
	26	72 ± 7^{a}	16 ± 5^{ab}	4 ± 3^{a}	4 ± 1^{a}	2 ± 1^{a}	82 ± 5
	28	79 ± 2^{a}	11 ± 4^{b}	2 ± 1^{a}	3 ± 1^{a}	2 ± 1^{a}	80 ± 9

[1]Arachidic acid arachidyl ester. [2]Arachidic acid behenyl ester. [3]Behenic acid behenyl ester. [4]Supplemented with n-hexacosane to a final concentration of 7×10^{-4} mol l^{-1}. Values are means ± SD of five independent experiments. Different letters within a column, with the same concentration and same compound class, indicate significant differences ($P < 0.05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test. The same superscript letter type (upper and lowercase, italic/bold/underlined) within a column and the same concentration indicates the dataset used for the respective one-way ANOVA. Prepenetration developmental stages of B. graminis conidia: ng, nongerminated conidium without a germ tube; pgt, germinated conidium with a primary germ tube formed; sgt, conidium with an additionally formed and elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, fully differentiated conidium with a

mature lobed and septated (sept) appressorium. Values resulting in differentiation rates (agt + app) ≥ 20% are highlighted in bold. nd, not determined.

Barley leaf wax stimulates in vitro prepenetration processes of *B. graminis* even in the presence of fatty acids

Because of the significantly elevated fatty acid content in the leaf cuticular wax of the barley mutant *cer-yj.667* and the reduced appressorium formation rates of *B. graminis* conidia on leaves of that mutant (Zabka *et al.*, 2008), one might speculate that free fatty acids could function as inhibitors of *B. graminis* differentiation. To further investigate this assumption, isolated barley leaf wax was spiked with 10% (w/w) of a fatty acid (C_{20}–C_{28}) into the Formvar® dipping solution. This treatment, however, resulted in only minor, nonsignificant changes with respect to germination and differentiation when compared with the Formvar®/barley wax-only glass slides (Figure 4).

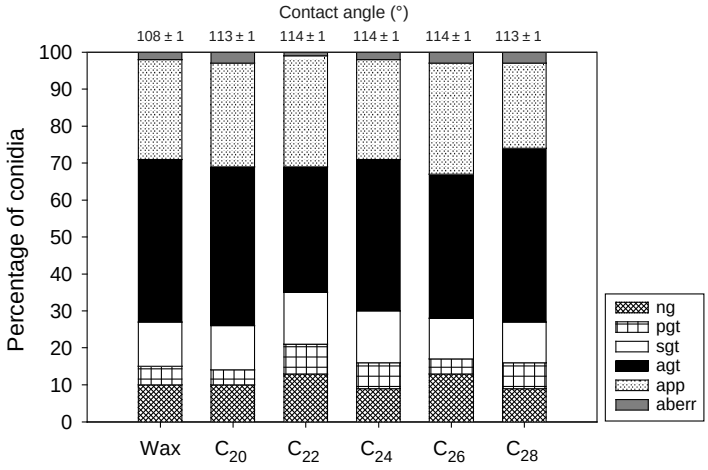

Figure 4 *Blumeria graminis* f. sp. *hordei* development on glass slides coated with Formvar®/barley leaf wax (480 µg barley wax ml^{-1} dipping solution) or Formvar®/barley leaf wax supplemented with 10% (w/w) of even-numbered fatty acids with chain lengths ranging from C_{20} to C_{28} and the corresponding glass slide contact angles. Values are given as the means of five independent experiments. A Mann–Whitney U-test was performed comparing each developmental category with the respective category of the wax control. No significant differences were found ($P \geq 0.05$). Prepenetration developmental stages of *B. graminis* conidia: ng, nongerminated conidium without a germ tube; pgt, germinated conidium with a primary germ tube formed; sgt, conidium with an additionally formed and elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, fully differentiated conidium with a mature lobed and septated (sept) appressorium; aberr, burst, dead conidia.

Very-long-chain aldehydes promote *in vitro* prepenetration processes in a dose- and chain length-dependent manner

In order to assay the qualitative and quantitative effects of very-long-chain aldehydes on powdery mildew prepenetration processes, glass slides covered with a Formvar®/*n*-hexacosane mixture, which had been supplemented with different quantities of single very-long-chain aldehydes (C_{22}–C_{30}) before coating, were

inoculated with *B. graminis* conidia (Tables 2 and 3). All of the tested aldehydes were capable of distinctly promoting *B. graminis* prepenetration processes. *n*-Hexacosanal was, by far, the most effective molecule tested. In the treatment with the lowest aldehyde proportion (7×10^{-8} mol l^{-1} aldehyde in the dipping solution), only *n*-hexacosanal had a significant positive impact on the germination and differentiation of *B. graminis* f. sp. *hordei* conidia (Table 2). In this treatment, *n*-docosanal, *n*-tetracosanal, *n*-octacosanal and *n*-triacontanal showed no significant differences from the otherwise largely inert alkane *n*-hexacosane with respect to germination and the formation of differentiated infection structures (secondary germ tube, appressorial germ tube and appressorium). A significant increase in germination and differentiation rates was found for *n*-tetracosanal and *n*-octacosanal in the treatment with a 10-fold increased amount of aldehydes. A 10-fold increase in the *n*-hexacosanal content led to an approximate doubling of the appressorium formation rate of *B. graminis* f. sp. *hordei*. However, only slight changes occurred on further increasing the n-hexacosanal proportion to 7×10^{-5} mol l^{-1}. Likewise, the presence of the other aldehydes led to an increase in appressorium formation, but only at elevated contents (7×10^{-6} and 7×10^{-5} mol l−1). In comparison with *n*-hexacosanal, the other tested aldehydes exhibited a gradual decrease in their differentiation-inducing capabilities with both decreasing and increasing chain lengths. With 20 ± 2% only, the treatment with the largest quantities of *n*-hexacosanal (7 × 10−5 mol l−1) approached the rate of appressorium formation found for barley leaf wax supplemented to Formvar® resin (22 ± 4%) (Figure 3b). Hence, the maximum rates of appressorium formation on these coated glass slides were significantly lower (P = 0.008) than on native leaf surfaces. Nevertheless, on Formvar®/*n*-hexacosane/*n*-hexacosanal-coated glass slides, as well as on the native leaf surfaces, 80–90% of the inoculated conidia differentiated a swollen appressorial germ tube or a fully mature appressorium.

Conidia of the wheat powdery mildew *B. graminis* f. sp. *tritici* generally showed a stronger response to the presence of very-long-chain aldehydes than did *B. graminis* f. sp. *hordei* (Table 3). However, *n*-hexacosanal was still the most effective aldehyde, particularly in the treatment with the lowest aldehyde amounts, where conidia of *B. graminis* f. sp. *tritici* formed 12% mature appressoria, whereas only 5% of the inoculated *B. graminis* f. sp. *hordei* conidia reached this advanced stage of prepenetration. At this low concentration, *n*-octacosanal – the most prevalent aldehyde of wheat leaf wax – significantly increased the differentiation of appressorial germ tubes and appressoria of *B. graminis* f. sp. *tritici*. In contrast with the barley powdery mildew, *B. graminis* f. sp. *tritici* showed no significant differences with respect to the formation of appressorial germ tubes and appressoria in the presence of *n*-octacosanal or *n*-hexacosanal in the 7×10^{-6} and 7×10^{-5} mol l^{-1} aldehyde treatments (Table 3). *Blumeria graminis* f. sp. *tritici*

exhibited a distinct shift towards elevated germination and differentiation capabilities in the presence of the longer chain aldehydes *n*-octacosanal and *n*-triacontanal, when compared with the barley powdery mildew fungus. Despite significant differences regarding surface contact angles, glass slides sprayed with isolated leaf wax or respective mixtures of *n*-hexacosane supplemented with different amounts of very-long-chain aldehydes in the absence of Formvar® exhibited very similar results to those obtained using the Formvar®-based *in vitro* system (Figure 5, Supporting Information Table S1). However, Formvar®/barley leaf wax-coated slides exhibited a significantly lower percentage of desiccated and burst conidia than did wax-sprayed glass slides (Figure 5).

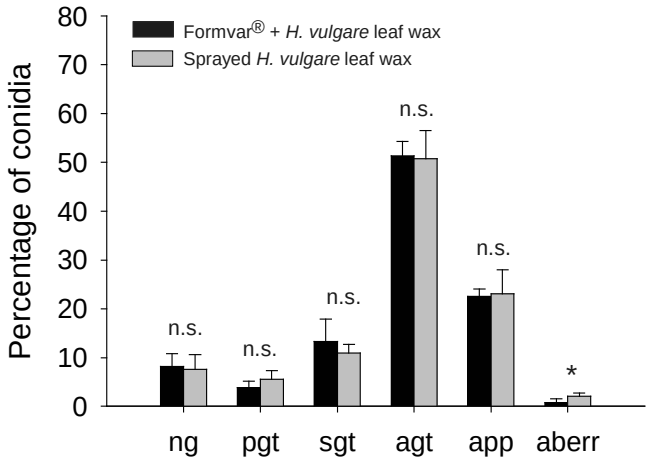

Figure 5 *Blumeria graminis* f. sp. *tritici* development on glass slides coated with Formvar® resin supplemented with barley leaf wax (black bars) and leaf wax-sprayed glass slides (gray bars). Prepenetration developmental stages of *B. graminis* conidia: ng, nongerminated conidium without a germ tube; pgt, germinated conidium with a primary germ tube formed; sgt, conidium with an additionally formed and elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, fully differentiated conidium with a mature lobed and septated (sept) appressorium; aberr, burst, dead conidia. *Significant differences ($P < 0.05$); ns, nonsignificant differences ($P \geq 0.05$; Mann–Whitney U-test).

Table 2 *B. graminis* f.sp. *hordei* development on Formvar®/*n*-hexacosane coated glass slides supplemented with very-long chain aldehydes (7 x10^{-8} to 7 x10^{-5} mol l^{-1} in the slide dipping solution) and corresponding surface contact angles.

Substratum		Percentage of conidia					Contact angle (deg)
		ng	pgt	sgt	agt	app	
0.5% Formvar®		22 ± 8^{A}	9 ± 4^{a}	6 ± 4$^{\underline{A}}$	4 ± 3^{A}	3 ± 2$^{\underline{A}}$	70 ± 3
0.5% Formvar® + 7 × 10^{-4} mol l^{-1} *n*-hexacosane		73 ± 3^{B}	18 ± 2^{b}	2 ± 1$^{\underline{A}}$	3 ± 1^{B}	1 ± 1$^{\underline{B}}$	114 ± 1
0.5% Formvar® membrane on 2% agarose		42 ± 3^{C}	28 ± 4^{c}	7 ± 2$^{\underline{A}}$	12 ± 3^{B}	9 ± 3$^{\underline{B}}$	nd
Concentration mol l^{-1}	Aldehyde						
7 × 10^{-8}	*n*-docosanal (C_{22})	73 ± 4^{a}	16 ± 1^{a}	4 ± 2^{a}	7 ± 3^{a}	1 ± 1^{a}	114 ± 1
	n-tetracosanal (C_{24})	70 ± 4^{a}	16 ± 2^{a}	5 ± 2^{a}	8 ± 2^{a}	1 ± 1^{a}	114 ± 1
	n-hexacosanal (C_{26})	45 ± 7^{b}	18 ± 3^{a}	12 ± 3^{b}	**20 ± 5^{b}**	**5 ± 1^{b}**	114 ± 1
	n-octacosanal (C_{28})	65 ± 2^{a}	18 ± 1^{a}	7 ± 2^{a}	9 ± 2^{a}	1 ± 1^{a}	114 ± 1
	n-triacontanal (C_{30})	67 ± 6^{a}	19 ± 4^{a}	6 ± 1^{a}	8 ± 3^{a}	1 ± 1^{a}	114 ± 1
7 × 10^{-7}	*n*-docosanal (C_{22})	69 ± 2^{a}	17 ± 1^{ab}	5 ± 1^{a}	8 ± 2^{a}	1 ± 1^{a}	114 ± 1
	n-tetracosanal (C_{24})	56 ± 2^{b}	16 ± 2^{ab}	8 ± 2^{a}	16 ± 3^{b}	2 ± 1^{a}	114 ± 1
	n-hexacosanal (C_{26})	22 ± 5^{c}	12 ± 2^{b}	17 ± 5^{b}	**37 ± 6^{c}**	**10 ± 2^{b}**	114 ± 1
	n-octacosanal (C_{28})	36 ± 2^{d}	18 ± 3^{a}	14 ± 3^{b}	**26 ± 4^{d}**	**6 ± 2^{b}**	114 ± 1
	n-triacontanal (C_{30})	66 ± 6^{a}	18 ± 3^{a}	5 ± 2^{a}	10 ± 2^{ab}	1 ± 1^{a}	114 ± 1
7 × 10^{-6}	*n*-docosanal (C_{22})	36 ± 5^{a}	26 ± 4^{a}	14 ± 4^{ab}	**20 ± 4^{a}**	**5 ± 1^{a}**	114 ± 1
	n-tetracosanal (C_{24})	19 ± 6^{b}	9 ± 3^{b}	19 ± 3^{b}	**41 ± 2^{b}**	**10 ± 2^{b}**	114 ± 1
	n-hexacosanal (C_{26})	12 ± 6^{b}	4 ± 2^{b}	17 ± 5^{ab}	**49 ± 3^{c}**	**17 ± 2^{c}**	114 ± 1
	n-octacosanal (C_{28})	13 ± 1^{b}	6 ± 2^{b}	16 ± 2^{ab}	**50 ± 3^{c}**	**14 ± 3^{c}**	114 ± 1
	n-triacontanal (C_{30})	38 ± 2^{a}	18 ± 3^{c}	11 ± 2^{a}	**27 ± 3^{d}**	**6 ± 1^{a}**	114 ± 1
7 × 10^{-5}	*n*-docosanal (C_{22})	12 ± 3^{ab}	11 ± 2^{a}	26 ± 5^{a}	**39 ± 5^{a}**	**11 ± 2^{a}**	114 ± 1
	n-tetracosanal (C_{24})	14 ± 6^{ab}	4 ± 1^{b}	15 ± 2^{b}	**50 ± 4^{bd}**	**16 ± 2^{bc}**	114 ± 1
	n-hexacosanal (C_{26})	9 ± 4^{b}	4 ± 1^{b}	12 ± 4^{b}	**54 ± 3^{bc}**	**20 ± 2^{d}**	113 ± 1
	n-octacosanal (C_{28})	9 ± 3^{b}	3 ± 1^{b}	11 ± 2^{b}	**58 ± 3^{c}**	**18 ± 1^{cd}**	114 ± 1
	n-triacontanal (C_{30})	18 ± 4^{a}	8 ± 1^{a}	14 ± 2^{b}	**45 ± 1^{ad}**	**13 ± 1^{ab}**	114 ± 1

Values are means ± SD of five independent experiments. Different letters within a column and the same concentration indicate significant differences ($P < 0.05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test. The same superscript letter type (upper and lowercase, italic/bold/underlined) within a column and the same concentration indicates the dataset used for the respective one-way ANOVA. Prepenetration developmental stages of *B. graminis* conidia: ng, nongerminated conidium without a germ tube; pgt, germinated conidium with a primary germ tube formed; sgt, conidium with an additionally formed and elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, fully differentiated conidium with a mature lobed and septated (sept) appressorium. Values resulting in differentiation rates (agt + app) ≥ 20% are highlighted in bold. nd, not determined.

Table 3 *Blumeria graminis* f. sp. *tritici* development on Formvar®/*n*-hexacosane-coated glass slides supplemented with very-long-chain aldehydes (7×10^{-8} to 7×10^{-5} mol l^{-1} in the slide dipping solution), and corresponding surface contact angles.

Substratum		Percentage of conidia					Contact angle (deg)
		ng	pgt	sgt	agt	app	
0.5% Formvar®		22 ± 8^{A}	9 ± 4^{a}	6 ± 4$^{\underline{A}}$	4 ± 3^{A}	3 ± 2$^{\underline{A}}$	70 ± 3
0.5% Formvar® + 7×10^{-4} mol l^{-1} *n*-hexacosane		73 ± 3^{B}	18 ± 2^{b}	2 ± 1$^{\underline{A}}$	3 ± 1^{B}	1 ± 1$^{\underline{B}}$	114 ± 1
0.5% Formvar® membrane on 2% agarose		42 ± 3^{C}	28 ± 4^{c}	7 ± 2$^{\underline{A}}$	12 ± 3^{B}	9 ± 3$^{\underline{B}}$	nd
Concentration mol l^{-1}	Aldehyde						
7×10^{-8}	*n*-docosanal (C_{22})	66 ± 4^{a}	27 ± 3^{a}	3 ± 1^{a}	4 ± 1^{a}	1 ± 1^{a}	114 ± 1
	n-tetracosanal (C_{24})	69 ± 5^{a}	22 ± 3^{a}	2 ± 1^{a}	6 ± 1^{a}	1 ± 1^{a}	114 ± 1
	n-hexacosanal (C_{26})	22 ± 6^{b}	21 ± 3^{a}	9 ± 1^{b}	**36 ± 6^{b}**	**12 ± 2^{b}**	114 ± 1
	n-octacosanal (C_{28})	41 ± 6^{c}	31 ± 3^{b}	6 ± 2^{c}	**16 ± 4^{c}**	**5 ± 2^{c}**	114 ± 1
	n-triacontanal (C_{30})	61 ± 4^{a}	23 ± 2^{a}	5 ± 2^{a}	9 ± 3^{a}	2 ± 1^{a}	114 ± 1
7×10^{-7}	*n*-docosanal (C_{22})	57 ± 6^{a}	34 ± 5^{ad}	3 ± 1^{a}	5 ± 2^{a}	1 ± 1^{a}	114 ± 1
	n-tetracosanal (C_{24})	51 ± 7^{a}	25 ± 4^{bd}	5 ± 2^{a}	**13 ± 4^{b}**	**6 ± 2^{b}**	114 ± 1
	n-hexacosanal (C_{26})	11 ± 3^{b}	9 ± 4^{c}	12 ± 2^{b}	**47 ± 7^{c}**	**19 ± 2^{c}**	114 ± 1
	n-octacosanal (C_{28})	17 ± 2^{b}	18 ± 3^{b}	12 ± 2^{b}	**36 ± 4^{d}**	**16 ± 3^{c}**	114 ± 1
	n-triacontanal (C_{30})	40 ± 3^{c}	29 ± 2^{d}	7 ± 2^{c}	19 ± 5^{b}	5 ± 2^{b}	114 ± 1
7×10^{-6}	*n*-docosanal (C_{22})	29 ± 4^{a}	45 ± 4^{a}	10 ± 3^{a}	10 ± 3^{a}	6 ± 2^{a}	114 ± 1
	n-tetracosanal (C_{24})	17 ± 4^{b}	14 ± 6^{b}	15 ± 2^{b}	**40 ± 7^{b}**	**14 ± 4^{b}**	114 ± 1
	n-hexacosanal (C_{26})	9 ± 2^{bc}	4 ± 3^{c}	9 ± 1^{a}	**54 ± 4^{c}**	**22 ± 2^{c}**	114 ± 1
	n-octacosanal (C_{28})	7 ± 1^{c}	5 ± 1^{c}	16 ± 4^{b}	**47 ± 2^{bc}**	**24 ± 1^{c}**	114 ± 1
	n-triacontanal (C_{30})	14 ± 5^{bc}	17 ± 4^{b}	15 ± 4^{b}	**38 ± 11^{b}**	**15 ± 2^{b}**	114 ± 1
7×10^{-5}	*n*-docosanal (C_{22})	24 ± 5^{a}	22 ± 4^{a}	16 ± 3^{a}	**27 ± 6^{a}**	**12 ± 1^{a}**	114 ± 1
	n-tetracosanal (C_{24})	12 ± 4^{b}	6 ± 1^{b}	13 ± 3^{ac}	**46 ± 3^{b}**	**23 ± 3^{b}**	114 ± 1
	n-hexacosanal (C_{26})	7 ± 3^{b}	2 ± 1^{b}	10 ± 3^{bc}	**51 ± 6^{b}**	**28 ± 5^{b}**	113 ± 1
	n-octacosanal (C_{28})	6 ± 1^{b}	3 ± 2^{b}	7 ± 2^{b}	**56 ± 3^{b}**	**27 ± 3^{b}**	114 ± 1
	n-triacontanal (C_{30})	7 ± 3^{b}	6 ± 3^{b}	14 ± 4^{ac}	**48 ± 8^{b}**	**24 ± 1^{b}**	114 ± 1

Values are means ± SD of five independent experiments. Different letters within a column and the same concentration indicate significant differences ($P < 0.05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test. The same superscript letter type (upper and lowercase, italic/bold/underlined) within a column and the same concentration indicates the dataset used for the respective one-way ANOVA. Prepenetration developmental stages of *B. graminis* conidia: ng, nongerminated conidium without a germ tube; pgt, germinated conidium with a primary germ tube formed; sgt, conidium with an additionally formed and elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, fully differentiated conidium with a mature lobed and septated (sept) appressorium. Values resulting in differentiation rates (agt + app) ≥ 20% are highlighted in bold. nd, not determined.

Fatty acids do not inhibit the stimulation of *B. graminis* prepenetration processes by the C_{26}-aldehyde *n*-hexacosanal *in vitro*

To demonstrate the germination- and differentiation-inducing activities of a single aldehyde wax compound, even in the presence of putative inhibitory fatty acids, Formvar®/*n*-hexacosane/fatty acid/*n*-hexacosanal-coated glass slides (7×10^{-6} mol l^{-1} aldehyde in the dipping solution) were subjected to inoculation with *B. graminis* (Figure 6). Irrespective of which fatty acid was applied, the addition of *n*-hexacosanal resulted in a significant increase in germination and differentiation rates when compared with the Formvar®/*n*-hexacosane/fatty acid-only treatments (Table 1), and reached similar values as on Formvar®/barley wax-coated glass slides. The slight differences between Formvar®/*n*-hexacosane/fatty acid/*n*-hexacosanal treatments and the Formvar®/barley wax treatment were all non-significant.

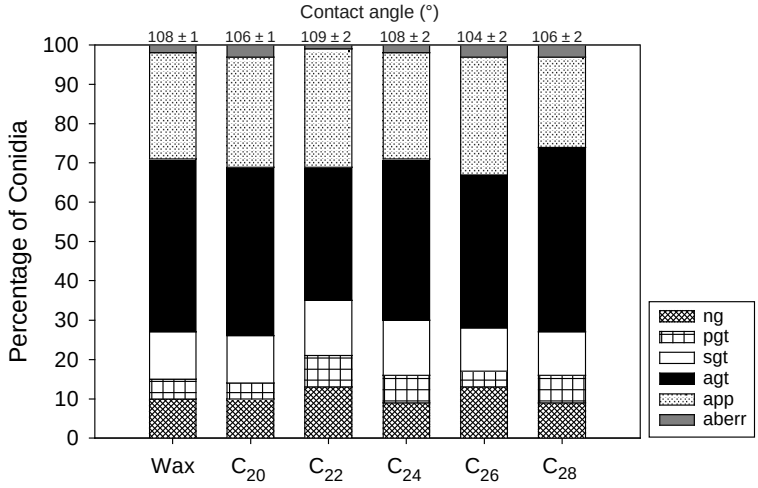

Figure 6 *Blumeria graminis* f. sp. *hordei* development on glass slides coated with Formvar®/barley leaf wax (480 µg barley wax ml^{-1} dipping solution) or Formvar®/n-hexacosane/fatty acid (7×10^{-5} mol l^{-1} of a C_{20}–C_{28} fatty acid) supplemented with 7×10^{-6} mol l^{-1} *n*-hexacosanal with the indicated contact angles. Prepenetration developmental stages of B. graminis conidia: ng, nongerminated conidium without a germ tube; pgt, germinated conidium with a primary germ tube formed; sgt, conidium with an additionally formed and elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, fully differentiated conidium with a mature lobed and septated (sept) appressorium; aberr, burst, dead conidia. A Mann–Whitney U-test was performed comparing each developmental category with the respective category of the wax control. No significant differences were found ($P \geq 0.05$).

2.5 Discussion

Cuticular waxes are well known to exert important general functions in the interplay of plants with their environment. (Müller & Riederer, 2005; Nawrath, 2006). This key adaptation in the evolution of plants (Raven & Edwards, 2004; Riederer, 2006) has been shown to affect plant–microbe interactions in several ways. Hence, the cuticle can be considered as a reservoir of signals telling phytopathogenic fungi that they have found an appropriate host to infect (Raffaele *et al.*, 2009). Cuticular wax

components, as well as cutin monomers, probably released by cutinases secreted by the conidium of *B. graminis*, have been shown to induce appressorium formation (Francis *et al.*, 1996; Tsuba *et al.*, 2002; Zabka *et al.*, 2008).

Apparently, *B. graminis* does not recognize all components present in the leaf wax, and therefore quite subtle differences in chemistry may have a relatively large effect (Carver & Gurr, 2006). The C_{26}-aldehyde (*n*-hexacosanal) present in barley leaf wax is known to induce differentiation of the secondary germ tube to a mature appressorium, whereas the C_{30}-aldehyde (*n*-triacontanal) is far less effective (Tsuba *et al.*, 2002). The alcohol analogs of these molecules are even less inductive (Tsuba *et al.*, 2002; Zabka *et al.*, 2008). Because of this clear-cut, cuticle-derived, chemical signaling, *B. graminis* appeared to be a suitable object for studying in vitro the action of different cuticular signals in triggering conidial germination and infection structure differentiation.

2.5.1 Prepenetration processes on wheat and barley leaves

Several earlier studies on the prepenetration stages of powdery mildew infection have found that fungal behavior is very similar on compatible and noncompatible grain hosts (Kunoh *et al.*, 1977; Willems, 2003). Therefore, it was not surprising that *B. graminis* f. sp. *hordei* conidia germinated and differentiated almost identically on the wheat and barley cultivars studied, as did the conidia of *B. graminis* f. sp. *tritici* – irrespective of differences concerning surface hydrophobicity and their assignment as incompatible nonhost or compatible host.

The differences found for the cuticular wax composition of secondary leaves of the assayed wheat and barley cv Winnetou and Bonus – primarily a shift in chain length for alcohols, aldehydes, fatty acids and esters – were generally in accordance with previous studies (von Wettstein–Knowles, 1971; Giese, 1975; Bianchi *et al.*, 1980; Baum *et al.*, 1989; Zabka *et al.*, 2008). Hence, the differences in wheat and barley leaf wax composition are apparently not sufficient to significantly affect the prepenetration processes of *B. graminis.*

2.5.2 The Formvar® resin-based *in vitro* system

The assessment of the biological activity of water-insoluble components of the epicuticular wax layer on the prepenetration processes of *B. graminis* is technically difficult, as coating of an artificial surface with isolated leaf waxes by simply evaporating the organic solvent results in an irregular distribution of the wax, even with uncoated regions, and therefore leads to highly variable results (Reisige *et al.*, 2006). Spraying chloroform-dissolved cuticular wax extracts onto hydrophilic glass slides, however, results in more homogeneous surfaces, providing distinctly

reproducible experimental data (Zabka *et al.*, 2008; Ringelmann *et al.*, 2009). Nonetheless, we developed a Formvar® resin-based surface coating procedure that leads to the deposition of a homogeneous layer on a hydrophilic glass surface providing highly uniform surface contact angles. Formvar® resin has been shown not to negatively affect the growth of powdery mildew germ tubes, and even to promote *B. graminis* differentiation at a moderate level (Kobayashi *et al.*, 1991; Kobayashi & Hakuno, 2003). This substance, in combination with the noninductive and more hydrophobic C_{26}-alkane *n*-hexacosane, appeared to be ideally suited for the establishment of a bioassay to characterize growth and developmental effects of specific wax components on the prepenetration processes of *B. graminis*.

The surface topology and physical structure of epicuticular waxes are known to play, at best, a minor role in conidial surface recognition processes leading to appressorium formation in *B. graminis* (Carver & Thomas, 1990; Rubiales *et al.*, 2001; Zabka *et al.*, 2008; Ringelmann *et al.*, 2009). A high density of epicuticular crystalline waxes is positively correlated with a high surface hydrophobicity (Holloway, 1970; Beattie & Marcell, 2002), which is commonly assessed by measuring the contact angle of a sessile water droplet on the respective surface.

The hydrophobic property of smooth layers of waxes depends on their chemical constitution and, almost certainly, on the orientation of constituent molecules in the solid state (Holloway, 1970). The Formvar®/*n*-hexacosane/aldehyde-coated glass slides had an average contact angle of 114°, which is slightly above the proposed maximum value of 110° representing a smooth wax surface (Holloway, 1970). Thus, an apparently marginal microroughness of the assayed surfaces must be responsible for their slightly higher contact angles (Holloway, 1970). However, more importantly, the contact angles were far above the *c.* 80° required for *n*-hexacosanal to trigger effectively the conidial prepenetration processes (Zabka *et al.*, 2008). The slight reduction of the contact angles on supplementing the 0.5% Formvar® solution with isolated barley or wheat leaf waxes might be attributable to the presence of large proportions of more polar and hence less hydrophobic *n*-alkanols in the isolated leaf wax fractions (Holloway, 1970). Contact angles are reduced even more when more polar alkanols or fatty acids are supplemented to the 0.5% Formvar® dipping solution.

The small protrusions with a slab- to plate-like outer appearance that appeared on the coated glass slides when extracted wheat or barley leaf wax was added to the 0.5% Formvar® solution might be the result of extracted plant wax recrystallization, although this did not further increase the surface hydrophobicity. In particular, the very-long-chain alkanol constituents of the wax are known to recrystallize as platelets on nonpolar surfaces (Koch *et al.*, 2006).

Generally, when compared with native leaf surfaces, glass slides coated with Formvar®/*n*-hexacosane/isolated leaf wax showed a slightly decreased germination

of *B. graminis* conidia, but, more prominently, a distinct decrease in the formation of mature appressoria, whereas the majority of the conidia remained in the appressorial germ tube stage. This difference could be explained by additional factors other than the wax composition and surface hydrophobicity involved in triggering appressorial differentiation present on barley epidermal surfaces (Carver *et al.*, 1996). However, the application of Formvar®-coated slides proved to be more efficient than using wax-sprayed glass surfaces for inoculation with *B. graminis* conidia. Formvar®/*n*-hexacosane-coated slides exhibited a significantly lower percentage of desiccated and broken conidia than did wax-sprayed glass slides. This improvement might be a consequence of the more uniformly coated surface.

The very-long-chain aldehydes accounted for *c.* 2% of the total wax extracts. Almost identical differentiation results on Formvar® surfaces spiked with leaf wax or *n*-hexacosane/*n*-hexacosanal (7×10^{-5} mol l^{-1} aldehyde treatment) endorse the notion that the Formvar®-based coating system constitutes an effective tool for assessing the effects of single wax components on the prepenetration processes of surface-penetrating plant pathogenic fungi.

2.5.3 Very-long-chain alkanes, alkanols, alkyl esters, fatty acids and the prepenetration processes

Among the assayed substances and substance classes, neither very-long-chain alkanes nor alkyl esters or fatty acids distinctly promoted the germination and/or differentiation of *B. graminis* conidia. The results of the present study therefore do not confirm very recent data suggesting a strong germination- and differentiation-promoting capability of C_{24} and C_{25} alkanes on *B. graminis* conidia (Feng *et al.*, 2009). However, in our hands, the most prominent barley leaf wax constituent *n*-hexacosanol resulted in significantly increased germination and differentiation rates, whereas the other *n*-alkanols remained essentially inert. The promoting effect, which is in full accordance with our previous study (Zabka *et al.*, 2008), was distinctly weakened when the concentration of the C_{26}-alkanol in the dipping solution was reduced to one tenth of the initial value, however, in the presence of the otherwise largely inert *n*-hexacosane, resulting in considerably higher contact angles. Hence, the present data suggest a dependence on concentration and chain length for the conidial germination- and differentiation-inducing activity of *n*-hexacosanol, even though an additional impact of a low surface hydrophobicity cannot be excluded.

The barley wax mutant *cer-yj.667* shows a significantly elevated fatty acid content of the leaf cuticular wax in combination with reduced appressorium formation rates on its leaf surfaces – particularly after removal of the epicuticular waxes (Zabka *et al.*, 2008). This led to the assumption that fatty acids might function

as inhibitors of *B. graminis* differentiation. However, our present data demonstrate that a fatty acid, even at a proportion of 10% with respect to the wax in the coating mixture, does not inhibit the in vitro germination and differentiation induced by isolated barley wax or synthetic n-hexacosanal.

The applied commercially available alkyl-esters did not result in a pronounced promotion of fungal germination and differentiation. As the naturally occurring primary alkanol-containing esters in barley and wheat leaf wax are characterized by hexacosanol (75%) or octacosanol (79%) as the predominant alcohol moiety and C_{16}, C_{18}, C_{20}, C_{22} and C_{24} as the major esterified fatty acids (Tulloch & Weenink, 1969; Giese, 1975), the applied alkyl-esters constitute only a minor fraction among the naturally occurring alkyl esters. Therefore, it cannot be excluded that other alkyl esters might nevertheless be capable of triggering germination and/or differentiation.

2.5.4 Very-long-chain aldehydes and *B. graminis* prepenetration processes

Knowledge about the specific function(s) exerted by very-long-chain aldehydes, as typical components of the outermost protective sheathing of the plant, is scarce. Only with respect to organismic interactions have some different tasks and roles for very-long-chain aldehydes been reported: The C_{28}-aldehyde octacosanal, for example, is known to act as an ovipositional stimulator for the Hessian fly (*Mayetiola destructor*) on wheat (Morris *et al.*, 2000), and is involved in host plant recognition and infection structure differentiation in the wheat stem rust fungus Puccinia graminis (Reisige *et al.*, 2006). These studies underline the relevance of very-long-chain aldehydes as cuticular signals in different pathogen–plant interactions.

Very-long-chain aldehydes as common wax constituents in many plant species are known to occur as monomers or in a polymeric state (Lamberton & Redcliffe, 1960). Epicuticular waxes on the leaf surfaces of sugar cane, rice and *Nepenthes* pitchers are, to some extent, insoluble at room temperature, whereas hot chloroform dissolves them and releases higher proportions of very-long-chain aldehydes (Haas *et al.*, 2001; Riedel *et al.*, 2003). Hence, the extraction of very-long-chain aldehydes by dipping leaves in chloroform at room temperature might result in an underestimation of the real aldehyde content, as some proportion could exist in an insoluble polymeric form. However, our data on wheat and barley are in accordance with previous studies (von Wettstein–Knowles, 1971; Giese, 1975; Bianchi *et al.*, 1980; Baum *et al.*, 1989; Zabka *et al.*, 2008).

Among the aldehydes present in the cuticular wax layers of wheat and barley leaves, the C_{26}-aldehyde *n*-hexacosanal, closely followed by the C_{28}-aldehyde

n-octacosanal, were the most effective molecules capable of promoting *B. graminis* prepenetration development. Depending on the amount of the C_{30}-aldehyde *n*-triacontanal present, rates of appressorium formation accounted for *c.* 10–50% of the values obtained with *n*-hexacosanal. These results are therefore in accordance with a previous study, although no explicit data concerning the amount of applied aldehydes is given (Tsuba *et al.*, 2002).

n-Hexacosanal is the main aldehyde component of barley leaf wax, whereas *n*-octacosanal is the most prevalent aldehyde constituent of wheat leaf wax (von Wettstein–Knowles, 1971; Bianchi *et al.*, 1980; Baum *et al.*, 1989; Tsuba *et al.*, 2002). As both plants are infected by a host-specific *forma specialis* of *B. graminis*, we also aimed to determine whether B. graminis f. sp. tritici might have specifically adapted to the distinct wax aldehyde spectrum of its specific host since the evolutionary divergence between the different *formae speciales* of grass powdery mildew (Wyand & Brown, 2003). Interestingly, the preference for *n*-hexacosanal was almost the same for barley and wheat powdery mildew. However, *B. graminis* f. sp. *tritici* showed a somewhat stronger response to *n*-octacosanal than did f. sp. *hordei*. This minor effect could be interpreted as a possible result of gradual adaptation to its specific host – although, so far, no distinct evidence for co-evolution between *B. graminis* ff. *spp*. and their specific hosts has been found (Wyand & Brown, 2003; Inuma *et al.*, 2007). Alternatively, this slight preference shift could simply be an isolate-specific trait that remains to be determined.

The complete absence of the most inductive *n*-hexacosanal from the abaxial leaf epicuticular waxes of ryegrass (*Lolium perenne*) results in drastically reduced *B. graminis* differentiation and infection rates on the abaxial leaf surface (Carver *et al.*, 1990; Ringelmann *et al.*, 2009). In combination with a distinctly reduced surface hydrophobicity, this specific feature widely protects the abaxial leaf surface of *L. perenne* from powdery mildew attack, which demonstrates and emphasizes the biological relevance of very-long-chain aldehydes in the powdery mildew–grass interaction.

To our knowledge, there are only two other known examples in which plant pathogenic fungi are known to exhibit a chain-length dependence with respect to appressorium formation triggered by constituents of the plant cuticle: the plant-pathogenic *Colletotrichum gloeosporioides* has a significant preference for very-long-chain primary alkanols (C_{24}–C_{32}), representing *c.* 5% of the cuticular wax of its host plant avocado (Podila *et al.*, 1993), and the rice blast fungus *Magnaporthe grisea* favors C_{16} and C_{18} cutin constituents for appressorium induction (Gilbert *et al.*, 1996).

In addition to the apparent relevance of the presence of a terminal carbonyl group for most effectively triggering germination and appressorium formation in *B. graminis* (Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Ringelmann *et al.*, 2009), the

concentration-dependent preference for very-long-chain aldehydes with chain lengths of C_{26} and C_{28} may suggest a protein with a size-selective hydrophobic patch or pocket to be involved in the perception of foliar surface chemistry. The weaker but still significant effect of the C_{26}-alkanol *n*-hexacosanol on fungal germination and differentiation underlines the relevance of chain length in combination with a hydroxyl or carbonyl head group of the inductive compound.

It has been speculated that fungal hydrophobins, such as MPG1 from *M. grisea* (Beckerman & Ebbole, 1996), known for their capability to form a hydrophobic coating on a surface, or other fungal surface-active proteins involved in hydrophobic surface sensing could play an important role in host recognition and pathogenesis via an interaction with fungal cutinase (Skamnioti & Gurr, 2008). This proposed interaction also seems plausible for *B. graminis*, as surface hydrophobicity and surface chemistry have been shown to share a distinct interdependence (Zabka *et al.*, 2008; Ringelmann *et al.*, 2009). Very recently, a secreted lipase (Lip1) from *B. graminis* was shown to possess lipolytic activity, releasing alkanes and alkanols from the leaf surface (Feng *et al.*, 2009). However, without considering the role of surface hydrophobicity, very-long-chain alkanes were reported to be the most efficient cues for appressorium formation released by Lip1 (Feng *et al.*, 2009). A strong differentiation-inducing capability of very-long-chain alkanes and alkanols is not confirmed by the present or previous studies on *B. graminis* differentiation (Tsuba *et al.*, 2002; Zabka *et al.*, 2008).

As anionic low-molecular-weight compounds can be taken up by the conidia of *B. graminis* before the formation of the primary germ tube (Nielsen *et al.*, 2000), the uptake of very-long-chain aldehydes or derivatives thereof might be involved in promoting germination and appressorium formation. However, it remains to be elucidated by which mechanism(s)/principle(s) plant pathogenic fungi are capable of solubilizing, binding, discriminating and perceiving very-long-chain aliphatic epicuticular wax constituents. Shedding further light on the signaling function(s) of cuticle-derived compounds and on the associated mechanism(s) is a major future challenge in the field of plant–pathogen interactions.

2.6 Acknowledgements

The authors thank Andrea Knorz for excellent and dedicated technical assistance, and Tim Carver (IGER, Aberystwyth, UK) and Professor R. Hückelhoven (Chair of Phytopathology, Technical University, Munich, Germany) for providing barley and wheat powdery mildew, respectively. This project was financially supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 567 (projects A5 and B8).

2.7 Supporting information

Table S1 *B. graminis* f.sp. *hordei* development on glass slides sprayed with *n*-hexacosane supplemented with very-long-chain aldehydes (7×10^{-8} to 7×10^{-5} mol l^{-1} in the spraying solution) and the corresponding surface contact angles.

Substratum		Percentage of conidia					Contact angle (deg)
Concentration mol l^{-1}	Aldehyde	ng	pgt	sgt	agt	app	
7×10^{-8}	*n*-docosanal (C_{22})	57 ± 6^{a}	26 ± 6^{a}	6 ± 2^{a}	8 ± 1^{a}	2 ± 1^{a}	102 ± 4
	n-tetracosanal (C_{24})	52 ± 2^{a}	29 ± 4^{a}	7 ± 1^{ab}	8 ± 3^{a}	1 ± 1^{a}	107 ± 3
	n-hexacosanal (C_{26})	15 ± 3^{b}	36 ± 8^{a}	11 ± 2^{b}	19 ± 4^{b}	7 ± 3^{b}	95 ± 8
	n-octacosanal (C_{28})	42 ± 10^{a}	36 ± 4^{a}	6 ± 4^{a}	5 ± 4^{a}	1 ± 1^{a}	82 ± 11
	n-triacontanal (C_{30})	43 ± 8^{a}	34 ± 8^{a}	7 ± 1^{ab}	8 ± 5^{a}	3 ± 1^{ab}	99 ± 6
7×10^{-7}	*n*-docosanal (C_{22})	47 ± 4^{a}	30 ± 5^{a}	6 ± 2^{a}	12 ± 3^{ab}	4 ± 2^{a}	107 ± 4
	n-tetracosanal (C_{24})	31 ± 5^{b}	28 ± 3^{ad}	12 ± 1^{bc}	19 ± 4^{ab}	6 ± 3^{a}	111 ± 1
	n-hexacosanal (C_{26})	9 ± 3^{c}	12 ± 3^{c}	20 ± 4^{b}	33 ± 4^{c}	13 ± 6^{b}	98 ± 5
	n-octacosanal (C_{28})	25 ± 5^{b}	21 ± 3^{ad}	15 ± 4^{bc}	24 ± 4^{b}	5 ± 1^{a}	93 ± 7
	n-triacontanal (C_{30})	29 ± 7^{b}	39 ± 5^{b}	10 ± 3^{ac}	13 ± 3^{a}	3 ± 1^{a}	96 ± 6
7×10^{-6}	*n*-docosanal (C_{22})	38 ± 6^{a}	27 ± 2^{a}	9 ± 3^{a}	21 ± 3^{a}	5 ± 3^{a}	111 ± 1
	n-tetracosanal (C_{24})	21 ± 3^{b}	22 ± 2^{a}	17 ± 2^{bc}	31 ± 4^{b}	8 ± 2^{a}	111 ± 1
	n-hexacosanal (C_{26})	8 ± 3^{c}	6 ± 4^{b}	21 ± 4^{c}	40 ± 5^{c}	18 ± 5^{b}	110 ± 2
	n-octacosanal (C_{28})	13 ± 4^{bc}	10 ± 3^{b}	15 ± 4^{ab}	50 ± 2^{d}	10 ± 2^{a}	111 ± 2
	n-triacontanal (C_{30})	20 ± 5^{b}	22 ± 3^{a}	15 ± 3^{ab}	33 ± 3^{bc}	8 ± 2^{a}	114 ± 2
7×10^{-5}	*n*-docosanal (C_{22})	13 ± 2^{a}	18 ± 3^{a}	21 ± 3^{a}	38 ± 2^{a}	9 ± 4^{a}	111 ± 1
	n-tetracosanal (C_{24})	11 ± 2^{a}	13 ± 2^{a}	21 ± 4^{a}	42 ± 3^{ab}	12 ± 2^{abc}	103 ± 3
	n-hexacosanal (C_{26})	6 ± 3^{a}	5 ± 2^{b}	16 ± 1^{ab}	42 ± 5^{ab}	25 ± 7^{c}	107 ± 7
	n-octacosanal (C_{28})	9 ± 2^{a}	7 ± 2^{b}	14 ± 2^{b}	49 ± 5^{b}	19 ± 6^{bc}	112 ± 2
	n-triacontanal (C_{30})	12 ± 4^{a}	14 ± 3^{a}	12 ± 1^{b}	45 ± 3^{ab}	15 ± 2^{abc}	109 ± 3

Values are means ± SD of five independent experiments. Different letters within a column and the same aldehyde concentration indicate significant differences ($P < 0.05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test. Abbreviations of the developmental categories are given in the legend of Figure 3. Values resulting in differentiation rates (agt + app) ≥ 20% are highlighted in bold.

3 Wax matters: absence of very-long-chain aldehydes from the leaf cuticular wax of the *glossy11* mutant of maize compromises the prepenetration processes of *Blumeria graminis*

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Published in *Plant Pathology* (2011) **60:** 1151-1161

3.1 Abstract

Conidial germination and differentiation, the so-called prepenetration processes, of the barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*) are triggered *in vitro* by very-long-chain aldehydes, minor constituents of barley leaf wax. However, until now it has not been demonstrated that these cuticle-derived molecules also play a significant role in the initiation and promotion of the fungal prepenetration processes *in vivo*, on the surface of a living plant leaf. In the maize (*Zea mays*) wax mutant *glossy11*, which is completely devoid of cuticular very-long-chain aldehydes, germination and appressorial differentiation of *B. graminis* were strongly impeded. Spraying the mutant leaf surface with aldehyde-containing wild-type wax or pure *n*-hexacosanal (C_{26}-aldehyde) fully restored fungal prepenetration, whereas maize wild-type leaf surfaces coated with *n*-docosanoic acid exhibited reduced conidial germination rates of 23%, and only 5% of the conidia differentiated infection structures. *In vitro* studies were performed to further corroborate the extensive prevention of fungal germination and differentiation in response to artificial surfaces coated with aldehyde-deficient maize wax. Because of its phenotype affecting the *B. graminis* prepenetration processes, the *glossy11* mutation of maize may become a valuable molecular target and genetic tool that could provide a means of developing basal powdery mildew resistance in the globally important crops wheat and barley.

Keywords: Blumeria graminis; cuticular wax; *glossy11*; maize; powdery mildew; very-long-chain aldehydes

3.2 Introduction

The plant cuticle is a protective sheathing produced by epidermal cells of aerial plant organs. Besides its central role in preventing non-stomatal water loss, the plant cuticle is also involved in protection against UV-radiation, reduction of deposition of dust and pollen, plant defence against fungal and bacterial pathogens, and participation in a variety of plant-insect interactions (Post-Beittenmiller, 1996). The cuticle provides the first barrier that fungi must overcome in order to gain access to the plant tissue, but it also provides chemical and physical cues that are necessary for the development of essential infection structures for many fungal pathogens (Carver & Gurr, 2006; Raffaele *et al.*, 2009). The cuticle is composed of two major types of lipids: the core structural cutin polymer mainly consisting of hydroxyl- and epoxy-C_{16} and C_{18} fatty acids, and the cuticular waxes, mostly comprised of aliphatic very-long-chain fatty acid (VLCFA) derivatives (e.g. primary and secondary alcohols, aldehydes, esters, ketones, alkanes) and often variable amounts of cyclic compounds such as triterpenoids and phenylpropanoids (Jetter *et al.*, 2006). The cuticular waxes can be divided into two spatially and sometimes chemically distinct layers: the intracuticular waxes embedded in the cutin matrix and the epicuticular waxes coating the surface. Hence, the first contact between an airborne pathogen and its host plant frequently takes place in an environment dominated by the presence of leaf cuticular waxes.

The obligate biotrophic ascomycete *Blumeria graminis* is one of the most destructive foliar pathogens of members of the Poaceae subfamily Pooideae, and therefore of the economically important cereals wheat and barley (Jørgensen, 1988; Murray & Brennan, 2010). Shortly after landing on a host plant surface, the wind-dispersed asexual conidia of *B. graminis* germinate and form a primary germ tube that attaches to the leaf surface and forms a short peg penetrating the cuticle (Edwards, 2002; Yamaoka *et al.*, 2006). Subsequently, a secondary germ tube elongates swells and differentiates into an appressorial germ tube, which matures and becomes a lobed, apical appressorium with a penetration peg formed to pierce both host cuticle and epidermal cell wall. These so-called prepenetration processes of B. graminis development are completed about 12–15 h after inoculation (Zhang *et al.*, 2005).

Several studies have demonstrated that, unlike wax crystal structure or surface topology, plant cuticular waxes can play important roles in chemically triggering germination and differentiation of the powdery mildew fungus *B. graminis* (Carver *et al.*, 1990; Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Feng *et al.*,

2009; Ringelmann *et al.*, 2009; Hansjakob *et al.*, 2010). Specifically very-long-chain aldehydes, present in many cuticular plant waxes, have turned out to be of particular significance for the *B. graminis* prepenetration processes. *In vitro*, even-numbered very-long-chain aldehydes (C_{22}–C_{30}) promote germination and differentiation of powdery mildew conidia in a dose and chain-length dependent manner (Hansjakob *et al.*, 2010). However, besides the chemical composition of a leaf surface, a multitude of environmental factors, such as surface hydrophobicity or atmospheric humidity, can significantly affect conidial prepenetration processes (Carver *et al.*, 1990; Zabka *et al.*, 2008; Ringelmann *et al.*, 2009). Therefore, despite evidence from in vitro studies, clear-cut proof for the crucial role of very-long-chain aldehydes in the initiation and promotion of *B. graminis* prepenetration processes on natural leaf surfaces is still lacking. To the authors' knowledge, a *B. graminis* host plant leaf surface completely devoid of very-long-chain aldehydes does not exist, or has not been characterized so far. However, the complete absence of very-long-chain aldehydes from the cuticular wax of the *glossy11* (*gl11*) wax mutant of the non-host Poaceae Zea mays (Sprague, 1938) has been demonstrated (Avato *et al.*, 1985). Therefore, this mutant appeared to be ideally suited to investigate the relevance of very-long-chain aldehydes for the successful initiation and promotion of *B. graminis* prepenetration processes. Germination and differentiation of *B. graminis* conidia were assayed on leaves of the aldehyde deficient *gl11* mutant, on the aldehyde surplus mutant *gl5* (Bianchi *et al.*, 1978), and on two non-glossy wild-type maize cultivars. *In vitro* as well as in vivo wax and/or aldehyde supplementation experiments were performed to elaborate and highlight the significant roles of these cuticle-derived signalling compounds in the successful initiation of *B. graminis* prepenetration processes.

3.3 Materials and Methods

Plant and pathogen material

Kernels of *non-glossy Zea mays* cv. Lambada (Pioneer Hi-Bred International) and C836B (wild-type), and of the glossy lines 428A (homozygous for the *gl5* mutation) and 215B (homozygous for the *gl11* mutation) (Maize Genetics COOP Stock Center) were sown in plastic pots (9 cm diameter) in standard potting soil (Type ED73; SteuderComp) and cultivated in growth chambers (AR-36L; CLF Plant Climatics) with 300 µmol m^{-2} s^{-1} light intensity in a 16/8 h photoperiod (26/20°C) at 70% relative humidity. For wax analysis and studies on *B. graminis* prepenetration processes, second and third leaves of maize plants at the four leaf stage were used.

Blumeria graminis f.sp. *hordei* (isolate CC1; originally obtained from Tim Carver, IGER, Aberystwyth, UK) was propagated on its host barley (*Hordeum vulgare* cv. Bonus) in a 16/8 h photoperiod (22/18°C) at 70% relative humidity until white

pustules became visible. In order to ensure uniform age and viability of conidia, colony-bearing leaves were shaken 1 day before conidia were required for experimentation. This treatment dislodged older conidia and promoted the formation of fresh conidia that were then available for subsequent assays.

Cuticular wax analysis

Total cuticular leaf wax extracts of second and third leaves (n = 5) were prepared by dipping the entire leaves, apart from cut edges, for 1·5 min into 30 mL of chloroform (99%; Carl Roth). Prior to solvent evaporation under a flow of nitrogen at 50°C, 5 µg of *n*-tetracosane (99%; Sigma Aldrich) was added to the extracts as internal standard. For gas chromatography (GC) analysis, hydroxyl groups were transformed into the corresponding trimethylsilyl derivates by reaction with bis-*N,O*-trimethylsilyltrifluoroacetamide (Machery-Nagel) in pyridine for 30 min at 70°C. Quantitative analysis was performed using capillary GC (6850; Agilent Technologies) and flame ionization detector; qualitative analysis was studied with mass spectrometric detection (m/z 50–750, MSD 5973; Agilent Technologies) under the same GC conditions (6890; Agilent Technologies). GC was carried out with on-column injection (30 m × 0·32 mm inner diameter, DB-1, d*f* 0·1 µm; J&W Scientific, Agilent Technologies). Oven temperature was programmed for 2 min at 50°C, 40°C min^{-1} to 200°C, 2 min at 200°C, 3°C min^{-1} to 320°C, 30 min at 320°C and He carrier gas inlet pressure was programmed for 5 min at 50 kPa, 3 kPa min^{-1} to 150 kPa, and 30 min at 150 kPa. Wax components were identified by comparison of their mass spectra with those of authentic standards and literature data (MSD ChemStation software package). For quantification of wax compounds GC was performed under the same conditions as described above with a flame ionization detector, but with H_2 as carrier gas programmed for 5 min at 5 kPa, 3 kPa min^{-1} to 50 kPa, and 30 min at 50 kPa.

Characterization of plant surfaces

Surface hydrophobicity was determined by measuring contact angles of sessile 6 µL droplets of distilled water (contact angle system OCA 15, software system SCA20; Dataphysics Instruments). In each case 50 measurements were performed on five independent surface samples.

For visualization of wax crystal structures, pieces of leaves were lyophilized at −52°C for 5 h at 5 Pa (Christ Alpha 1–2), mounted on aluminium holders and sputter coated with gold palladium (Bal-Tec SC005, 25 mA, 300 s; Balzers). Specimens were examined by SEM (Zeiss DSM 962, 15 KV; Zeiss). The sputtering conditions, depositing an alloy layer with a thickness of approximately 20 nm on the tissue samples, were optimized for the acceleration voltage used in the SEM.

Coating of glass slides and manipulation of maize leaf surfaces

Histobond® glass slides (Marienfeld) were coated using a 0·5% Formvar® resin (Polyvinyl formal, CAS 63450-15-7; Applichem) solution in chloroform, supplemented with total leaf wax extract or pure n-hexacosanal adjusted to 250 µg mL^{-1} in the dipping solution. If applicable, wax extracts were spiked with 2% (w/w) of pure *n*-hexacosanal (Hansjakob *et al.*, 2010). This Formvar® resin-based in vitro system provides highly homogeneous hydrophobic surfaces that exhibit essentially uniform contact angles. It permits the specific analysis of the effects of bioactive lipophilic extracts or single wax constituents on the prepenetration processes *of B. graminis*, excluding secondary effects due to variations in surface hydrophobicity (Hansjakob *et al.*, 2010). The wax coverage of each of the coated glass slides amounted to 0·6 µg cm^{-2}. Surface contact angles of coated glass slides were determined as described above.

Adaxial surfaces of detached second and third leaves were sprayed with total leaf wax extracts adjusted to 380 µg mL^{-1} in chloroform (99%; Carl Roth) or solutions (10 mg mL^{-1}) of pure *n*-docosane, *n*-docosanol, *n*-docosanoic acid, docosanoic acid docosanoyl ester (C_{44}-alkyl ester) (99%; Sigma Aldrich), *n*-hexacosanal (C_{26}-aldehyde) or combinations thereof in chloroform using a glass chromatography sprayer from a distance of approximately 30 cm. Control leaves were sprayed with an equal volume of chloroform only. The amount of the wax coating deposited on the leaf surfaces was determined by dipping the sprayed leaves into 30 mL of chloroform and subsequently quantified by GC analysis as described in the previous section. The spraying experiments were repeated three times independently.

Studies on *B. graminis* prepenetration processes

Adaxial surfaces of detached maize leaves or glass slides were fixed at the base of a settling tower. Conidia from infected barley leaves were blown into the tower by pressurized air to ensure even distribution at a density of approximately 20 conidia mm^{-2}. Leaves and artificial surfaces were incubated for 16 h in darkness at 20°C on moist filter paper in a sealed glass Petri dish to maintain a relative humidity of at least 90%.

For microscopic analysis, leaves were initially bleached on filter paper moistened with ethanol:acetic acid (3:1, v/v) and were then transferred to filter paper soaked with fixation solution (lactic acid:glyercol:water (1:1:1, v/v/v)) for 5 h (Lyngkjær & Carver, 1999). To visualize the fungal structures leaf surfaces were stained with droplets of 0·05% (w/v) trypan blue (Merck) in acetic acid:glycerol:water (1:1:1, v/v/v) for 30 min. Developmental stages of conidia inoculated onto artificial glass slide surfaces were observed directly without prior staining. In total 2500 conidia were analysed for each surface in five independent experiments with 500 conidia per repetition. Individual conidia were analysed by

light microscopy (Leica DMR). Micrographs were acquired with a digital camera (Zeiss Axio Cam MRc) and Axio Vision software package 4·8·1 (Zeiss). Analysis was undertaken to determine whether the conidia had not germinated (ng), had formed only a primary germ tube (pgt), a secondary non-swollen germ tube (sgt), a swollen, secondary appressorial germ tube (agt) or a fully differentiated hooked appressorium with a septum (app). Additionally, the amount of conidia apparently damaged during incubation was recorded. Only single, well separated conidia were evaluated at each observation to exclude possible inhibitory effects due to crowding.

Statistical analysis

The basis for statistical analyses was n = 5 independent experiments, where n = 1 represents 500 examined conidia. Significant differences ($P < 0{\cdot}05$) between multiple datasets were tested by one-way anova followed by Tukey HSD *post hoc* test. In case of significant differences ($P < 0{\cdot}05$; Levene test) of homogeneity the data were transformed. The analyses were performed with STATISTICA 8 (StatSoft).

3.4 Results

3.4.1 Cuticular leaf wax coverage and composition

The non-glossy maize lines C836B and cv. Lambada had almost identical cuticular wax coverages of about 8 µg cm^{-2} whereas the glossy lines 215B, homozygous for the *gl11* mutation, and 428A, homozygous for the *gl5* mutation, showed distinctly reduced wax accumulations of only 1·3 ± 0·3 and 4 ± 2 µg cm^{-2}, respectively. When compared to the non-glossy lines, the maize line 215B exhibited a complete absence of very-long-chain aldehydes and of fatty acids, with distinctly increased levels of alkyl esters, particularly of molecules with chain lengths ranging from C_{40} to C_{48} (Figure 1). In addition, this mutant line showed a slight reduction in the relative proportion of its most prominent wax constituent *n*-dotriacontanol (C_{32}-alkanol), a constituent which was also dominant (up to 75%) in the cuticular waxes of the non-glossy lines C836B and Lambada. The maize line 428A exhibited greatly elevated levels of very-long-chain aldehydes. In this glossy mutant the C_{32} aldehyde *n*-dotriacontanal accounted for up to 60% of the wax constituents, whilst the proportion of *n*-dotriacontanol was reduced to roughly 15%. The different maize lines assayed displayed only minor differences in the relative proportion of *n*-alkane constituents. However, the *gl11* mutant showed slightly increased overall proportions of n-alkanes ranging from C_{27} to C_{37} whereas in Lambada and 428A, *n*-hentriacontane (C_{31} alkane) formed the most prominent *n*-alkane constituent. Roughly 5% of the maize wax constituents were not identified.

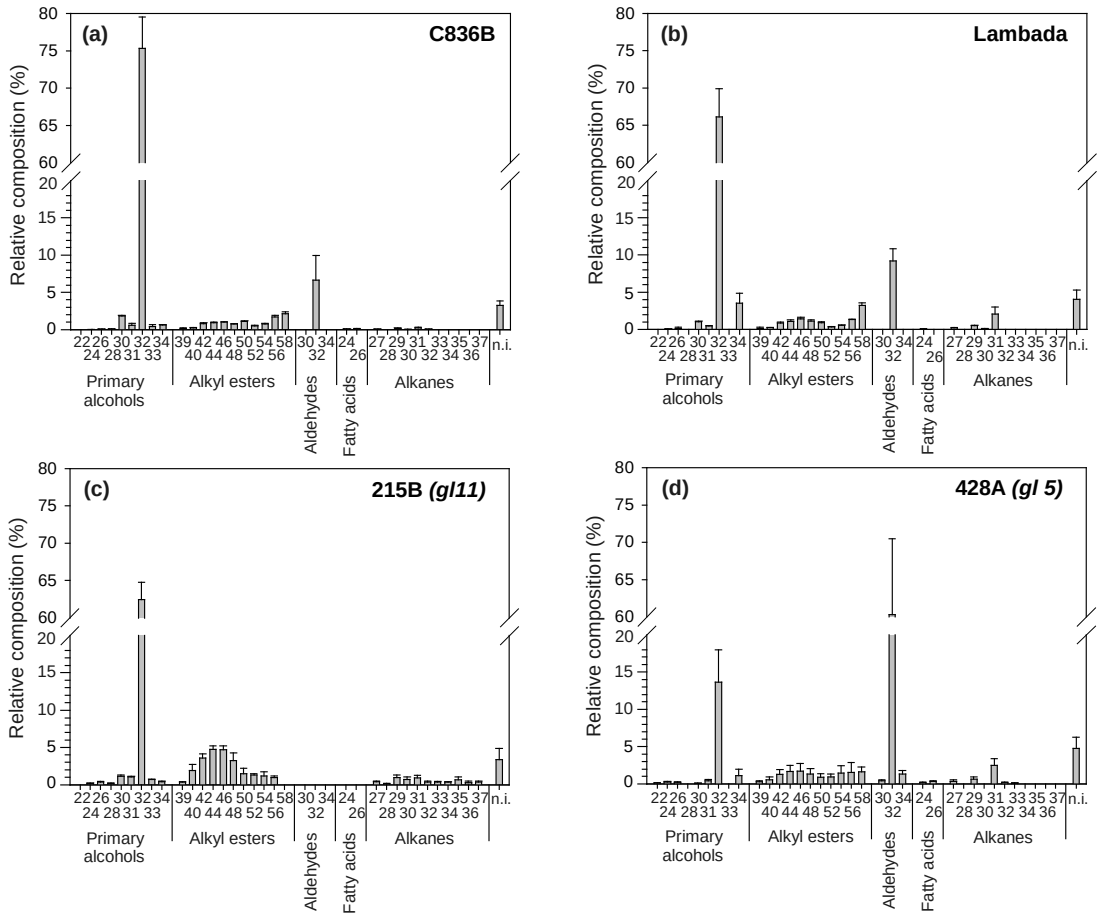

Figure 1 Relative composition (%) of total leaf wax from (a) Zea mays line C836B, (b) cv. Lambada, (c) glossy mutant line 215B (homozygous for the *gl11* mutation) and (d) line 428A (homozygous for the *gl5* mutation). Carbon chain lengths are indicated for each chemical compound class. n.i., not identified. Data are given as the mean of five repetitions ± SD.

3.4.2 Leaf surface wettability and wax crystal structure

The mutant lines 215B and 428A showed distinctly reduced surface contact angles of 123 ± 4° and 144 ± 5°, respectively, whereas the non-glossy maize cv. Lambada and C836B exhibited superhydrophobic surfaces with contact angles of 156 ± 5° to 167 ± 6°. The differences in wax composition and leaf surface hydrophobicity of maize non-glossy and *glossy*-mutant cuticular waxes were reflected by different epicuticular wax crystal morphologies (Figure 2). The adaxial epicuticular wax layer of cv. Lambada leaves was characterized by abundant wax crystal platelets, approximately 1–1·5 µm in length. However, line C836B exhibited smaller platelets that barely exceeded a length of 1 µm. For both lines the smooth surface of the epicuticular wax film was visible only in comparably small areas as the crystals formed a dense and relatively thick network (Figure 2a,b). Unlike the wild-type, the aldehyde surplus line 428A exhibited a flaky mixture of smaller and larger platelets (1·3–2·0 µm in length) that sparsely protruded from a matrix of apparently horizontally oriented and more or less coalesced platelets (Figure 2c). Line 215B

exhibited single, irregularly scattered wax bodies, smaller (0·2–0·8 µm in length) and much less abundant than the platelets on non-glossy leaves (Figure 2d).

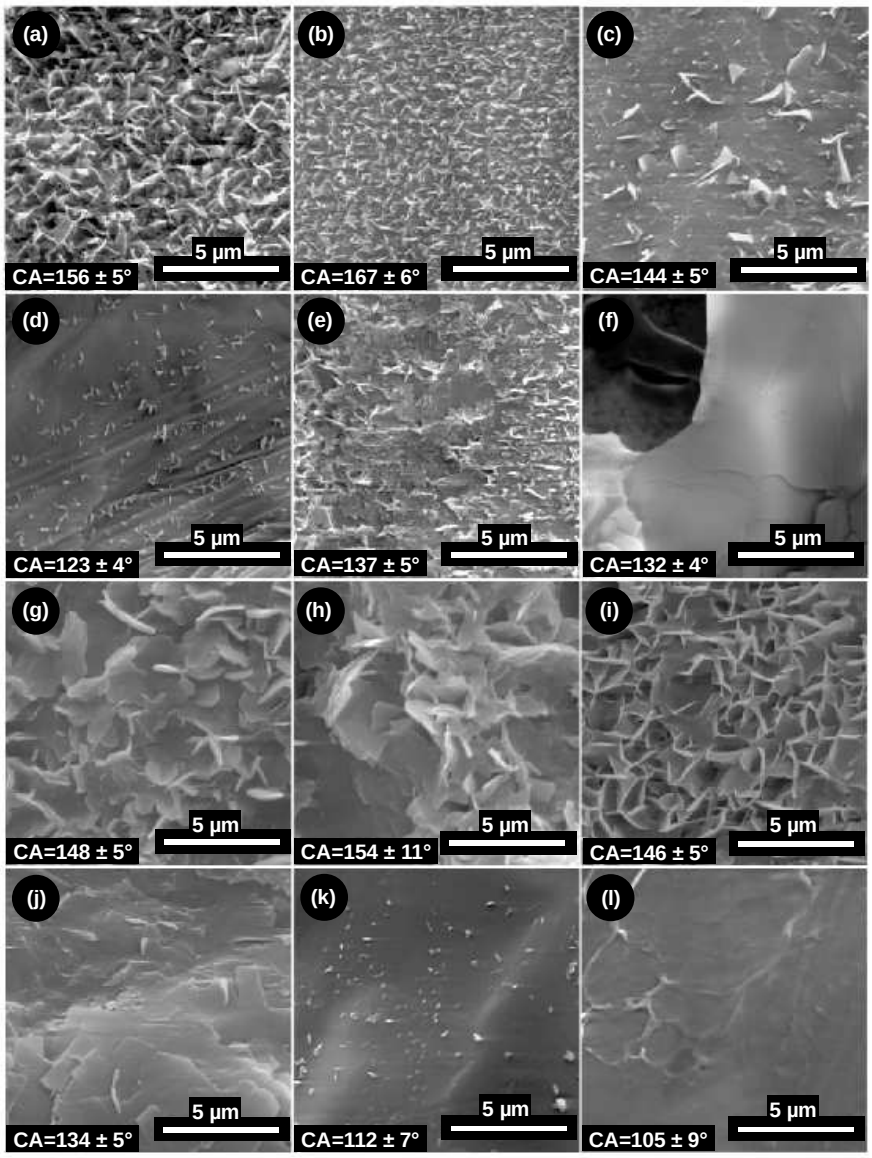

Figure 2 Scanning electron microscopy micrographs of maize leaf surfaces. Native adaxial leaf surfaces of (a) *Zea mays* cv. Lambada, (b) C836B, (c) *glossy* mutants 428A (*gl5*) and (d) 215B (*gl11*). Adaxial leaf surfaces of *Zea mays* cv. Lambada sprayed with (e) chloroform only, (f) *n*-docosane, (g) *n*-docosanol or (h) *n*-docosanoic acid, (i) docosanoic acid docosanoyl ester [C44-alkyl ester] and (j) *n*-hexacosanal. Adaxial leaf surfaces of *glossy* mutant 215B (*gl11*) sprayed either with (k) chloroform only or with (l) total leaf wax extract from C836B. Contact angles (CA) ± SD are indicated at the bottom of each micrograph.

In order to demonstrate the significance of the cuticular leaf wax composition of the different maize lines in the in vivo studies, in vitro bioassays using glass slides coated with Formvar® resin supplemented with the respective leaf wax extracts were performed (Figure 3c). Despite a general reduction in germination and differentiation (agt + app), the results obtained from the in vitro glass slide assays almost fully reflected the differences in fungal germination and differentiation seen on the different adaxial maize leaf surfaces (Figure 3b). Surface contact angles of

Formvar®/wax coated glass slides were generally lower than those of detached maize leaves and did not reflect the leaf surface wettability of the different maize lines (Table 1). The surface contact angles of all coated glass slide surfaces ranged from 91 ± 2° for the C836B wax coating up to 102 ± 4° for wax coating of the *gl11* mutant line 215B.

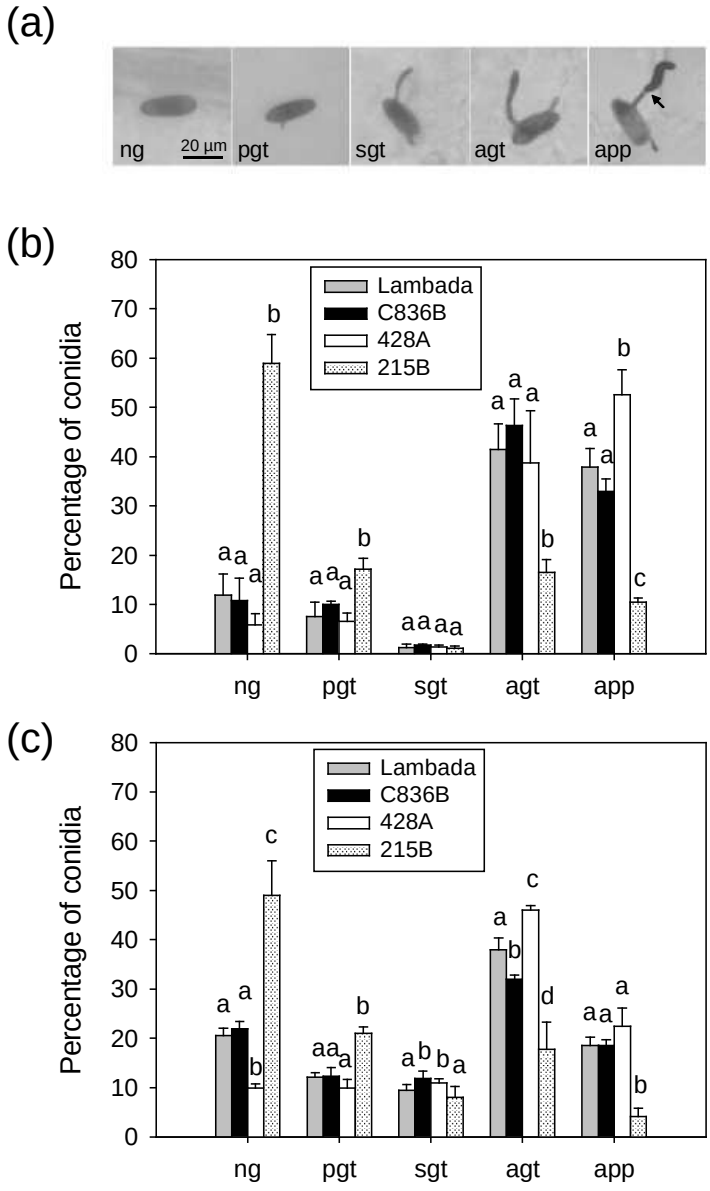

Figure 3 (a) Prepenetration stages of *Blumeria graminis* conidia on maize leaf surfaces: non-germinated conidium (ng), conidium with only a primary germ tube (pgt), conidium with a secondary elongated germ tube (sgt), conidium with a swollen appressorial germ tube (agt), fully differentiated conidium with a lobed appressorium (app) and a septum (arrow). Development of B. graminis conidia on (b) adaxial leaf surfaces of different maize lines and (c) on glass slides covered with Formvar® resin supplemented with the corresponding total leaf wax extracts from *Zea mays* cv. Lambada (grey bars), C836B (black bars), *glossy* mutant lines 428A (*gl5*, white bars) and 215B (*gl11*, striated bars). Data are given as the mean (n = 5) ± SD. Different letters within a developmental category in (b) and (c) indicate significant differences (P < 0·05) determined in a one-way ANOVA followed by a Tukey *post hoc* test.

Table 1 Contact angles of glass slides coated with total leaf wax extracts and/or *n*-hexacosanal

Wax extract/wax constituent	Contact angle (°) ± SD
C836B	91 ± 2
Lambada	99 ± 2
215B (*gl11*)	102 ± 4
215B (*gl11*) + 2%*n*-hexacosanal	102 ± 2
428A (*gl5*)	95 ± 2
n-hexacosanal	101 ± 1

3.4.3 Masking of the *gl11* phenotype

As the mutation responsible for the *gl11* phenotype has not yet been identified, a molecular complementation was not feasible. Therefore, spraying wax extracts or single wax constituents onto living maize leaves was chosen as an appropriate methodology of surface manipulation in order to modify the chemical surface characteristics in a reasonable manner. Spraying aldehyde-containing maize leaf wax extracts onto the surface of the *gl11* mutant line 215B that is fully devoid of very-long-chain aldehydes was intended to override the *gl11* phenotype concerning the distinct impediment of *B. graminis* germination and differentiation (Figure 4a). Spraying chloroform only onto the leaf surfaces of cv. Lambada and 215B (*gl11*) distinctly modified the epicuticular leaf surface structures, resulting in eroded and coalesced wax crystals (Figure 2e,k). This modification resulted in reduced surface contact angles. However, the chloroform treatment alone had no significant effects on the subsequent prepenetration processes of *B. graminis*, hence the procedure was generally regarded as being applicable. Spraying leaves of 215B with a wax extract from leaves of C836B resulted in almost smooth leaf surfaces devoid of any protruding wax platelets, a nearly doubled total wax coverage of 2·8 ± 0·4 µg cm^{-2}, and in contact angles of only 105 ± 9° (Figure 2l). The *B. graminis* germination rates on these leaves (Figure 4a) approximated those on untreated C836B leaves (Figure 3b), resulting in about 90% of germinated conidia. Most strikingly, the differentiation rate (agt + app) of about 80% fully matched the one on leaves of C836B (Figure 3b). Spraying 215B and cv. Lambada leaves with the C26-aldehyde *n*-hexacosanal, a potent inducer of B. graminis prepenetration processes (Hansjakob *et al.*, 2010), led to total wax coverage of 4 ± 0·9 and 19 ± 6 µg cm^{-2}, respectively, and surfaces exhibiting scaly, horizontally oriented platelets (shown for cv. Lambada in Figure 2j). Irrespective of plant genotype, this treatment resulted in distinctly increased germination and differentiation rates when compared to native 215B and cv. Lambada leaves (Figure 4b).

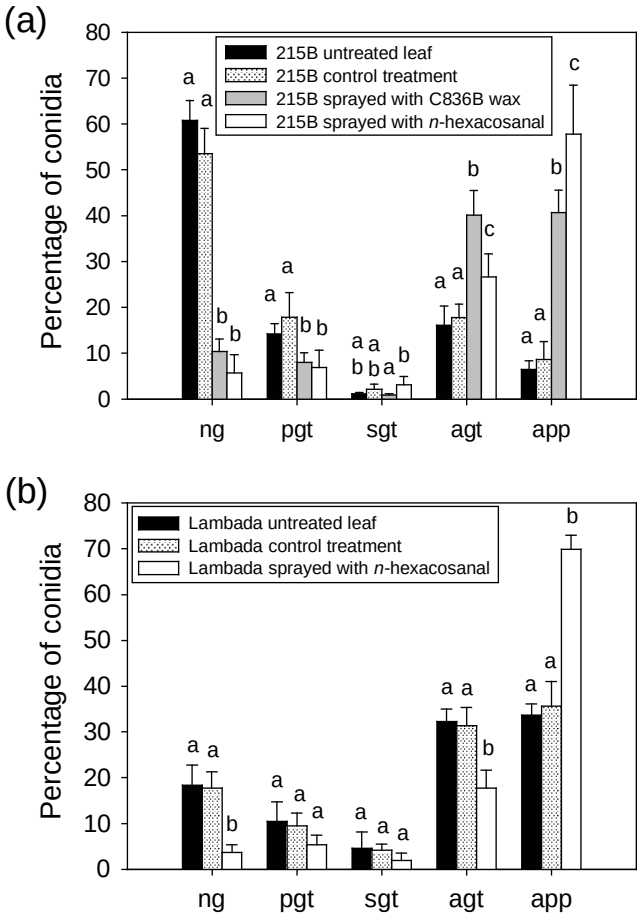

Figure 4 Development of *Blumeria graminis* conidia on (a) adaxial leaf surfaces of maize line 215B (gl11) and (b) cv. Lambada. Leaves were sprayed with solutions of C836B wax (grey bars) or n-hexacosanal (white bars) in chloroform, with chloroform only (control treatment, shaded bars) or untreated (black bars). Data are given as the mean (n = 5) ± SD. Different letters within a developmental category indicate significant differences ($P < 0{\cdot}05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test.

3.4.4 Mimicking of the *gl11* phenotype

Spraying the largely inert alkane *n*-docosane (C_{22} alkane) onto leaves of maize cv. Lambada (a germination and differentiation supportive surface) resulted in a total wax coverage of 132 ± 11 µg cm^{-2}, and surfaces with a crustaceous outer appearance, exhibiting a network of large horizontally and vertically protruding wax plates (Figure 2f). Inoculation of these surfaces led to substantially reduced fungal germination and differentiation rates (Figure 5a). Only 50% of the inoculated conidia germinated on the treated leaf surfaces while the proportion of differentiated conidia decreased to values below 30%. Spraying cv. Lambada leaves with *n*-docosanol (C_{22} alkanol) or *n*-docosanoic acid (C_{22} fatty acid) led to total wax coverage of 58 ± 7 and 127 ± 7 µg cm^{-2}, respectively. Both treatments resulted in surfaces exhibiting large rounded platelets (2–4 µm in length) causing surface contact angles of roughly 150° (Figure 2g,h). Inoculation of these modified leaf surfaces resulted in a further significant reduction in germination and

differentiation rates. The treatment with *n*-docosanoic acid resulted in a germination rate of 23%, while only about 5% of the inoculated conidia differentiated infection structures (Figure 5a). Spraying adaxial cv. Lambada leaf surfaces with a C_{44}-alkyl ester (docosanoic acid docosanoyl ester) led to total wax coverage of 56 µg cm^{-2}, and surfaces exhibiting rounded and somewhat coalescent platelets of 1–3 µm in length (Figure 2i). This treatment resulted in a drastic increase in the proportions of non-germinated conidia and in differentiation rates that accounted for <10% of the inoculated conidia (Figure 5b). However, spraying line 215B adaxial leaf surfaces with the C_{44}-alkyl ester resulted in only a slight further decrease of fungal differentiation rates (Figure 5c). In combination with 2% *n*-hexacosanal, the *gl11* phenotype mimicking effect of the C_{44}-alkyl ester was completely neutralized on sprayed leaf surfaces of cv. Lambada (58 ± 10 µg cm^{-2} total wax coverage) with nearly identical germination and differentiation rates as on native leaves (Figure 5b).

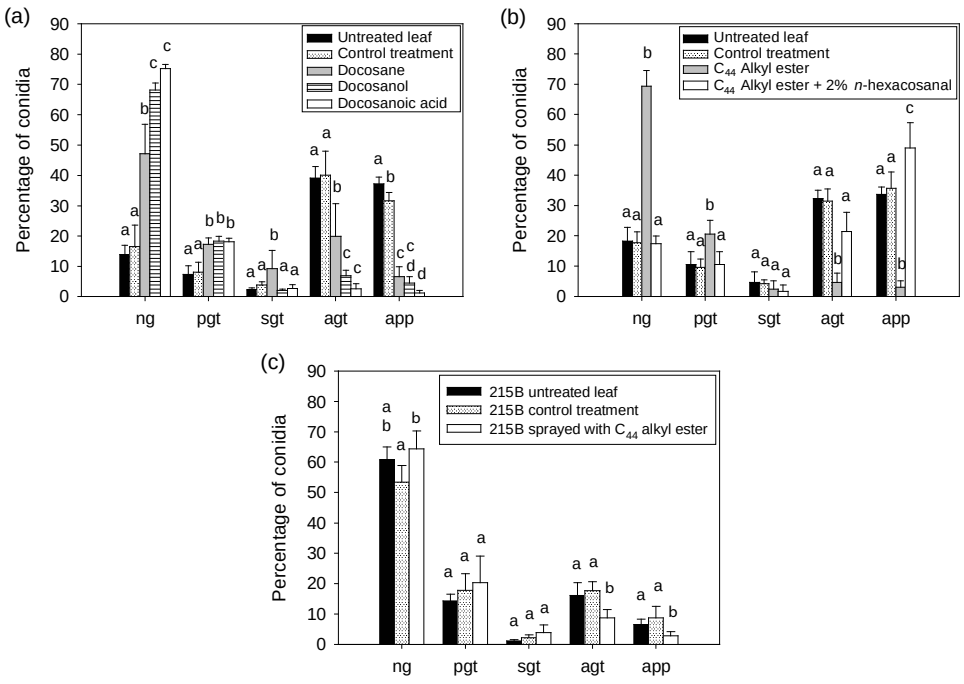

Figure 5 Development of *Blumeria graminis* conidia on adaxial *Zea mays* cv. Lambada and 215B (*gl11*) leaf surfaces sprayed with different single wax constituents dissolved in chloroform. (a) Development of conidia on cv. Lambada leaves: native leaves (black bars), leaves sprayed with chloroform only (shaded bars), leaves sprayed with *n*-docosane (grey bars), *n*-docosanol (striated bars) or *n*-docosanoic acid (white bars). (b) Development of conidia on cv. Lambada leaves: native leaves (black bars), leaves sprayed with chloroform only (shaded bars), leaves sprayed with a C_{44}-alkyl ester [docosanoic acid docosanoyl ester] (grey bars) and sprayed with a mixture of the C_{44}-alkyl ester with 2% *n*-hexacosanal (white bars). (c) Development of conidia on leaves of 215B (*gl11*): native leaves (black bars), leaves sprayed with chloroform only (shaded bars), leaves sprayed with a C_{44}-alkyl ester [docosanoic acid docosanoyl ester] (white bars). Data are given as the mean (n = 5) ± SD. Different letters within a developmental category indicate significant differences ($P < 0{\cdot}05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test.

3.4.5 Supplementing *gl11* wax with *n*-hexacosanal

Spiking leaf wax extracts from the *gl11* mutant maize line 215B with 2% *n*-hexacosanal resulted in vitro in similar proportions of non-germinated conidia as with leaf wax extracts from C836B (Figure 6) or cv. Lambada (Figure 3c). The treatment with *n*-hexacosanal led to a significant reduction in the proportions of conidia in the primary and secondary germ tube stages of development and even out-performed the C836B wax with respect to appressorial germ tube and appressorium formation, but had no significant impact on surface contact angles (Table 1). Formvar® glass slides supplemented with pure *n*-hexacosanal exhibited a slight increase in the proportion of appressorial germ tubes when compared with slides coated with C836B wax or a mixture of 215B wax with 2% *n*-hexacosanal (Figure 6).

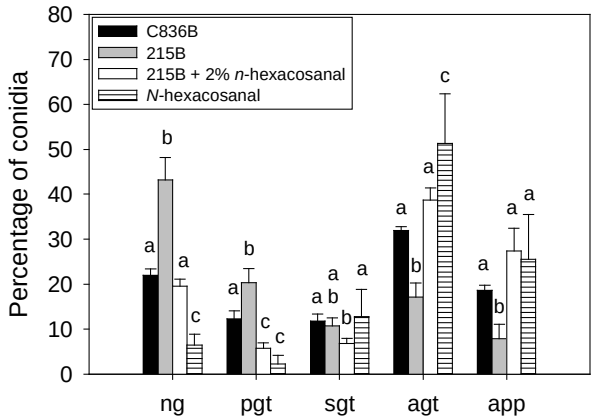

Figure 6 Development of *Blumeria graminis* conidia on glass slides coated with Formvar® resin supplemented with extracted wax from C836B (black bars) or 215B (*gl11*, grey bars), with 215B wax spiked with 2% *n*-hexacosanal (white bars) or with pure *n*-hexacosanal only (striated bars). Data are given as the mean (n = 5) ± SD. Different letters within a developmental category indicate significant differences ($P < 0{\cdot}05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test.

3.5 Discussion

Several loci in maize, the *Glossy* or *Gl* loci, have been found to affect the quantity and/or the composition of cuticular waxes on the surface of seedling leaves (Neuffer *et al.*, 1997). While the *gl5* mutant has been described as predominantly containing very-long-chain aldehydes in its cuticular waxes (Bianchi *et al.*, 1978), the *gl11* mutant was characterized as completely lacking them (Avato *et al.*, 1985). As several very-long-chain aldehyde molecules were demonstrated to act as potent inducers of the B. graminis prepenetration processes (Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Ringelmann *et al.*, 2009; Hansjakob *et al.*, 2010), the above mentioned very-long-chain aldehyde deficient and surplus glossy mutants of maize were used to explicitly test the relevance of the presence of very-long-chain aldehydes for the

initiation of *B. graminis* prepenetration processes *in vivo*, on surfaces of living plant leaves.

The two non-glossy lines C836B and cv. Lambada showed very similar overall cuticular wax compositions, as in other non-glossy wild-type lines (Bianchi & Salamini, 1975; Avato *et al.*, 1985). As expected, the cuticular wax of the *gl11* mutant line 215B was completely devoid of very-long-chain aldehydes although, with about 23% the proportion of alkyl esters, this deficiency was not as pronounced as previously described (Avato *et al.*, 1985). In addition, the cuticular wax of line 215B contained no free fatty acids, whereas the *gl11* mutant in the inbred WF9 genetic background assayed by Avato *et al.* (1985) had 5% free fatty acids. The substantial reduction in the proportion of primary alkanols described for the *gl11* mutation in the WF9 genetic background was not seen with line 215B. The *gl5* mutation in the WF9 genetic background accumulated 83·5% very-long-chain aldehydes and only 8·7% primary alkanols as constituents of the cuticular leaf wax (Bianchi *et al.*, 1978). However, the 428A mutant line used in the present study showed a very-long-chain aldehyde proportion of only 65% and a primary alkanol proportion of 13%. These deviations from the initial descriptions of the *gl11* and *gl5* wax compositional phenotype may be attributable to different genetic backgrounds and culture conditions.

Surface hydrophobicity and the presence of very-long-chain aldehydes are substantially responsible for providing initial cues promoting *B. graminis* prepenetration processes (Zabka *et al.*, 2008). The contact angles of the native and modified maize leaf surfaces and of the artificial glass slide surfaces used in the present study were all above the critical value of 80°, therefore permitting the effective promotion of B. graminis prepenetration processes in the presence of very-long-chain aldehydes (Zabka *et al.*, 2008; Ringelmann *et al.*, 2009).

The results of the *in vivo* and *in vitro* inoculation experiments unequivocally corroborate the importance of very-long-chain aldehydes for the successful initiation of the barley powdery mildew prepenetration processes. The aldehyde-deficient *gl11* mutant line 215B showed a dramatic reduction of conidial germination and differentiation when compared to aldehyde-containing leaf surfaces of other maize lines. However, the differentiation on the *gl11* mutant was still higher than one might have expected from the lack of very-long-chain aldehydes in its cuticular wax (Hansjakob *et al.*, 2010). This suggests that other, probably still unidentified, constituents of the maize leaf wax might also contribute to the stimulation of *B. graminis* prepenetration processes. However, such compounds remain to be identified and their active principle elucidated. Despite its drastically increased very-long-chain aldehyde content, the native leaf surfaces or glass slides coated with *gl5* wax extracts were only slightly more inductive than the non-glossy lines C836B or cv. Lambada. This indicates that when the aldehyde

proportion in the wax exceeds a certain threshold no additional stimulation of prepenetration can be achieved by further increasing the amount or proportion of very-long-chain aldehydes, which is fully in accordance with previous data (Hansjakob *et al.*, 2010). However, on the non-host maize leaves the proportion of fully mature appressoria was approximately only half of that found on host barley leaves (Hansjakob *et al.*, 2010). In contrast, rates of appressorial germ tube and appressorium formation on wax-coated glass slides were comparable between maize and barley leaf wax extracts, indicating that factor(s) other than wax composition might be involved.

The importance of chemical leaf surface features for the establishment of *B. graminis* suggests that interference with cuticular wax chemistry will disrupt pathogen development and consequently reduce infestation. Research on a variety of film-forming polymer agents that coat the leaf surface has demonstrated that coating leaf surfaces with such compounds can indeed lead to reductions in *B. graminis* infection (Sutherland & Walters, 2002; Walters, 2006). Likewise, it has been hypothesized here that an inductive very-long-chain aldehyde-containing maize wax overlay could mask the *gl11* phenotype of the 215B maize leaf surface with respect to restoration of germination and differentiation behaviour. In fact, spraying 215B leaves with wax from C836B resulted in substantially increased germination and differentiation rates, demonstrating that the modified wax composition of the leaf surface was responsible for that restorative effect. This effect was further corroborated by spraying 215B and cv. Lambada leaves with pure *n*-hexacosanal, which resulted in even higher *B. graminis* germination and differentiation rates. Likewise, adding 2% of the C_{26}-aldehyde *n*-hexacosanal to extracted wax of line 215B led to even higher in vitro differentiation rates than with the C836B wax alone. From these experiments one may conclude that in the case of maize leaves and maize wax the presence of very-long-chain aldehydes contributes up to 70% to the success of conidial differentiation, thereby underlining the importance of these compounds for the prepenetration processes and consequently for fungal infection in a compatible fungus/host interaction.

The reverse experiment, coating the naturally inductive leaf surfaces of cv. Lambada with different non-inductive wax constituents (Hansjakob *et al.*, 2010), resulted in a *B. graminis* developmental phenotype that greatly resembled that of line 215B, exhibiting substantially decreased germination and differentiation rates. The fact that the presence of only 2% *n*-hexacosanal in combination with the non-inductive C_{44}-alkyl ester was fully sufficient to revert the induction masking effect of the C_{44}-alkyl ester on naturally inductive leaf surfaces suggests that the substantial proportion of alkyl esters present in the aldehyde-deficient wax of line 215B may not be primarily responsible for the *gl11* prepenetration defective phenotype.

The significant differences in *in vitro* germination and differentiation rates among the C_{22} molecules of different substance classes could be explained by a miscellaneous molecular orientation of the distinct compounds on the sprayed leaf surface (Holloway, 1970), resulting in putatively non-homogeneously coated leaf surface sections that could permit differential access to the inducing aldehyde compounds below the artificial wax coating. In field trials, a water stable emulsion with *n*-dodecanol as the major component has been demonstrated to substantially reduce infestation of powdery mildew on wheat (Han, 1990). However, the extent to which this compound might exert an aldehyde-masking effect on sprayed wheat leaf surfaces remains speculative.

Interestingly, the wax phenotype of the aldehyde surplus mutant line 428A (*gl5*) exhibiting horizontally oriented platelets strongly resembled the artificially modified leaf surfaces of cv. Lambada sprayed with *n*-hexacosanal. Likewise, the barley *eceriferum (cer)* wax mutant *cer-yp.949* showing a significantly reduced proportion of primary alkanols, down to nearly 50%, in combination with a 5-fold increase of very-long-chain aldehydes in its cuticular wax, also exhibited horizontally oriented epicuticular wax platelets (data not shown).

It has been shown for wax constituent recrystallization that substrate polarity influences the spatial orientation of the molecule layer adhering first on the substrate (Koch *et al.*, 2006). Wax crystals preferentially grew in a horizontal (polar substrate) or vertical (non-polar substrate) direction. Hence differences in the polarity of the cuticle proper might explain why coating the 215B leaf surface with extracted leaf wax from C836B resulted in a smooth horizontally orientated wax layer, while covering the natural wax crystals of cv. Lambada with *n*-docosane, *n*-docosanol, *n*-docosanoic acid or the C_{44}-alkyl ester resulted in fairly different wax crystal morphologies. In general, wax platelet morphologies based on or modified by alkane, aldehyde, ester, ketone secondary alkanol and fatty acid constituents are comparatively poorly defined with only weak discrimination between plate morphological variants or their chemical basis (Jeffree, 2006). Nevertheless, the different amounts of wax and of wax constituents applied to the respective leaf surfaces might have also contributed to the generation of the highly divergent epicuticular wax crystal morphologies. However, from previous studies it is known that presence or absence of epicuticular wax crystals on the leaf surface and modifications of the wax crystal morphology can at best exert minor effects on the prepenetration processes of *B. graminis* (Carver & Thomas, 1990; Zabka *et al.*, 2008).

To the authors' knowledge, this is the first study that demonstrates the in vivo relevance of the presence of cuticle-derived very-long-chain aldehydes for the prepenetration processes of the barley powdery mildew fungus *B. graminis*. So far, the mutation responsible for the *gl11* phenotype has not been identified, although

its locus has been mapped to chromosome 2 (Krakowsky *et al.*, 2006). This is the only known mutation from a member of the Poaceae that is devoid of very-long-chain aldehyde cuticular wax constituents. The molecular identity and the exact role(s) of an assumed Gl11 gene product remain to be elucidated. Nevertheless, this well-known mutation that exhibits a unique wax compositional phenotype resulting in substantial disruption of *B. graminis* prepenetration processes could be investigated in subsequent studies aimed at its molecular identification and in unravelling the maize wax biosynthetic pathways in general. The *glossy 11* mutant of maize also offers the rare chance to study the biological relevance of aldehydes as typical cuticular wax constituents of a multitude of plant species and may open avenues for establishing potentially more durable resistance mechanisms in other crop plants such as wheat and barley to specialized fungal pathogens (Niks & Rubiales, 2002).

3.6 Acknowledgements

The authors thank Andrea Knorz for excellent and dedicated technical assistance and Tim Carver (IBERS, Aberystwyth University, UK) for providing barley powdery mildew. This project was financially supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 567 (project A5).

4 Appressorium morphogenesis and cell cycle progression are linked in the grass powdery mildew fungus *Blumeria graminis*

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Fungal Biology (in revision)

Abbreviations:
DAPI: 4′,6-diamidino-2-phenylindole; DMF: dimethylformamide; DMSO: dimethyl sulfoxide; HU: hydroxyurea; LatA: latrunculin A

4.1 Abstract

Conidial germination and differentiation - the so-called prepenetration processes - of the barley powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) are essential prerequisites for facilitating penetration of the host cuticle. Although the cell cycle is known to be pivotal to cellular differentiation in several phytopathogenic fungi there is as yet no information available concerning the relationship between cell cycle and infection structure development in the obligate biotroph *B. graminis*. The timing of specific developmental events with respect to nuclear division and morphogenesis was followed on artificial and host leaf surfaces by DAPI (4′,6-diamidino-2-phenylindole) staining in combination with a pharmacological approach applying specific cell cycle inhibitors. It was found that the uninucleate conidia germinated and then underwent a single round of mitosis five to six hours after inoculation. During primary germ tube formation the nucleus frequently migrated close to the site of primary germ tube emergence. This nuclear repositioning was distinctly promoted by very-long-chain aldehydes that are common host cuticular wax constituents known to induce conidial differentiation. The subsequent morphogenesis of the appressorial germ tube preceded mitosis that was spatially uncoupled from subsequent cytokinesis. Blocking of S-phase with hydroxyurea did not inhibit formation of the appressorial germ tube but prevented cytokinesis and

appressorium maturation. Benomyl treatment that arrests the cell cycle in mitosis inhibited nuclear separation, cytokinesis and formation of mature appressoria. Thus, we conclude that a completed mitosis is not a prerequisite for the formation and swelling of the appressorial germ tube, which normally provides the destination for one of the daughter nuclei, while appressorium maturation depends on mitosis.

4.2 Introduction

The obligate biotrophic ascomycete *B. graminis* is one of the most destructive foliar pathogens of grain (Jørgensen, 1988; Murray and Brennan, 2010). *B. graminis* conidia must complete a specific set of developmental stages on the host surface before they can penetrate into host tissue: shortly after landing on a host plant surface, the wind-dispersed asexual conidia of *B. graminis* germinate by forming an aseptate, primary germ tube that attaches to the leaf surface and forms a short peg penetrating the cuticle but only touching the plant cell wall. This primary germ tube is most likely involved in the absorption of water and solutes from the plant cell wall (Edwards, 2002; Yamaoka *et al.* 2006). Subsequently, a secondary germ tube elongates, swells and differentiates into the appressorial germ tube, which matures and becomes a septate, lobed apical appressorium with a penetration peg formed to pierce both host cuticle and cell wall. These prepenetration processes of fungal development are completed about 12–15 h after inoculation.

The prepenetration processes are a crucial stage in the fungal life cycle. Therefore, fungi have evolved mechanisms to ensure that conidia will respond to specific signals that are indicative of favourable growth conditions. In *B. graminis*, germination and differentiation of infection structures can be induced by plant-derived substances or signals, such as contact with a solid hydrophobic surface and/or presence of host cuticle constituents (Francis *et al.* 1996; Tsuba *et al.* 2002; Zabka *et al.* 2008). Plant cuticular waxes are known to play important roles in triggering germination and differentiation of the grass powdery mildew *B. graminis* (Carver *et al.* 1990; Tsuba *et al.* 2002; Zabka *et al.* 2008; Ringelmann *et al.* 2009; Feng *et al.* 2009; Hansjakob *et al.* 2010; Hansjakob *et al.* 2011). Very-long-chain aldehydes – present in many cuticular plant waxes – have turned out to be of particular significance for the *B. graminis* prepenetration processes. Presence of very-long chain aldehydes considerably promotes germination and differentiation of powdery mildew conidia in a dose and chain-length dependent manner (Hansjakob *et al.* 2010; Hansjakob *et al.* 2011). Besides the chemical composition of a leaf surface a multitude of environmental factors can significantly affect conidial germination and subsequent differentiation (Carver *et al.* 1990; Ringelmann *et al.* 2009).

In many plant pathogenic fungi the short period of growth on the plant surface during prepenetration is characterized by a tight coupling of morphogenesis and cell cycle progression. Infectious dikaryotic hyphae of the basidomycetous smut fungus *Ustilago maydis* are arrested in the G_2 phase of the cell cycle (Banuett, 1992). Only after appressorium formation and host cuticle penetration the cell cycle arrest is released resulting in proliferation of the fungus in the host tissue (Banuett and Hershkowitz, 1994). A similar cell cycle arrest is also present in rust fungi such as *Uromyces appendiculatus* (Kwon and Hoch, 1991). In the rice blast fungus *Magnaporthe oryzae,* the completion of mitosis in the germ tube is considered a prerequisite for appressorium morphogenesis (Veneault-Fourrey *et al.* 2006; Saunders *et al.* 2010a, 2010b). Shortly after germination a single round of nuclear division takes place in *M. oryzae* conidia. One of the daughter nuclei moves to the tip of the germ tube, where the appressorium is formed, while the other migrates back into the conidial cell. Mitosis and nuclear migration precede cytokinesis, which is required for appressorium differentiation. By contrast, the differentiation of infection structures including appressoria in the anthracnose fungus *Colletotrichum gloeosporioides* can occur without previous mitosis and nuclear division (Nesher *et al.* 2008).

Until now its obligate biotrophy, the lack of a stable transformation system and its susceptibility to free water during prepenetration have vastly hindered the cytological analysis of nuclear division during the essential phase of infection structure formation of *B. graminis* (Manners and Hossain 1963; Sivapalan 1994; Iwamoto *et al.* 2002; Zhang *et al.* 2005). By using a distinct nuclear staining procedure the present study investigated the timing of specific developmental events on artificial and host leaf surfaces as well as the association of nuclear division and morphogenesis during *B. graminis* prepenetration processes by applying a pharmacological approach. Application of an improved *in vitro* system - based on a Formvar® membrane containing differentiation inducing host wax - allowed the application of various cell cycle inhibitors omitting a possible interference with enzymatic activities and/or chemical properties of the otherwise underlying plant tissue.

4.3 Material and Methods

Pathogen and plant material

Barley (*Hordeum vulgare* L. cv. Stendal, IG Pflanzenzucht, Munich, Germany) was sown in plastic pots (diameter 9 cm) filled with standard potting soil (Typ ED73; SteuderComp, Schermbeck, Germany) and kept in growth chambers with 300 µmol photons m^{-2} s^{-1} light intensity in a 16 h : 8 h photoperiod at 22°C : 18°C and 70% relative humidity.

Blumeria graminis (DC.) Speer f. sp. *hordei* Em. Marchal (isolate CC1, obtained from Tim Carver, Institute of Grassland and Environmental Research (IGER, Aberystwyth, UK) was propagated on its host plant *H. vulgare* until distinct white powdery pustules appeared. One day before conidia were required for experimentation, spore-bearing leaves were shaken to remove older conidia so that freshly emerged conidia were available for subsequent assays.

Preparation of epidermal strips and artificial surfaces

In order to facilitate the microscopic analysis of the prepenetration processes of *B. graminis* f.sp. *hordei* on its natural host surface, abaxial epidermal strips from primary leaves of 12 day old barley plants were used. For that purpose the epidermal cell layers were carefully stripped off using a scalpel and fine forceps and immediately transferred to Petri dishes containing 20 ml of flotation buffer (10 mM Tris/Cl, pH 8, 1 mM $CaCl_2$).

For *in vitro* prepenetration studies, total leaf wax extracts of secondary leaves of 14 d old barley plants were prepared in chloroform and sprayed onto glass slides (Elka, Sondheim, Germany) using a glass chromatography sprayer (Zabka *et al*., 2008). The wax coated glass slides were stored for at least 16 hours at room temperature, to ensure complete solvent evaporation. As surface hydrophobicity is known to play an important role for the initiation of *B. graminis* prepenetration processes the contact angles of 1-µl droplets of distilled water deposited on the coated glass surfaces were determined (contact angle system OCA 15, software system SCA20; Dataphysics Instruments, Filderstadt, Germany). A total of 20 measurements on each of at least five independent surface samples were performed. Only glass slides exhibiting contact angles above 100° were subjected to further experimentation.

For cell cycle inhibitor studies on the host plant surface, epidermal strips of primary leaves of 12 d old barley plants were placed in a plastic Petri dish onto 0.5% Bacto-Agar (10 mM Tris/Cl, pH 8, 1 mM $CaCl_2$), supplemented with the corresponding cell cycle inhibitor, or as control, with an equivalent volume of the solvent, only.

In order to investigate *B. graminis* haustoria development we used adaxial epidermal cell layers of 12 day old barley coleoptiles as these - after fungal penetration - more effectively support fungal development than stripped barley leaf epidermal tissues (Bushnell *et al.* 1967). Coleoptile epidermal cell layers, prepared according to Bushnell *et al.* (1967), were floated with their adaxial side up on 0.2 ml buffer (10 mM Tris/Cl, pH 8.0, 1 mM $CaCl_2$) on microscope glass slides. The buffer was supplemented with the corresponding cell cycle inhibitor, or as control, with an equivalent volume of the solvent, only.

For *in vitro* cell cycle inhibitor studies standard microscopy glass slides (Elka, Sondheim, Germany) were dipped into a solution of 1% (w/v) Polyvinylpyrrolidone 40 (PVP40) (Sigma Aldrich) and dried completely for 24 hours at room temperature. The PVP-coated slides were then dipped into a 0.5% Formvar® (Polyvinyl formal, CAS 63450-15-7, Applichem) solution in chloroform supplemented with total barley leaf wax extract (500 µg ml^{-1}) and dried for 16 hours at room temperature (Hansjakob *et al*., 2010). The edges of the slides were then carefully scratched with fine sandpaper and the slides were slowly immersed in distilled water in an angle of about 45°. This treatment led to solubilisation of the PVP40 coating and the subsequent release of Formvar®/wax membranes floating on the water surface. One side of a glass slide was covered with 665 µl of 1% Bacto water agar (supplemented with inhibitor substance or solvent only) and was submersed below the floating membrane. The agar-covered slide was carefully pulled out again in a 45° angle, resulting in the transfer of a Formvar®/wax membrane onto the agar surface. Excess water was dabbed off by gently touching the edges of the slide with a paper towel.

Epidermal strips with their adaxial surface up, wax-coated glass slides and Formvar®/wax membranes were fixed at the base of a settling tower. Conidia from infected barley leaves were blown into the tower using pressurized air to ensure their even distribution at a density of approximately 2 x 10^3 conidia cm^{-2}. Artificial surfaces and epidermal strips were kept in a humid atmosphere with wet filter paper applied underneath to achieve a relative humidity of at least 90%. The samples were incubated in darkness at 20°C.

Inhibitors

Hydroxyurea (HU) (Sigma Aldrich) was prepared as 1 M stock solution in ddH_2O and was used to inhibit S-phase at a final concentration of 500 mM for *in vitro* experiments and 100 mM for studies on the host plant surface. Benomyl (Sigma Aldrich) was prepared as 17 mM stock solution in Dimethylformamide (DMF) to inhibit cytokinesis of nuclei at a final concentration of 170 µM. Lantrunculin A (LatA) (Calbiochem) was prepared as 1 mM stock solution in dimethyl sulfoxide (DMSO) to inhibit microtubule polymerization at a working concentration of 50 µM. Cycloheximide (Sigma Aldrich) was purchased as 1 mg ml^{-1} stock solution in DMSO and was used at a final concentration of 50 µg ml^{-1} to inhibit protein biosynthesis. The appropriate amount of inhibitor substance was added to the Bacto agar after cooling to approximately 50°C. In each experiment an equivalent volume of the corresponding solvent was added to the agar as control.

Post-incubation processing of artificial surfaces and epidermal strips

After incubation the Formvar®/wax membranes were carefully re-floated on distilled water by submerging the wax membrane agar glass slide stack into water at an acute angle of approximately 10° to prevent displacement of conidia. The floating Formvar®/wax membranes were then carefully transferred onto positively charged glass slides (Menzel Superfrost Plus, Braunschweig, Germany) ensuring the firm electrostatic attraction of the membranes.

Leaf epidermal strips were carefully transferred from the agar onto plastic foils. Glass slides and foils with the leaf epidermal strips were flash frozen for 15 min in a beaker immersed in liquid nitrogen to prevent direct contact of the liquid nitrogen with the samples and subsequently lyophilized for 1.5 h at 5 Pa and -52°C (Christ Alpha 1-2). Studies on moisture relations in powdery mildews have shown that free water is inhibitory to *B. graminis* germination (Manners & Hossain, 1963; Sivapalan 1994). During prepenetration conidia are extremely sensitive towards the presence of free water, resulting in burst conidia. Therefore, the lyophilization step was a necessary prerequisite for the analysis of number and position of nuclei in B. graminis conidia during prepenetration.

DAPI staining

Lyophilized samples were covered with several droplets of freshly prepared staining solution (50 mM Tris/malic acid, pH 5.2; 20 mg ml^{-1} PVP40; 0.4% TritonX-100 (v/v); 15% (v/v) Glycerol; 4% formaldehyde (v/v); 0.6 µg ml^{-1} 4′,6-Diamidin-2-phenylindol) and were incubated with a coverslip at 4°C for 30 min prior to observation.

Light and epifluorescence microscopy

Microscopic analysis was performed to determine whether conidia subjected to different surfaces and treatments had remained non germinated (ng), had formed a primary germ tube only (pgt), a secondary non-swollen germ tube (sgt), a swollen secondary germ tube (agt) or a fully differentiated hooked appressorium with septum (app). Additionally, position and number of nuclei were recorded for each conidium. 250 conidia were observed in at least three independent replications for each treatment and time point. In addition, the quality of fungal conidia was verified by detecting the loss rate of those apparently damaged or desiccated during the inoculation procedure. Only single, well-separated conidia were counted to eliminate the possibility of inhibition as a result of crowding.

Microscopic analysis was performed with an epifluorescence microscope (Leica DMR) with excitation and emission wavelength of 355-425 nm and 455 nm, respectively. Micrographs were taken using a digital camera (Zeiss AxioCam MRc) and processed with AxioVison 4.8 software package.

4.4 Results

4.4.1 Spatiotemporal behaviour of *B. graminis* nuclei during prepenetration

To investigate the processes of cell and nuclear division during *B. graminis* prepenetration we first analyzed nuclear behaviour on epidermal strips of primary barley leaves within a 10 hours period following inoculation (Tab. 1). The ungerminated conidia were always uninucleate and the vast majority of the nuclei (99%) resided in a more or less central position within the conidium (Fig. 1a). After 2 h, 90 % of the conidia had germinated by forming a short aseptate primary germ tube (Fig. 1b; Tab. 1). In 85% of the germinated conidia the nucleus had moved close to the site of primary germ tube emergence (Supplementary Table 1, Fig. 1b). After 4 h 70% of the conidia had started to form a non-swollen secondary germ tube (Tab. 1; Fig. 1c) and in 90% of the conidia in this stage the nucleus had migrated close to the site of secondary germ tube emergence (Supplementary Table 1). Five hours post inoculation 58% of the inoculated conidia had differentiated a swollen appressorial germ tube (Tab. 1) and 87% of these conidia were still uninucleate (Fig. 1d). However, 5.5 h post inoculation already 71% of the conidia were in the appressorial germ tube stage (Tab. 1) of which then 41% exhibited two separate nuclei close to the site of emergence of the appressorial germ tube (Supplementary Table 1, Fig. 1e). Half an hour later, 37% of the conidia showed a fully mature appressorium with the typical apical hook and the characteristic septum. In each of these conidia one of the daughter nuclei had migrated into the newly formed appressorial cell (Fig. 1f; Supplementary Table 1). After 7 hours 70% of the inoculated conidia had formed a mature appressorium. The frequencies of the distinct fungal developmental stages at 8 and 10 hours after inoculation were not recorded, as, according to previous prepenetration studies (Hansjakob *et al.*, 2010) a further significant increase in the frequencies of differentiated conidia could not be expected.

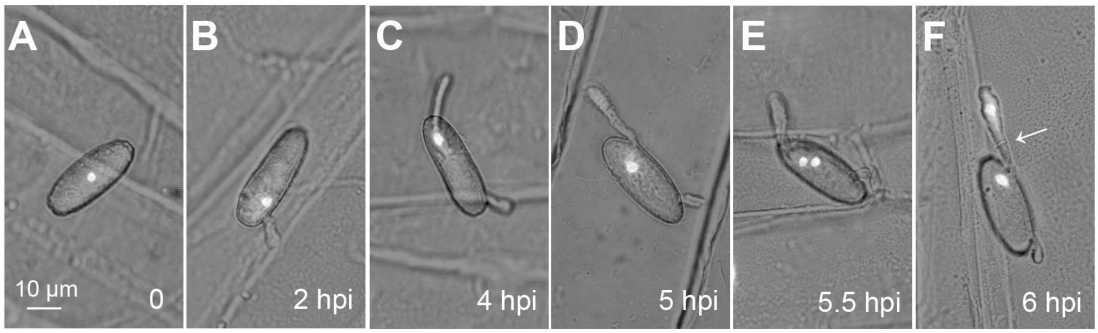


Figure 1 Development, nuclear division and cytokinesis of DAPI-stained *Blumeria graminis* f.sp. *hordei* conidia during prepenetration on a host plant surface. Conidia were inoculated on abaxial barley leaf epidermal strips and incubated for up to six hours post inoculation (hpi). (a) Conidium directly after inoculation; (b) emergence of the primary germ tube and migration of the nucleus to

the site of pgt emergence (2 hpi); (c) secondary germ tube formation and migration of nucleus to the site of sgt emergence (4 hpi); (d) appressorial germ tube formation, showing the nucleus in proximity to the site of agt emergence (5 hpi); (e) appressorial germ tube formation and nuclear division in proximity to the agt (5.5 hpi); (f) appressorium formation and migration of one nucleus towards the apex of the agt and subsequent septum formation (6 hpi). The arrow points to the septum between appressorium and conidium..

Table 1 Development of *Blumeria graminis* f.sp. *hordei* conidia on barley wax coated glass slides and on epidermal strips 0 – 10 hpi.

hpi	Developmental stage									
	ng		pgt		sgt		agt		app	
	Substratum									
	Wax coated glass slide	Epidermal strip	Wax coated glass slide	Epidermal strip	Wax coated glass slide	Epidermal strip	Wax coated glass slide	Epidermal strip	Wax coated glass slide	Epidermal strip
	Percentage of conidia (± SD)									
0	100	100	0	0	0	0	0	0	0	0
2	29 (± 5)	7 (± 3)	70 (± 5)	90 (± 2)	0	3 (± 1)	0	0	0	0
4	19 (± 3)	3 (± 2)	66 (± 2)	21 (± 4)	15 (± 1)	70 (± 6)	0	6 (± 3)	0	0
4.5	12 (± 5)	6 (± 1)	61 (± 5)	15 (± 1)	24 (± 6)	38 (± 5)	3 (± 2)	41 (± 5)	0	0
5	14 (± 3)	5 (± 1)	54 (± 3)	12 (± 2)	21 (± 3)	23 (± 3)	7 (± 2)	58 (± 6)	4 (± 3)	0
5.5	12 (± 3)	6 (± 1)	43 (± 9)	10 (± 3)	32 (± 7)	10 (± 3)	9 (± 3)	71 (± 3)	3 (± 3)	2 (± 3)
6	15 (± 2)	6 (± 2)	55 (± 5)	7 (± 3)	16 (± 5)	6 (± 1)	11 (± 4)	33 (± 2)	4 (± 2)	3 7 (± 2)
6.5	20 (± 2)	n/d	51 (± 2)	n/d	10 (± 1)	n/d	5 (± 2)	n/d	14 (± 3)	n/d
7	15 (± 4)	7 (± 2)	46 (± 6)	7 (± 2)	19 (± 5)	5 (± 1)	8 (± 3)	8 (± 1)	12 (± 4)	70 (± 3)
8	15 (± 4)	n/d	50 (± 6)	n/d	10 (± 4)	n/d	6 (± 2)	n/d	19 (± 7)	n/d
10	19 (± 3)	n/d	42 (± 9)	n/d	9 (± 2)	n/d	9 (± 2)	n/d	20 (± 9)	n/d

n/d: not done; ng: non germinated; pgt: primary germ tube only; sgt: non-swollen secondary germ tube only; agt: appressorial germ tube; app: fully differentiated appressorium with a septum

On wax-sprayed glass slides (Fig. 2) the development of *B. graminis* conidia followed almost the same time schedule as on leaf epidermal strips even though the overall frequencies of germination and differentiation were significantly decreased (Tab. 1). On leaf epidermal strips70% of the conidia formed a mature appressorium while only 20% reached this developmental stage on the wax sprayed glass slides 10 hours after inoculation. Generally, the development of conidia on wax coated glass slides was slightly retarded and more asynchronous. On epidermal strips the formation of secondary germ tubes peaked at 4 hours after inoculation whereas on wax-sprayed glass slides the maximum value occurred after 5.5 hours. Likewise, the maximum values for appressorium formation (20%) were attained one hour later than on epidermal strips.

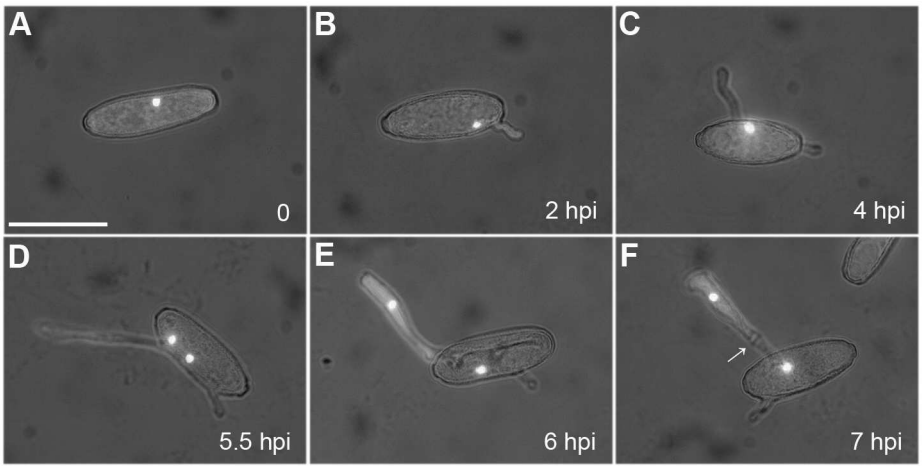


Figure 2 Development, nuclear division and cytokinesis of DAPI-stained *Blumeria graminis* conidia during prepenetration *in vitro*. Glass slides were sprayed with barley leaf wax extract and incubated for up to seven hours post inoculation (hpi). (a) Conidium immediately after inoculation; (b) emergence of the primary germ tube and migration of the nucleus to the site of pgt emergence (2 hpi); (c) secondary germ tube formation and migration of nucleus to the site of sgt emergence (4 hpi); (d) appressorial germ tube formation and nuclear division in proximity to the agt (5.5 hpi); (e) migration of one nucleus towards the apex of the agt (6 hpi); (f) appressorium formation and cytokinesis (7 hpi). The arrow points to the septum between the conidium and appressorium. Scale bar represents 25 µm.

The application of leaf epidermal strips, however, might pose technical difficulties in pharmacological studies. For example, in experiments in which conidia on the barley epidermis are treated with pharmacological agents from underneath, the epidermal cells may alter the chemical in some way or may prevent it from coming into contact with the germling (Kinane *et al.* 2000). In order to circumvent such obstacles we developed an improved Formvar®/wax/agar system that resulted in substantially increased rates of appressorium formation of up to 70% and moreover allowed investigating the effects of water soluble pharmacological agents (Fig. 3). Supplementing the underlying water agar with 50 µg ml^{-1} cycloheximide demonstrated that the Formvar®/wax membrane is indeed permeable for solutes, as fungal development was strongly affected upon exposure with this potent inhibitor of protein biosynthesis. Less than 5% of the conidia showed the formation of an appressorium or an appressorial germ tube. The presence of DMSO had no significant effects.

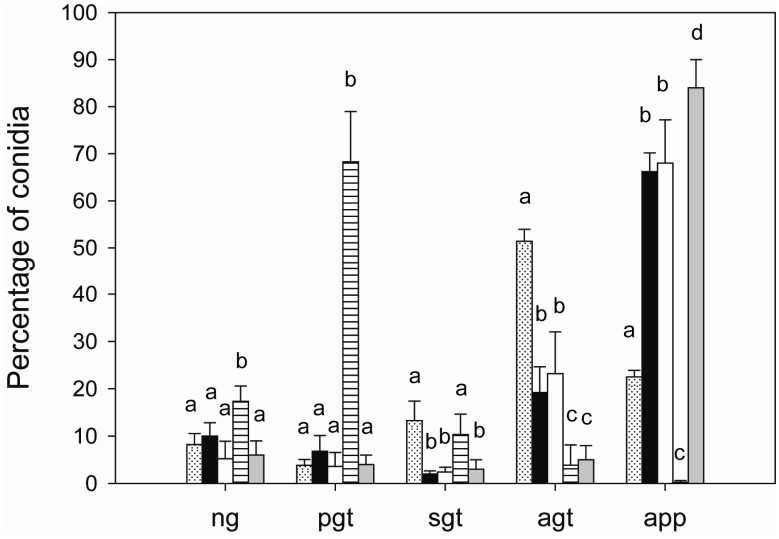


Figure 3 Development of *Blumeria graminis* f.sp. *hordei* conidia on different artificial substrata and on epidermal strips after 16 hpi. Prepenetration processes on glass slides covered with Formvar® resin supplemented with barley leaf wax extract (shaded bars), on Formvar®/barley leaf wax membranes on 1% water agar as control (black bars), on Formvar®/barley leaf wax membranes on 1% water agar DMSO control (white bars), on Formvar®/ barley leaf wax membranes on 1% water agar supplemented with 50 µg ml^{-1} cycloheximide solved in DMSO (striated bars) and on epidermal strips placed on 0.5% agar (grey bars). Prepenetration developmental stages: ng, non-germinated conidium without a germ tube; pgt, germinated conidium with primary germ tube formed; sgt, conidium with additionally elongated secondary germ tube; agt, conidium with a swollen elongated secondary germ tube; app, fully differentiated conidium with a mature lobed and septated appressorium. Values are given as mean ±SD of five independent experiments. Different letters within a developmental category indicate significant differences ($P < 0.05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test.

4.4.2 The presence of very-long-chain aldehydes affects nuclear migration in *B. graminis* conidia

Previous investigations have demonstrated that very-long-chain aldehydes considerably promote germination and differentiation of *B. graminis* conidia. In order to find out whether the absence of very-long-chain aldehydes would affect the early nuclear behaviour, conidia were incubated on Formvar®-coated glass slides supplemented with the non-promoting C_{26}-alkane *n*-hexacosane (Hansjakob *et al*. 2010). 280 On Formvar slides supplemented with very-long-chain aldehyde containing barley leaf wax extract or with the C_{26}-aldehyde *n*-hexacosanal more than 80% of the conidia developed a primary germ tube at 2 h post inoculation while on Formvar® slides supplemented with *n*-hexacosane only 24% had formed a primary germ tube (Tab. 2; Supplementary Figure 1). Almost 90% of the germinated conidia on aldehyde containing surfaces showed the migration of the nucleus from a central position close to the site of primary germ tube emergence, whereas only 20% of the germinated conidia on Formvar®/n-hexacosane slides showed this migration (Tab. 3; Supplementary Figure 1). The nuclei of all conidia that were non-germinated at 2 h post inoculation on Formvar®/*n*-hexacosane slides occupied a

rather central position in the conidium, whereas around 15% of the nuclei of non-germinated conidia on very-long-chain aldehyde containing surfaces had a position close to one of the conidial poles (Tab. 3; Supplementary Figure 1). At 16 h post inoculation only 7% of the conidia on *n*-hexacosane were differentiated while more than 80% showed the formation of an appressorial germ tube or of an appressorium on very-long-chain aldehyde containing surfaces (Supplementary Table 4). However, the appressorium bearing conidia on *n*-hexacosane exhibited the same nuclear positioning as in presence of very-long-chain aldehydes (Supplementary Table 5). Thus, absence of very-long-chain aldehydes results in a modified fungal behaviour in the first two hours after inoculation.

Table 2 Development of *Blumeria graminis* f.sp. *hordei* conidia on Formvar®-coated glass slides supplemented with extracted barley leaf wax or wax constituents at 2 h post inoculation.

Substratum	Developmental stage				
	ng	pgt	sgt	agt	app
	Percentage of conidia (± SD)				
Barley leaf wax	16 (± 1)	84 (± 1)	0	0	0
2% (n/n) *n*-hexacosanal	17 (± 2)	83 (± 2)	0	0	0
N-hexacosane	76 (± 4)	24 (± 4)	0	0	0

ng: non germinated; pgt: primary germ tube only; sgt: non-swollen secondary germ tube only; agt: appressorial germ tube; app: fully differentiated appressorium with a septum

Table 3 Positions of nuclei in conidia of the corresponding developmental stages on Formvar® coated glass slides supplemented with barley leaf wax or wax constituents at 2 h post inoculation. (Data are given in %).

Substratum	Developmental stage			
	ng		pgt	
	Position of nuclei			
				
Barley leaf wax extract	87	13	89	11
2% (n/n) *n*-hexacosanal	85	15	87	13
N-hexacosane	100	0	20	80

ng: non germinated; pgt: primary germ tube only

4.4.3 Spatial uncoupling of nuclear division and cytokinesis

On native and artificial surfaces nuclear division always occurred in the primordial conidium close to the site of secondary germ tube emergence, 5 to 6 h after inoculation (Figs. 1 and 2). Within the following 30 to 60 min one of the two resulting daughter nuclei moved towards the swollen hyphal tip of the appressorial germ tube, while the other remained within the primordial conidium. The subsequent cytokinesis occurred within the proximal third of the appressorial germ tube approximately at the midpoint between the two daughter nuclei, 6 to 7 h after inoculation. Hence, during appressorium differentiation, nuclear division and cytokinesis were spatially separated and the transit of the nucleus to the tip of the appressorial germ tube always preceded cytokinesis and appressorium maturation.

4.4.4 Cell cycle and morphogenesis

The link between cell cycle and morphogenesis was investigated using cell cycle inhibitors in combination with the improved Formvar®/wax/agar in *vitro* system. In order to test the possible effect of polar growth prevention on the cell cycle, we treated conidia with latrunculin A (LatA), which disrupts actin polymerization (Spector *et al.* 1983). LatA treatment only slightly affected the formation of the primary germ tube, but it strongly inhibited the development of the appressorial germ tube (Fig 4b). Consequently, 77% of the conidia remained in the uninucleate primary germ tube stage, whereas only 7% exhibited nuclear division and formed a mature appressorium (Tab. 4). Without LatA, however, 76% of the conidia formed a mature appressorium and only 2% remained in the primary germ tube stage.

Hydroxyurea (HU), frequently used as a cell cycle inhibitor, causes an immediate inhibition of DNA synthesis by acting as a ribonucleotide reductase inhibitor without interfering with the synthesis of ribonucleic acid or of protein. HU-treated conidia formed a primary germ tube and as expected, most of the nuclei did not divide, thus indicating that the cell cycle was arrested (Fig. 4d). Despite the lack of nuclear division, 80% of the conidia formed an appressorial germ tube that reached the same size as developed by untreated conidia (Fig. 4d; Tab. 4). Nevertheless, 9% of the conidia passed through nuclear division and differentiated an appressorium. Thus, treatment with 500 mM HU widely prevented nuclear division in the conidia but had almost no effect on germination and formation of the appressorial germ tube. Most of the conidia treated with benomyl, which causes disassembly of the microtubule cytoskeleton, thereby arresting the cell cycle in mitosis and hence blocking nuclei separation (Bergen and Morris, 1983), also formed primary germ tubes and swollen appressorial germ tubes before growth

was arrested. 81% of the conidia formed an appressorial germ tube, while only 2% exhibited nuclear division and differentiated a septate appressorium (Tab. 4). In 96% of the conidia in the appressorial germ tube stage only one nucleus was observed positioned within the proximal third of the appressorial germ tube indicating the inhibition of nuclear separation, but apparently not of nuclear migration (Fig.4f; Supplementary Table 2). Thus, inhibition of nuclear division may prevent subsequent cytokinesis and formation of mature appressoria.

Table 4 Development of *Blumeria graminis* f.sp. *hordei* conidia on Formvar®/wax membranes in presence of cell cycle inhibitors at 16 hpi.

Inhibitor	Developmental stage				
	ng	pgt	sgt	agt	app
	Percentage of conidia (± SD)				
LatA	14 (± 7)	77 (± 6)	1 (± 1)	1 (± 1)	7 (± 7)
LatA control	5 (± 2)	2 (± 2)	2 (± 2)	17 (± 7)	76 (± 4)
HU	6 (± 2)	5 (± 4)	4 (± 2)	80 (± 5)	9 (± 2)
HU control	9 (± 3)	3 (± 4)	2 (± 2)	19 (± 5)	69 (± 5)
Benomyl	6 (± 2)	6 (± 2)	5 (± 3)	81 (± 6)	2 (± 3)
Benomyl control	3 (± 2)	3 (± 3)	0 (± 0)	25 (± 9)	69 (± 8)

ng: non germinated; pgt: primary germ tube only; sgt: non-swollen secondary germ tube only; agt: appressorial germ tube; app: fully differentiated appressorium with a septum

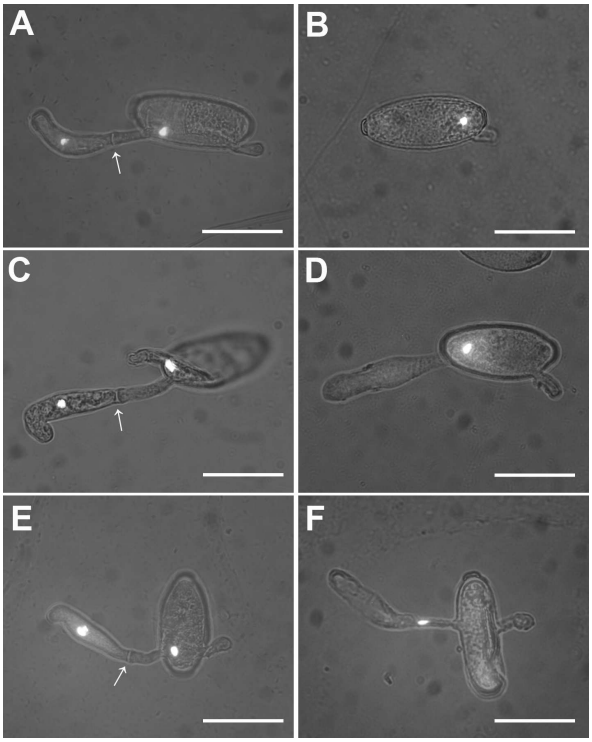


Figure 4 Effects of cell cycle inhibitors on morphogenesis and nuclear division of *Blumeria graminis* f.sp. *hordei* conidia *in vitro*. Formvar®/wax membranes were placed on 1% agar containing the respective supplements, inoculated with conidia and then incubated for 16 h. (a) LatA solvent

control (DMSO); (b) 50 µM LatA; (c) HU solvent control (H_20); (d) 500 mM HU; (e) benomyl solvent control (DMF); (f) 170 µM benomyl. Conidia were stained with DAPI. Arrows point to the septum between appressorium cells and conidium. Scale bar represents 20 µm.

In vitro, LatA prevented the polarized growth of the appressorial germ tube after formation of the primary germ tube (Fig. 4). Hence, as LatA was expected not to result in the formation of infection structures, it was omitted from the subsequent experiments with leaf epidermal strips. However, the results for HU and benomyl were further corroborated by experiments using barley leaf epidermal strips placed on 0.5% agar supplemented with the respective cell cycle blocker (Fig. 5; Tab. 5). Incubation of the conidia on epidermal strips in the presence of only 100 mM HU or 170 µM benomyl resulted in roughly the same distribution of developmental stage frequencies (Tab. 5) as obtained with the Formvar®/wax/agar *in vitro* system (Tab. 4). As expected, the control treatments showed distinctly higher percentages of differentiated conidia, while the frequencies of non-germinated conidia remained constant throughout the different treatments. In accordance with the results from the Formvar®/wax/agar *in vitro* system, treatments with HU or benomyl resulted in prevention of nuclear division and of cytokinesis but not of appressorial germ tube formation (Fig. 5; Tab. 5). The presence of DMF or DMSO had no effect in these treatments. (Tables 4 and 5).

Table 5 Development of *Blumeria graminis* f.sp. *hordei* conidia on epidermal strips in presence of cell cycle inhibitors at 16 hpi.

Inhibitor	Developmental stage				
	ng	pgt	sgt	agt	app
	Percentage of conidia (± SD)				
HU	6 (± 4)	4 (± 1)	2 (± 2)	80 (± 8)	10 (± 8)
HU control	6 (± 3)	4 (± 2)	3 (± 2)	5 (± 3)	84 (± 6)
Benomyl	7 (± 2)	7 (± 5)	7 (± 0)	86 (± 6)	0
Benomyl control	8 (± 1)	5 (± 2)	2 (± 2)	4 (± 2)	84 (± 3)

ng: non germinated; pgt: primary germ tube only; sgt: non-swollen secondary germ tube only; agt: appressorial germ tube; app: fully differentiated appressorium with a septum

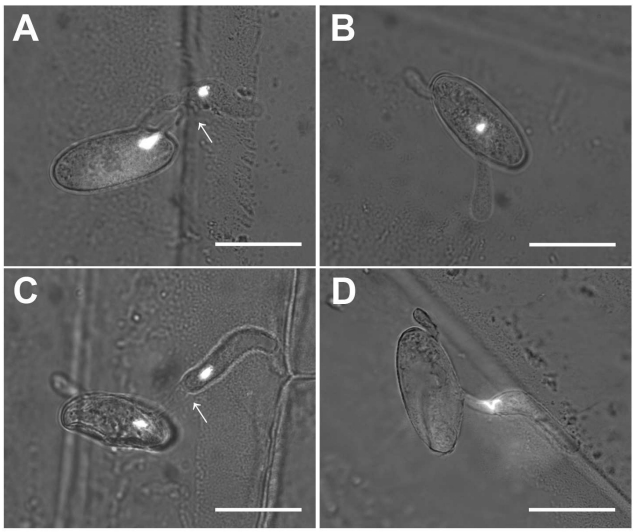


Figure 5 Effects of cell cycle inhibitors on morphogenesis and nuclear division of *Blumeria graminis* f.sp. *hordei* conidia stained with DAPI on a host plant surface. Barley epidermal strips were placed on 0.5% agar containing the respective supplements, inoculated with conidia and then incubated for 16 h. (a) HU solvent control (H_20); (b) 100 mM HU; (c) benomyl solvent control (DMF); (d) 170 µM benomyl. Arrows point to the septum between appressorium cell and conidium. Scale bar represents 20 µm

In order to find out whether conidia affected by the applied cell cycle inhibitors were still capable of infecting their hosts, we used adaxial epidermal strips of barley coleoptiles (Bushnell *et al.* 1967). In the HU control treatment 83% of the inoculated conidia differentiated an appressorial germ tube (agt) or a mature appressorium (app) at 18 h post inoculation, but only 14% of the conidia formed a haustorium (Tab. 6). The benomyl control treatment exhibited 70% differentiated conidia and 9% formed a haustorium, whereas the application of 25 mM HU or 170 µM benomyl resulted in a distinct decrease in the number of differentiated conidia to 47 and 39%, respectively. The cell cycle inhibitor treatments completely inhibited the formation of haustoria in barley coleoptile epidermis cells.

Table 6 Differentiation of *Blumeria graminis* f.sp. *hordei* conidia and formation of haustoria on adaxial coleoptile epidermal strips in presence of cell cycle inhibitors at 18 h post inoculation.

Inhibitor	Developmental stage	
	Differentiated (agt + app)	haustorium
	Percentage of conidia (± SD)	
HU (50 mM)	24 (± 7)	0
HU (25 mM)	47 (± 9)	0
HU control	83 (± 5)	14 (± 6)
Benomyl (170 µM)	39 (± 8)	0
Benomyl control	70 (± 4)	9 (± 5)

agt: appressorial germ tube; app: fully differentiated appressorium with a septum

4.5 Discussion

In many plant pathogenic fungi, the capability to cause disease relies on the ability to elaborate specialized infection structures that enable direct penetration of the plant cuticle (Tucker and Talbot, 2001). The coordination of cell cycle progression and morphogenesis during prepenetration is of particular importance for the pathogenicity of several hemibiotrophic plant pathogenic fungi (Veneault-Fourrey *et al.* 2006; Nesher *et al.* 2008; Saunders *et al.* 2010a, 2010b). However, there is at present almost no information available on nuclear division and cytokinesis during the prepenetration processes of the obligately biotrophic grass powdery mildew fungus *B. graminis*. Therefore, the present study aimed at investigating the spatiotemporal course of events concerning nuclear division and cytokinesis during germination and appressorium formation of this agriculturally important cereal pathogen. We and others have shown that - among additional factors - the presence of very-long-chain aldehydes in the host cuticular wax is of major importance for the initiation of conidial germination and efficient differentiation of infection structures (Tsuba *et al.* 2002; Hansjakob *et al.* 2010; Hansjakob *et al.* 2011). However, how these signals are connected to the developmental biology of *B. graminis* as it undergoes the characteristic morphogenetic transitions to form appressoria remains largely unclear.

One major goal of the present study was to visualize and characterize the timing of events regarding nuclear behaviour in germinating and differentiating *B. graminis* conidia. In order to bypass the extreme sensitivity of living grass powdery mildew conidia to the presence of free water we developed a nuclear staining protocol based on lyophilisation of the conidia and subsequent DAPI staining of the nuclei. For the first time this enabled us to follow the nuclear behaviour in prepenetration stage *B. graminis* conidia. Unfortunately, the applied protocol did not support a live imaging approach due to its technical specifications. Nevertheless, the resulting data clearly show that - on differentiation inducing native and artificial surfaces - uninucleate *B. graminis* conidia germinate and then undergo a single round of mitosis, which is completed 5 to 6 h post inoculation (Fig. 6).

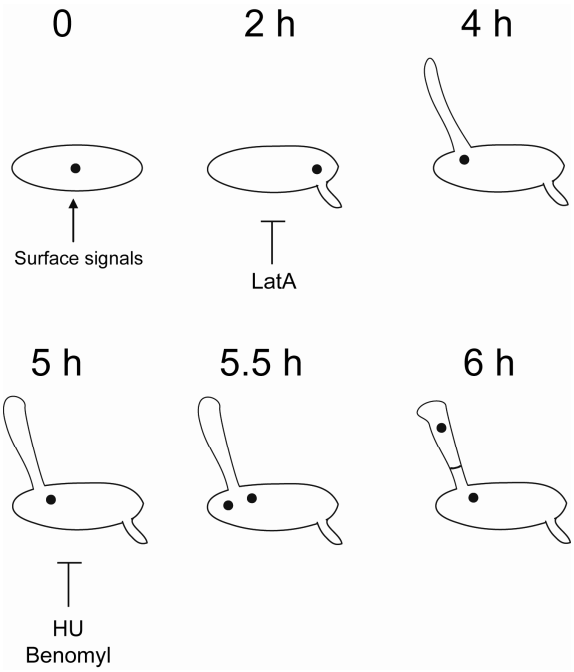


Figure 6 Summarizing model depicting cell cycle and morphogenesis during *B. graminis* prepenetration. Surface signals induce germination of a resting uninucleate conidium. The nucleus then migrates from the centre of the conidium towards the site of emergence of the primary germ tube (2 h). When the secondary germ tube starts to be formed, the nucleus is relocated in proximity to the site of secondary germ tube emergence (4 h). The secondary germ tube swells and develops into the appressorial germ tube (5 h), before mitosis occurs in the conidium (5.5 h). After nuclear division the appressorium cell further matures and the septum is formed between the two cells (6h). LatA treatment prevents emergence of the secondary germ tube and further cell proliferation. HU does not prevent emergence and differentiation of the secondary germ tube into the appressorial germ tube, but inhibits appressorium formation. Benomyl does not suppress the development of appressorial germ tube formation, but prevents maturation of the appressorium.

Our results with artificial surfaces lacking very-long-chain aldehydes suggest that the nuclear migration during primary germ tube formation, which directs the nucleus towards the site of primary germ tube emergence is strongly promoted by the presence of very-long-chain aldehydes. Nuclear migration during the prepenetration phase is also observed in other pathogenic fungi. Similarly, the single nucleus in the conidium of the the entomopathogenic ascomycete *Metarhizium anisopliae* usually moves to a position close to the developing germ tube before the first mitotic division (St. Leger *et al.* 1989). When *M. oryzae* conidia are in contact with a hydrophobic inductive surface a nucleus migrates into the developing germ tube where subsequently mitosis occurs (Veneault-Fourrey *et al.* 2006). The nuclear migration in *B. graminis* conidia during primary germ tube formation might be a comparatively early and microscopically visible manifestation of fungal response(s) to its host indicating the perception of physicochemical stimulants triggering or promoting germination and differentiation of infection

structures. It, however, remains to be determined, whether this nuclear repositioning might indicate a possible re-entry of the conidium into the cell cycle.

In *B. graminis* the nuclear division preceding formation of a mature appressorium always occurred within the primordial conidium close to the site of secondary germ tube emergence and not within the appressorial germ tube as in *M. oryzae* and *C. gloeosporioides* (Nesher *et al.* 2008; Saunders *et al.* 2010b). In *B. graminis*, the movement of a nucleus from the conidium into the appressorial germ tube had been briefly mentioned already, however, without referring to the previous mitosis (McKeen 1972a). Cytokinesis, marked by the formation of a distinct septum in the appressorial germ tube of *B. graminis*, occurred at the midpoint between the daughter nuclei in the proximal third of the appressorial germ tube, about one hour later, 6 to 7 h after inoculation. The formation of a septum to separate the differentiated appressorium cell from the conidium is likely to be essential for the generation of the turgor pressure built up during cuticle penetration (Pryce-Jones *et al.* 1999; Iwamoto *et al.* 2007). Nuclear separation and migration of one daughter nucleus into the swollen appressorial germ tube always preceded cytokinesis and hence final differentiation of the appressorium. A similar developmental pattern showing a spatial uncoupling of nuclear division and cytokinesis was recently demonstrated for appressorium formation in the hemibiotrophic plant pathogen *M. oryzae* (Saunders *et al.* 2010a, 2010b), whereas in the anthracnose fungus *C. gloeosporioides* nuclear division and cytokinesis were shown to be spatially coupled during appressorium formation (Nesher *et al.* 2008). Our results strongly indicate that in *B. graminis* the site of appressorial septation is spatially separated from the previous nuclear division. This feature is not observed in hyphae and generative cells of *B. graminis* (McKeen, 1972b).

With our improved Formvar®/wax/agar system it was now possible to analyse the effects of water-soluble compounds/pharmaceuticals on the prepenetration processes of B. *graminis*, omitting the possible interference with enzymatic activities and/or chemical properties of living plant tissue. This improved in vitro system resulted in even higher frequencies of fully differentiated conidia than reported for a comparable system based on differentiation inducing cellulose membranes (Kobayashi *et al*. 1991; Kinane *et al*. 2000). Our system even approached almost those values recorded for appressorium differentiation on epidermal strips and on native barley leaves (Hansjakob *et al*. 2010). As the primary germ tube is supposed to play an important role in water uptake from the host during prepenetration (Carver and Bushnell 1983), it is tempting to speculate that supporting the Formvar®/wax membrane with a layer of water agar could result in an improved water supply to serve the needs of the germlings and hence in more vital germlings that more frequently complete the differentiation of infection structures. This, however, remains to be demonstrated.

In order to investigate the importance of the cell cycle progression for appressorium development, we applied different water soluble cell cycle inhibitors to our improved *in vitr*o system: the DNA synthesis inhibitor hydroxyurea (HU) that blocks cell cycle in the S-phase (Bachewich *et al*. 2005), the inhibitor of actin polymerization latrunculin A (LatA) that leads to cell cycle arrest at the G_2-phase (Rupeš *et al*. 2001), and benomyl that causes disassembly of the microtubule cytoskeleton, arresting the cell cycle in mitosis and preventing separation of nuclei (Bergen and Morris 1983; Spector *et al.* 1983; Li and Murray 1991). However, since the first contact between conidia and cell cycle inhibitors takes place after inoculation onto the Formvar®/wax surface, we cannot simply deduce when the pharmaceutical compounds enter the cell and when they start affecting their target processes.

In presence of LatA the largely unimpaired formation of the short primary germ tube that emerges potentially prior to the disruption of actin polymerization might be due to a delay in the uptake of that compound. The inhibition of the subsequent polarized growth of the appressorial germ tube indicates that LatA exerts its effects at least after approximately 2 h, while effects of HU and benomyl treatment become apparent after about 5 to 5.5 h after inoculation (Fig. 6). After addition of HU to germinating conidia of *M. oryzae* they predominantly arrest development with undifferentiated germ tubes, whereas benomyl treatment still allows the formation of swollen germ tube tips (Veneault-Fourrey *et al.* 2006; Saunders *et al.* 2010). In contrast to *M. oryzae* germ tubes of HU-treated conidia of *C. gloeosporioides* reach the same size as germ tubes of untreated spores and form similar numbers of fully developed appressoria (Nesher *et al.* 2008). In *Candida albicans*, Treatment with HU or with nocodazole, which depolymerizes microtubules and locks cells in mitosis, results in cells that continue to elongate despite their inability to divide (Bachewich *et al*. 2005; Berman, 2006). In *B. graminis,* however, HU and benomyl treatment did not result in failure of appressorial germ tube formation but in failure to form fully developed appressoria. Interestingly, benomyl treatment resulted in migration of the unseparated nuclei into the appressorial germ tube to a position where septation would have occurred. Incompletely separated daughter nuclei as a result of benomyl treatment have also been described for the necrotrophic fungal pathogen *Botrytis cinerea* (Richmond and Phillips, 1975). As benomyl also affects cytoplasmic microtubules the effects on morphogenesis may not be directly related to nuclear division, but could be due to cytoplasmic events.

Our results obtained with coleoptile epidermal strips subjected to cell cycle inhibitors may suggest that only fully mature *B. graminis* appressoria are capable of host penetration, subsequent haustorium formation and consequently infection. However, we generally cannot exclude a possible interference with enzymatic

activities and/or specific properties of the coleoptile epidermis cells. In contrast to the situation on leaf epidermal strips and on Formvar®/wax membranes the percentage of differentiated conidia was distinctly reduced upon exposure to HU or benomyl which demonstrates that the type and/or condition of underlying tissue can modify the fungal response towards these chemical agents.

4.6 Conclusions

Nuclear migration during formation of the primary germ tube is strongly promoted by the presence of very-long-chain aldehydes, which further corroborates the germination inducing effect of these stimulants (Zabka *et al.* 2008). Moreover, we conclude that a completed mitosis is not a prerequisite for the formation and swelling of the appressorial germ tube, which normally provides the destination for one of the daughter nuclei, while appressorium maturation depends on mitosis and subsequent cytokinesis. Taken together our results suggest that in *B. graminis*, like in *M. oryzae* (Veneault-Fourrey *et al.* 2006), mitosis and subsequent cytokinesis serve as preconditions for appressorium maturation, host cuticle penetration and thus for pathogenicity. In *C. gloeosporioides,* however, all stages of pathogenic germination, including appressoria formation, are independent of mitosis (Nesher *et al.* 2008). Comparable to early events in *M. oryzae* one could propose that in germinating *B. graminis* conidia the entry of the cell cycle into S-phase might lead to the initiation of appressorial germ tube development, which remains independent of mitosis or subsequent cytokinesis (Saunders *et al.*, 2010a, 2010b). Likewise, the spatial uncoupling of nuclear division and cytokinesis might be specifically associated with the morphogenetic program for appressorium differentiation in *B. graminis.* Interfering with early processes of plant infection appears to be one of the most promising means of controlling fungal plant pathogens. For example, conditional mutation of the *SEPTATION-ASSOCIATED1* (*SEP1*) gene in *M. oryzae* prevented the rice blast fungus from causing disease. This mutation perturbed the spatial regulation of septation and prevented the development of infection-competent appressoria (Saunders *et al.*, 2010b). In the case of *B. graminis* a more profound understanding of the fungal development during prepenetration might prove essential particularly for future studies e.g. focussing on specific transcriptional or enzymatic properties of the appressorial cell. Due to presently lacking efficient mutagenesis and transformation protocols and its obligate biotrophy it remains a challenging task to gain more detailed knowledge and a deeper understanding of the processes and events in this crucial stage of barley powdery mildew development.

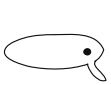
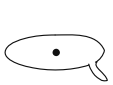
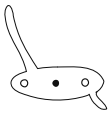
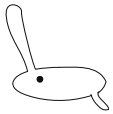
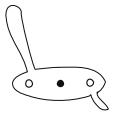
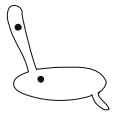
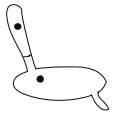
4.7 Acknowledgements

The authors thank Andrea Knorz for her excellent and dedicated technical assistance. This project was financially supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 567 (project A5).

4.8 Supporting information

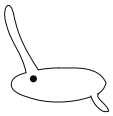
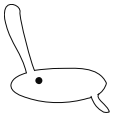
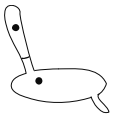
See the next pages

Table S1 Positioning of nuclei in conidia of the corresponding developmental stages between 0 hpi and 10 hpi on glass slides coated with barley wax and on barley epidermal strips (ES).

	Developmental stage																							
	ng				pgt				sgt								agt							
	Position of nuclei																							
hpi																										
	Substratum																							
	Wax	ES	Wax	ES	Wax	ES	Wax	ES	Wax	ES	Wax	ES	Wax	ES	Wax	ES	Wax	ES	Wax	ES	Wax	ES	Wax	ES
	Percentage of nuclei																							
0	99	99	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	95	96	5	4	85	85	15	15	0	30	0	70	0	0	0	0	0	0	0	0	0	0	0	0
4	92	90	8	10	94	90	6	10	91	90	9	10	0	0	0	0	0	72	0	28	0	0	0	0
4.5	83	94	17	6	95	94	5	6	71	91	20	9	9	0	0	0	70	96	0	2	27	2	3	0
5	86	85	14	15	99	85	1	15	49	81	5	17	40	0	6	2	18	82	0	5	40	11	42	2
5.5	90	81	10	19	96	100	4	0	56	92	2	8	36	0	6	0	14	41	0	2	46	41	40	16
6	85	92	15	8	98	94	2	6	64	96	6	4	18	0	12	0	10	46	0	0	5	29	85	25
6.5	89	n/d	11	n/d	97	n/d	3	n/d	73	n/d	4	n/d	19	n/d	4	n/d	8	n/d	0	n/d	0	n/d	92	n/d
7	93	94	7	6	95	92	5	8	80	100	7	0	9	0	4	0	7	46	0	0	0	18	93	36
8	82	n/d	18	n/d	95	n/d	5	n/d	53	n/d	16	n/d	19	n/d	12	n/d	4	n/d	0	n/d	4	n/d	92	n/d
10	90	n/d	10	n/d	98	n/d	2	n/d	67	n/d	0	n/d	31	n/d	2	n/d	12	n/d	0	n/d	2	n/d	86	n/d

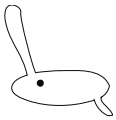
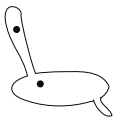
n/d: not done; ng: non germinated; pgt: primary germ tube only;sgt:non-swollen secondary germ tube only; agt: appressorial germ tube; app: fully differentiated appressorium with a septum

Table S2 Positioning of nuclei in conidia of the corresponding developmental stages 16 hpi in presence of latrunculin A (LatA), hydroxyurea (HU) and benomyl *in vitro*. (Data are given in %)

Developmental stage	Position of nuclei	Inhibitor		
		LatA	HU	benomyl
ng		93	72	56
		7	28	44
pgt		79	45	57
		21	55	43
sgt		100	100	100
agt		100	100	4
		0	0	96
app		100	100	100

ng: non germinated; pgt: primary germ tube only; sgt: non-swollen secondary germ tube only; agt: appressorial germ tube; app: fully differentiated appressorium with a septum

Table S3 Conditions of nuclei in percent in agt stage conidia 16 hpi in presence of hydroxyurea (HU) and benomyl on barley epidermal strips. (Data are given in %)

Developmental stage	Position of nuclei	Inhibitor	
		HU	benomyl
agt		100	100
		n/a	5
		n/a	95

n/a not applicable, n/a not applicable; agt: appressorial germ tube

Table S 4 Development of *Blumeria graminis* f.sp. *hordei* conidia on Formvar®-coated glass slides supplemented with wax constituents at 16 hpi.

Substratum	Developmental stage				
	ng	pgt	sgt	agt	app
	Percentage of conidia (± SD)				
Barley leaf wax	9 (± 4)	4 (± 1)	6 (± 2)	52 (± 5)	29 (± 4)
2% (n/n) *n*-hexacosanal	5 (± 2)	4 (± 1)	4 (± 1)	50 (± 6)	37 (± 6)
N-hexacosane	66 (± 3)	21 (± 4)	6 (± 3)	4 (± 1)	3 (± 1)

ng: non germinated; pgt: primary germ tube only; sgt: non-swollen secondary germ tube only; agt: appressorial germ tube; app: fully differentiated appressorium with a septum

Table S5 Conditions of nuclei in conidia of the corresponding developmental stages on Formvar®-coated glass slides supplemented with barley leaf wax and wax constituents at 16 hpi. (Data are given in %).

Substratum	Developmental stage									
	ng		pgt		sgt		agt		app	
	Position of nuclei									
Barley leaf wax extract	90	10	53	47	52	48	5	7	88	100
2% (n/n) *n*-hexacosanal	87	13	50	50	57	43	8	5	87	100
N-hexacosane	96	4	11	89	70	30	27	73	0	100

ng: non germinated; pgt: primary germ tube only; sgt: non-swollen secondary germ tube only; agt: appressorial germ tube; app: fully differentiated appressorium with a septum

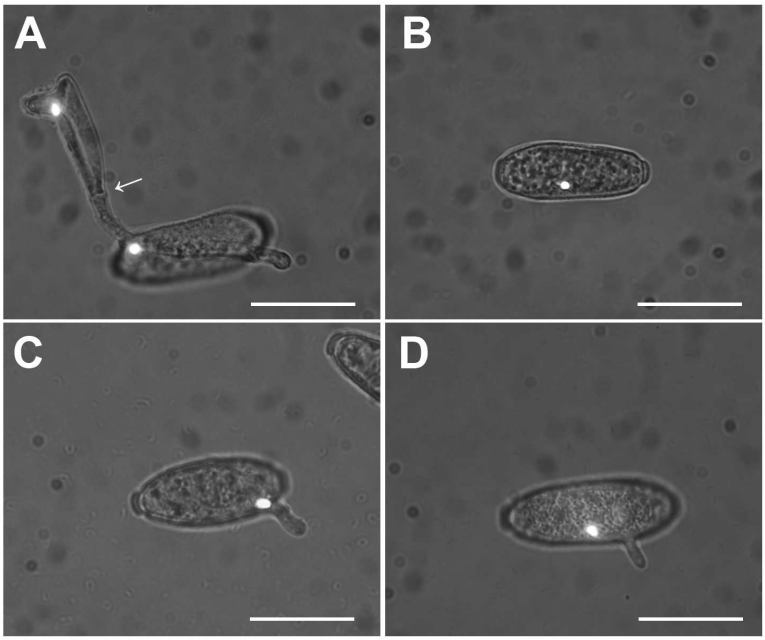


Figure S1 Effects of different substrata on morphogenesis and nuclear division of *Blumeria graminis* f.sp. *hordei* conidia stained with DAPI in vitro. Glass slides were coated with Formvar® resin supplemented with inducing and non-inducing cuticular wax constituents. (a) Fully differentiated conidium on 2% (n/n) *n*-hexacosanal and (b) non-germinated conidium on *n*-hexacosane at 16 hpi; conidia with primary germ tubes at 2hpi on (c) 2% (n/n) n-hexacosanal and (d) on n-hexacosane. Arrow points to the septum between appressorium cell and conidium. Scale bar represents 20 µm.

5 Surface dependent gene expression of barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* during the prepenetration processes

5.1 Introduction

Blumeria graminis f.sp. *hordei* is an obligate biotrophic plant pathogen that causes powdery mildew disease on barley and requires a living host to complete its life cycle (Bélanger, 2002). After landing on the host plant the asexual conidia follow a highly ordered synchronous morphogenetic development (Edwards, 2002; Green *et al*., 2002). Immediately after contact with the plant surface a primary germ tube is formed that attaches to the surface and penetrates only the cuticle (Yamaoka *et al*., 2006). Later a secondary germ tube emerges, which elongates and forms a swollen and hooked appressorium. Underneath the appressorium, a penetration peg is formed that breaks the cuticle and the plant cell wall by enzymatic activities in combination with high turgor pressure (Francis *et al*., 1996; Pryce-Jones *et al*., 1999).

Host cuticular waxes have the capabilities to induce germination and differentiation of *B. graminis* conidia (Tsuba *et al*., 2002; Zabka *et al*., 2008; Ringelmann *et al*., 2009). *In vitro* very-long-chain aldehydes with chain lengths between C_{22} to C_{30} were able to promote conidia germination and differentiation in a dose and chain-length dependent manner (Chapter 2). *In vivo*, the absence of very-long-chain aldehydes in the cuticular wax strongly impedes germination and differentiation of *B. graminis* conidia (Chapter 3). Additionally, cutin monomers and cellulose films stimulated the differentiation process in a host free artificial system (Kobayashi *et al*., 1991; Francis *et al*., 1996). The initiation of the morphogenetic development is a highly ordered sequence and a crucial step in the fate of the conidium, which is accompanied by changes in cellular metabolism (Both *et al*., 2005ab). Early germination and differentiation of *B. graminis* conidia are regulated by cAMP dependent protein kinase A (PKA) signalling pathway, which responds to plant derived surface properties (Hall *et al.*, 1999; Hall & Gurr, 2000; Kinane *et al*., 2000). Several studies with *B.graminis* on the transcriptional level were performed in order to trace metabolic changes during the life cycle and the expression of virulence determinants using SAGE (serial analysis of gene expression) or cDNA microarrays. (Thomas *et al*., 2001; Thomas *et al*., 2002; Grell *et al*., 2003; Both *et al*, 2005ab). These experimental approaches are suitable for studying global gene

expression profiles and do not necessarily consider low expressed genes. *B. graminis* was sampled at different pre- and postpenetrative developmental stages by lifting the fungal conidia or hyphae with cellulose acetate from native leaf surfaces (Zhang *et al*., 2005). So far, no studies were performed that considered changes in *B. graminis* gene expression according to an important signal molecule of cuticular wax, such as *n*-hexacosanal (Chapter 2, 3; Tsuba *et al*., 2002; Zabka *et al*., 2008; Ringelmann *et al*., 2009).

In order to identify transcripts that are upregulated in response to contact with inducing cuticular wax constituents, a strategy based on suppression subtractive hybridization of cDNA (SSH) was chosen (Diatchenko *et al*., 1996). The SSH approach is a powerful tool to compare two populations of mRNA, whereas low and high abundant differentially expressed transcripts are efficiently enriched and selectively amplified. The Formvar® resin based *in vitro* system provides a stable experimental platform for generation of two RNA populations of *B. graminis* conidia that were subtracted from each other. The RNA was derived from conidia inoculated on a surface coated with the inductive very-long-chain aldehyde *n*-hexacosanal and the non-inductive alkane *n*-hexacosane, respectively. The analysis of differentially expressed sequences might identify putative genes, which are induced in response to *n*-hexacosanal and may have a critical role in the differentiation of *B. graminis* conidia. Otherwise, the SSH-based experimental approach helps to identify genes, which are involved in perception of plant surface properties or those which are involved in intracellular signal transduction pathways.

Among several candidate clones an upregulated transcript was identified and its expression was confirmed by quantitative real time PCR (RT-qPCR). This putative novel *n*-hexacosanal responsive gene was cloned by 3’ and 5’ RACE and showed no homologies to genes of known function in fungal development and pathogenicity. However, a multitude of related transcripts were identified in EST databases that are expressed during all developmental stages. Searching the genome of *B. graminis* f.sp. *hordei* DH14 revealed a large set of homologous sequences related to the aldehyde responsive transcript. These findings probably suggest a unique role of a larger set of genes, which are expressed during the pathogenic development of *B. graminis*.

5.2 Material and Methods

Plant and pathogen material

Hordeum vulgare L. cv. Bonus (obtained from the Nordic Gene Bank, Alnarp, Sweden) was sown in plastic pots (9 cm diameter) filled with standard potting soil (Typ ED73; SteuderComp, Schermbeck). The seedlings were kept in growth

chambers with 300 µmol photons m^{-2} s^{-1} light intensity in a 16 h/8 h light/dark period at 22°C/18°C and 70% relative humidity.

Blumeria graminis (DC.) E.O. Speer f. sp. *hordei* Em. Marchal (isolate, CC1 originally obtained from Tim Carver, Institute of Grassland and Environmental Research, Aberystwyth, UK) was propagated on its host barley under the same conditions as described above until white powdery pustules developed. One day before conidia were required for experiments leaves were shaken to remove older conidia so that freshly emerged conidia were available for the experiments.

Coating of glass slides

Histobond glass slides (Marienfeld, Lauda-Königshofen) were cleaned with Deconex® 11 UNIVERSAL detergent (Applichem, Darmstadt), rinsed with deionized water, immersed in isopropanol and were finally rinsed again in water. The air dried slides were coated using a 0.5% Formvar® resin solution (Polyvinyl formal, CAS 63450-15-7, Applichem) in chloroform supplemented with 6.8×10^{-4} mol l^{-1} *n*-hexacosane or in combination with 5% (n/n) of *n*-hexacosanal and were dried over night. The Formvar® resin based *in vitro* system provides highly homogenous hydrophobic surfaces with uniform contact angles. The *n*-hexacosane coated surfaces were chosen, since it does not exhibit an inductive effect on the differentiation processes of *Blumeria graminis*, whereas the *n*-hexacosanal coated surface significantly triggers the prepenetration processes of *B. graminis* (Chapter 2).

Kinetics of fungal prepenetration processes

In order to study the kinetics of conidia germination and differentiation, glass slides covered with Formvar®/*n*-hexacosane or *n*-hexacosanal were fixed at the base of a settling tower. Conidia from infected barley leaves were blown into the tower by pressurized air to ensure an even distribution at a density of approximately 20 conidia mm^{-2}. After seven minutes, one glass slide was transferred into a plastic Petri dish with a moist filter paper for each sampling time point and was sealed with Parafilm“M”® to maintain a relative humidity of at least 90%. The slides were incubated at 20°C in darkness. Of each time point, the developmental stages of 100 conidia were observed by light microscopy (Leica DMR, Wetzlar). It was determined whether the conidia had not germinated (ng), had formed only primary germ tube (pgt), a secondary non-swollen germ tube (agt) or a fully differentiated hooked appressorium with a septum (app). Only well separated conidia were evaluated to exclude possible inhibitory effects due to crowding. The experiments were repeated three times independently.

RNA isolation

Adaxial sides of detached leaves of barley (*H. vulgare* cv. Bonus) or glass slides coated with Formvar®/*n*-hexacosane or *n*-hexacosanal were inoculated in a settling tower with pressurized air at a density of 50 conidia mm^{-2} to provide enough biological material for the subsequent nucleic acid isolation. After allowing the conidia to rest for seven minutes, the glass slides or leaves were incubated for 15 minutes in a sealed glass Petri dish on moist filter paper in darkness at 20°C. After incubation the inoculated surfaces were painted with 5% (w/v) cellulose acetate (Roth, Karlsruhe) in acetone. The acetone treatment leads to a fast desiccation of the conidia and conservation of the nucleic acids (Both *et al.*, 2005). After complete evaporation of the solvent the cellulose acetate foils were stripped carefully with forceps and were frozen in liquid nitrogen. For each extraction 24 cellulose acetate strips were pooled from the same surfaces and stored at -80°C until used.

The frozen cellulose acetate strips were carefully ground in liquid nitrogen with sea sand (Merck, Darmstadt). After adding 10 ml freshly prepared nucleic acid extraction buffer (50 mM Tris/Cl, pH 9; 150 mM NaCl; 5 mM EDTA pH 9; 5% SDS) the grinding was continued until the buffer was completely thawed. The suspension was transferred to reaction tubes and centrifuged twice for 5 min at full speed (5417C, Eppendorf, Hamburg) at room temperature to clear the suspension of debris. The supernatant was transferred to fresh reaction tubes and an equivalent volume of phenol/chloroform/isoamyl alcohol (Roti PCI, 24:24:1, Roth, Karlsruhe) was added. The tubes were carefully inverted for 2 min and the phases were separated by centrifugation at full speed at 4°C for 15 min (5415R, Eppendorf, Hamburg). The upper phase was recovered, an equivalent volume of chloroform isoamyl alcohol (24:1, v/v) was added and the tubes were inverted for 2 min. The phases were again separated by centrifugation and the nucleic acids of the supernatant were precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol (Applichem, Darmstadt) at -20°C over night. The precipitate was recovered by centrifugation at full speed (at 4°C) and the pellet was washed twice with 80% ethanol. The pellets were dried in the SpeedVac® (Thermo Fisher Scientific, Schwerte) for 10 min at 35 mbar and resuspended in 10 µl diethyl pyrocarbonate (DEPC) treated double deionized water (ddH_2O). The resuspended pellets were pooled and the genomic DNA was digested with DNAseI according to the manufacturers protocol in the reaction tube (Qiagen, Hilden).

The total RNA used for the suppression subtractive hybridization was purified after the DNAseI digestion with RNeasy® Plant Mini Kit (Qiagen, Hilden) according to the manufacturers protocol and was eluted in 15 µl of RNAse free ddH_2O. The RNA used for quantitative reverse transcription polymerase chain reaction (RT-qPCR) was purified with PCI and chloroform/isoamylalcohol and was

recovered after the DNAseI digestion with 3 M sodium acetate and ethanol as described above. The pellets were resuspended in 12 µl DEPC treated ddH_2O.

RNA quality control

The concentrations of total RNA in the preparations were determined with a Nanodrop spectral photometer (Peqlab, Erlangen). The integrity of RNA used for SSH library construction was confirmed with the ExperionTM system (BioRad, München) according to the manufacturers protocol (Figure 1).

The integrity of total RNA used as template for the quantitative reverse transcription polymerase chain reaction (RT-qPCR) was confirmed by denaturing agarose gel electrophoresis. For one gel of 100 ml 1.7% (w/v) of agarose (Peq Gold Universal agarose; Peqlab, Erlangen) was boiled in deionized water and diluted with 10 x MOPS buffer (0.4 M 3-[N-morpholino] propanesulfonic acid; 0.1 M sodium acetate; 10 mM EDTA, pH 7.0) and 7% (v/v) formaldehyde (Applichem, Darmstadt). One µl total RNA was diluted with 5 µl DEPC treated ddH_2O and 5 µl RNA loading buffer (Crystal 2x RNA loading buffer with ethidium bromide; Bioline, Luckenwalde). Samples were denatured in a thermoblock for 5 min at 65°C and chilled on ice for 5 min. The electrophoresis was performed in 1 x MOPS buffer at 120 volts for 30 min. The gel was visualized and documented in a GelDoc EQ station in combination with Quantity One 1-D analysis software (Biorad, München).

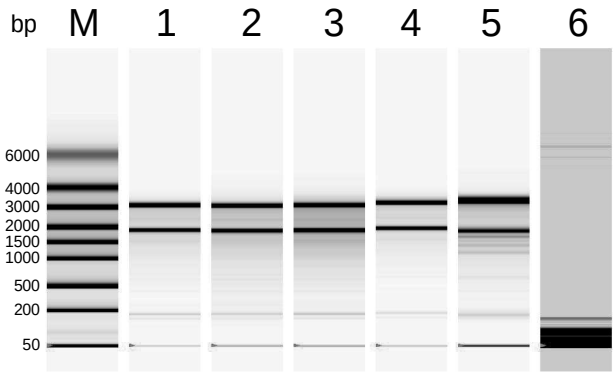


Figure 1 Experion^{TM} system simulated picture of gel electrophoresis of *B. graminis* total RNA used for suppression subtractive hybridization. Single RNA preparations were pooled and 500 pg of RNA was loaded. Lane (M) Experion^{TM} RNA ladder (Biroad, München), lane (1) and (3) are RNA preparations from conidia incubated on *n*-hexacosanal coated surfaces (548 ng μl^{-1} and 262 ng μl^{-1}); lane (2) and (4) are RNA preparations from conidia incubated on *n*-hexacosane coated surfaces (250 ng μl^{-1} and 263 ng μl^{-1}). In lane (5) a positive control was loaded (RNA preparation of Arabidopsis leaves; M. Baumann), lane (6) water control.

The mechanism of suppression subtractive hybridization (SSH)

The first step in the SSH procedure is a full length cDNA synthesis of both RNA populations and, subsequently, an endonucleolytic digestion with *Rsa*I. The tester cDNA (*n*-hexacosanal) is divided in two portions and each is ligated with two

different cDNA adaptors, which only attaches to the 5’ end of the cDNA, since the ends of the adaptors are dephosphorylated. During the first hybridization the driver cDNA (*n*-hexacosane) is added in excess to each adaptor-ligated tester cDNA. The DNA strands are denatured by heat and the formation of hybrid molecules is possible, when a specific sequence was expressed in the tester and the driver cDNA population (Figure 2, molecule type c). Hence, these DNA molecules are not available any more for the second hybridization step when both tester portions are pooled, whereas the remaining single stranded cDNA sequences with different adaptors can form hybrids (Figure 2, molecule type e). Single stranded molecules and homodimers with the same adaptors (Figure 2, molecule types a, b, and d) are not any more available for hybridization. For further enrichment of tester hybrids with different adaptors, freshly denatured driver is added, without prior denaturing the previously formed hybrids. After filling in the ends of the adaptors by DNA polymerase, the differentially expressed tester hybrids (Figure 2, molecule type e) have different primer binding sites on their 5’ and 3’ ends, allowing only an exponential amplification of the hybrid molecules during the suppression PCR step. For further amplification of hybrid molecules and reduction of background a second nested PCR step is performed. For the reverse subtraction, the *n*-hexacosane cDNA is used as tester and the *n*-hexacosanal cDNA as driver, which enriches sequences that were differentially expressed in conidia inoculated on the *n*-hexacosane surface.

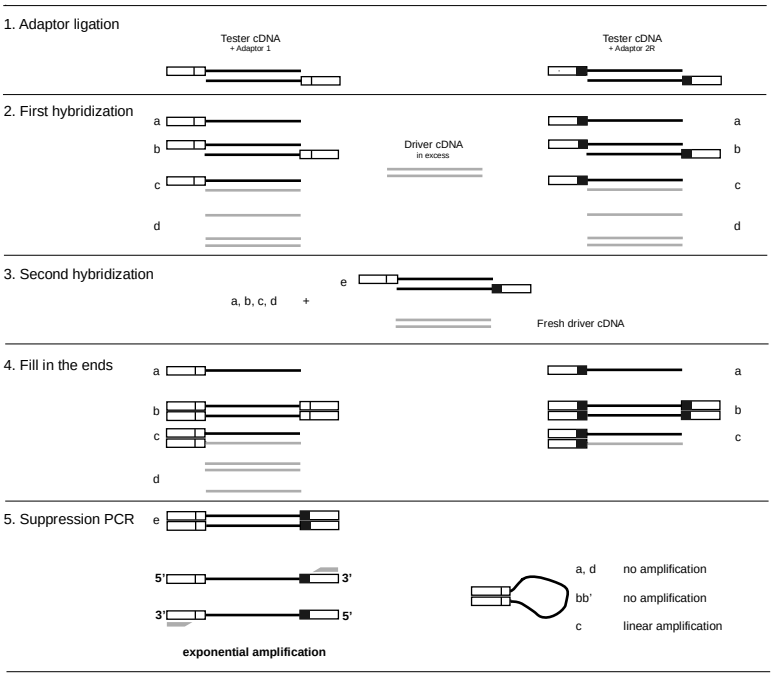


Figure 2 Mechanism of the suppression subtractive hybridization. For forward subtraction tester cDNA is derived from the inductive *n*-hexacosanal and driver cDNA from the non-inductive *n*-hexacosane coated surface. For details see text.

Synthesis and amplification of cDNA

For first strand synthesis, the SMART™ PCR cDNA synthesis kit (Clontech, Saint-Germain-en-Laye, France) was used according to the manufacturers protocol, which generates a double stranded full length cDNA out of total RNA due to the **s**witching **m**echanism **a**t 5' end of **R**NA **t**emplate (SMART). The Moloney Murine leukemia virus reverse transcriptase (MMLV-RT) used for reverse transcription provides a terminal transferase activity, which incorporates a stretch of dCTPs at the 5' end of the newly synthesized cDNA strand. These dCTPs overhangs form hybrids with the SMART™ oligonucleotide, which serves as primer for the second strand synthesis.

For SMART™-cDNA synthesis 1 µg total RNA was adjusted to a total volume of 3 µl with DEPC ddH_2O. Each 5 µl reaction contained 1 µl of 3'SMART ORF Primer II A (10 µM), 1 µl of SMART II A oligonucleotide (10 µM) and was incubated at 72°C for 2 min (Mastercycler gradient, Eppendorf, Hamburg). After the reaction was chilled for 2 min in an ice bath, 2 µl of 5 x first strand buffer, 1 µl DTT (20 mM), 1 µl 50 x dNTP mix (10 mM) and 1 µl PowerScript™ reverse transcriptase (TaKaRa Bio, Otsu, Shiga, Japan) were added. The samples were incubated at 42°C for 1 h, and filled up to a final volume of 50 µl with TE-buffer (10 mM Tris/Cl; 1 mM EDTA, pH 7). The reaction was terminated at 72°C for 7 min and the SMART™ cDNA was stored at -20°C. Prior to amplification of the SMART™-cDNA by long distance PCR (LD-PCR), the appropriate number of cycles for the amplification step was determined. The SMART™ cDNA was diluted 1:10 with ddH_2O and 3 µl were added to 30 µl of a PCR mastermix [10 x Advantage® 2 PCR buffer; 2 µl 50 x dNTP mix (10 mM); 2 µl 5' PCR-primer II A (12 µM); 2 µl 50 x Advantage® 2 Polymerase mix (Clontech Saint-Germain-en-Laye, France)]. The reaction was incubated at 95°C for 1 min as initial denaturation, 15-21 cycles at 95°C for 1 min, 65°C for 30 s and an elongation at 68°C for 6 min. The samples were stored on ice and 10 µl of each reaction was separated on a 1% agarose gel (Figure 3). According to the gel electrophoresis, 19 cycles were determined as optimum, since the reactions were still in the exponential amplification phase, whereas with 21 cycles the plateau phase of the PCR reaction was already reached. For each cDNA population (*n*-hexacosanal and *n*-hexacosane) six reactions, each with a total volume of 100 µl, were amplified with the cycle sequence described above.

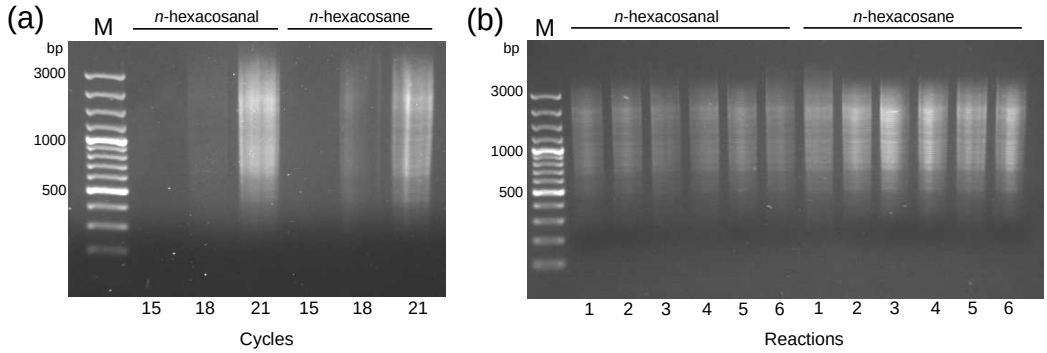


Figure 3 Long-distance PCR for amplification of SMART^{TM}-cDNA. Test PCR for determination of optimum cycle number (a) and PCR for amplification of SMART^{TM}-cDNA with the optimum 19 cycles (b).

*Rsa*I digestion and adaptor ligation

The LD-PCR products were pooled and purified with the QIAquick PCR-Purification Kit (Qiagen, Hilden) according to the manufacturers protocol. The purified PCR-products were digested with *Rsa*I (25 U, Clontech, Saint-Germain-en-Laye, France), 43.5 µl LD-PCR product, 5 µl 10 x *Rsa*I restriction buffer at 37°C for 1.5 h. After digestion the reactions were pooled to one fraction for each surface. For control purposes 5 µl from each reaction step were separated on a 1.5% agarose gel (Figure 4).

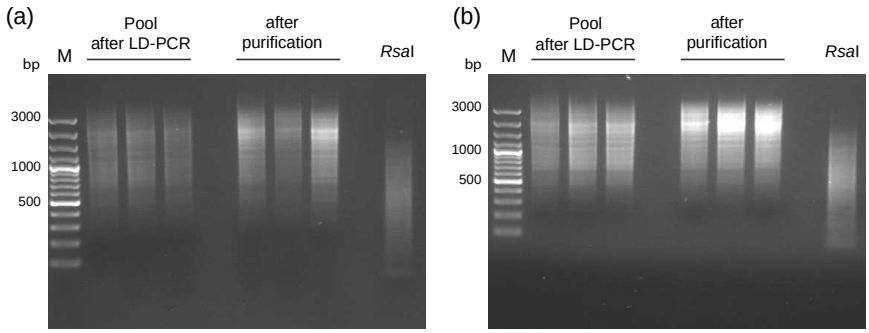


Figure 4 Controls of LD-PCR purification and of *Rsa*I endonuclease digestion of cDNA from conidia incubated on Formvar®/*n*-hexacosanal (a) or *n*-hexacosane (b) coated glass slides. LD-PCR products were pooled and purified with QIAquick PCR purification kit. The three purified fractions were again pooled and digested with *Rsa*I.

The remaining 145 µl of the *Rsa*I digest were inverted in an equal volume of phenol/chloroform/isoamyl alcohol (Roti PCI, 24:24:1, Roth, Karlsruhe) for 2 min. After phase separation at full speed for 15 min at 4°C (5415R, Eppendorf, Hamburg), the upper phase was purified with an equal volume of chloroform/isoamylalcohol (24:1 v/v). The aqueous phase was precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol at -20°C overnight. The precipitate was recovered by centrifugation at full speed and 4°C for 20 min (5415R,

Eppendorf, Hamburg), washed twice with 80% ethanol and dried in the vacuum centrifuge (10 min, 35 mbar). Finally, the pellet was resuspended in 6 µl ddH_2O.

The concentration of the *Rsa*I digested double stranded cDNA was adjusted to 300 ng μl^{-1} with ddH_2O. For forward and reverse subtraction the following tester adapter ligation reactions were set up, whereas the nomenclature of the PCR-select™ cDNA subtraction kit user manual (Clontech; Saint-Germain-en-Laye, France) was retained.

Forward subtraction	
Tester 1-1	Tester 1-2
2 µl *Rsa*I digested cDNA (*n*-hexacosanal)	2 µl cDNA *Rsa*I digested (*n*-hexacosanal)
2 µl Adaptor 1	2 µl Adaptor 2R
6 µl Mastermix	6 µl Mastermix

Reverse subtraction	
Tester 2-1	Tester 2-2
2 µl *Rsa*I digested cDNA (*n*-hexacosane)	2 µl *Rsa*I digested cDNA (*n*-hexacosane)
2 µl Adaptor 1	2 µl Adaptor 2R
6 µl Mastermix	6 µl Mastermix

The Mastermix contained for five reactions 15 µl ddH_2O, 10 µl 5 × ligation buffer, and 5 µl T4 DNA-Ligase. The reactions were incubated at 16°C in a thermocycler (Mastercycler gradient; Eppendorf, Hamburg) over night. The enzyme was heat inactivated at 72°C for 5 min and the addition of 1 µl of 20 × EDTA/Glycogen-Mix (Clontech; Saint-Germain-en-Laye, France).

Hybridizations and amplification of subtracted cDNA

In the following two hybridization steps, the driver cDNA was added in excess to each adapter ligated tester cDNA.

(a) *First hybridization*

For the first hybridizations the following reactions were set up:

Hybridization	H1	H2
*Rsa*I-digested driver cDNA (*n*-hexacosane)	1.5 µl	1.5 µl
Adaptor 1-ligated tester 1-1	1.5 µl	-
Adaptor 2-ligated tester 1-2	-	1.5 µl
4 × Hybridization buffer	1.0 µl	1.0 µl

Hybridization	H3	H4
*Rsa*I-digested driver cDNA (*n*-hexacosanal)	1.5 µl	1.5 µl
Adaptor 1-ligated tester 2-1	1.5 µl	-
Adaptor 2-ligated tester 2-2	-	1.5 µl
4 × Hybridization buffer	1.0 µl	1.0 µl

The reactions were denatured at 98°C in a thermocycler (Mastercycler gradient; Eppendorf, Hamburg) for 1.5 min and incubated for 8 h at 68°C.

(b) *Second hybridization*

For the second hybridization the samples of the first hybridization reactions were mixed (H1+H2 and H3+H4) and freshly denatured driver cDNA was added to enrich differentially expressed sequences. One µl driver cDNA and 1 µl of 4 × Hybridization buffer were filled up to a final volume of 4 µl with ddH_2O. One µl of this mixture was covered with mineral oil and denatured at 98°C for 1.5 min. The products of the first hybridizations (H2 or H4) were drawn up into a 10 µl pipette tip. Then a small amount of air and freshly denatured driver was drawn up. The air bubble in the tip prevents mixing of tester and driver before the next step. Then the content of the pipette tip was transferred to the first hybridization sample. The reaction was incubated at 68°C over night and was terminated by adding 200 µl dilution buffer.

(c) *Suppression PCR*

To selectively enrich differentially expressed cDNA sequences, two consecutive PCR reactions were necessary. For the suppression PCR 2.5 µl of 10 × Advantage® 2 PCR-buffer, 0.5 µl dNTPs (10 mM), 1 µl PCR-primer 1 (10 µM), 0.5 µl 50 × Advantage® 2 polymerase mix (Clontech; Saint-Germain-en-Laye, France) were at first incubated in a final volume of 24 µl for 5 min at 75°C in a thermocycler to fill up the adaptors. Subsequently the suppression PCR was continued immediately (initial denaturation at 94°C for 30 s; 27 cycles 66°C for 30s, 72°C for 1.5 min).

The products of the first PCR were diluted 1:10 with ddH_2O and the nested-PCR for amplification of the differentially expressed sequences was performed. For the nested PCR 1 µl of diluted suppression PCR product, 2.5 µl 10 x Advantage® 2 PCR buffer, 0.5 µl dNTPs (10 mM), 1 µl nested PCR primer 1 (10 µM), 1 µl nested PCR primer 2R, 50 x Advantage® 2 polymerase mix (Clontech; Saint-Germain-en-Laye, France) were filled up to 24 µl with ddH_2O. The suppression PCR included an initial denaturation at 94°C for 30 s followed by 10 cycles at 68°C for 30 s and 72°C for 1.5 min. The final products of the suppression and the nested PCR show an enrichment of DNA sequences in the range between 500 and 1000 bp (H1 and H3) compared to the unsubtracted controls (Figure 5). Additionally the control suppression and nested PCR reaction of the PCR-Select™ cDNA subtraction kit was treated analogously.

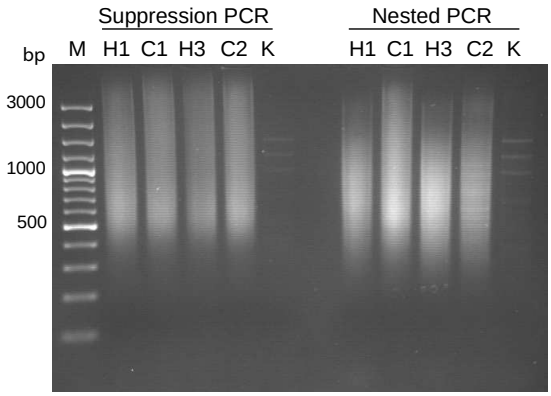


Figure 5 Control of first PCR (suppression PCR) and nested PCR after hybridization steps. Forward subtraction (H1), reverse subtraction (H3), unsubtracted forward control (C1), unsubtracted reverse control (C2) and control subtraction of SSH kit (Clontech) (K).

Establishment of cDNA libraries

The nested PCR products were ligated to pGEM®-T Easy (Promega, Madison, WI, USA). The ligation reactions (5 µl 2 × rapid ligation buffer, 1 µl PCR product, 1 µl T4 DNA ligase, 2 µl ddH_2O) were incubated for 16 h at 4°C. *Escherichia coli* JM 109 highly competent cells (New England Biolabs, Frankfurt a. M.) were thawed on ice for 10 min and 2 µl of the ligation reaction was added and incubated for 30 min on ice. After a heat shock at 42°C for 45 s the transformation reactions were chilled on ice for 5 min. After addition of 900 µl prewarmed (37°C) SOC medium, the cells were incubated for 1.5 h at 37°C under shaking at 180 rpm. From 1:10 and 1:100 dilutions in SOC medium, 100 µl were streaked onto lysogeny broth Isopropyl β-D-1-thiogalactopyranoside/ampicilline/5-bromo-4-chloro-indolyl-β-D-galactopyranoside (LB-IPTG/ampicilline/X-Gal) plates. The plates were incubated over night at 37°C. Single colonies were stored in Hogness–Freezing (100 µg ml^{-1} ampicilline) medium at -80°C.

SOC medium (for 100 ml): 2 g Tryptone, 0.5 g Yeast extract, 1 ml 1 M NaCl, 0.25 ml 1M KCl.
Final volume 97 ml with deionized H_2O. After autoclaving (121°C, 20 min) 1 ml of sterile filtered 2 M Mg^{2+}-solution ($MgCl_2 \cdot 6\ H_20$ and $MgSO_4 \cdot 7\ H_20$) and 1 ml sterile filtered 2 M Glucose were added and filled up to 100 ml with sterile deionized H_2O.

LB-IPTG/ampiclilline/XGal plates (for 1 l): 10 g Tryptone, 5 g Yeast extract 5 g NaCl, 15 g Agar
After autoclaving (121°C, 20 min) and cooling to approximately 50°C, 100 µg ml^{-1} ampicilline, 0.3 mM IPTG and 80 µg ml^{-1} X-Gal were added.

10 × Hogness-Freezing Medium (HFM) (for 100 ml): *HFM-solution 1* (80 ml): 62.2 g 87 % (v/v) glycerine, 0.5 g sodium citrate dihydrat, 0.9 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7\ H_2O$. *HFM-solution 2* (20 ml): 6.3 g $K_2HPO_4 \cdot 3\ H_2O$, 1.8 g KH_2PO_4. Final volume 20 ml with deionized H_2O. After autoclaving (121°C, 20 min) the HFM-solutions 1 and 2 were pooled. For 1 × HFM the 10 × HFM solution was diluted with LB-liquid broth.

LB liquid broth (for 1 l): 10 g Tryptone, 0.5 g Yeast extract, 5 g NaCl. After autoclaving (121°C, 20 min) and cooling to approximately 50°C, 100 µg ml^{-1} ampicilline were added.

Isolation of plasmids and sequencing

Liquid cultures of *E. coli* were incubated in LB liquid medium (100 µg ml^{-1} ampicilline) at 37°C and 200 rpm for 16 h. The plasmids were isolated with peqGold-Plasmid-Kit (Peqlab, Erlangen) according to the manufacturers protocol. The plasmids were sequenced with the dideoxy nucleotide method according to Sanger (1977) using standard sequencing primer SP6 (5'-ATTTAGGTGACACTATAG-3') or T7 (5'-TAATACGACTCACTATAGGG-3') (initial denaturation at 95°C for 5 min, 34 cycles of denaturation at 95°C for 1 min, primer annealing at 54°C for 30 s following elongation at 72°C for 1.5 min) using an ABI Prism® sequencer.

Agarose gel electrophoresis

Agarose (Peq Gold Universal agarose; Peqlab, Erlangen) gels were prepared at 1% - 2% (w/v) concentrations with 0.1 µg ml^{-1} of ethidumbromide (Applichem, Darmstadt) according to the DNA fragment lengths to be separated in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA-Na_2, pH 8). The samples were spiked with 6 x DNA loading buffer (40% w/v sucrose, 0.25% w/v bromphenol blue). For fragment size estimation 5 µg of a standard DNA ladder was loaded (100 bp plus or 1 kb ladder [Genecraft, Köln], 50 bp O'GeneRuler™ [Fermentas, St. Leon-Rot]). The electrophoresis was performed at 90V. Visualisation and documentation were done with a GelDoc EQ station in combination with Quantity One 1-D analysis software (BioRad, München)

Reverse Northern analysis with digoxigenine labelled SMART™-cDNA

For screening of differentially expressed sequences, a filter hybridization assay with digoxigenine labelled total cDNA, derived from conidia incubated for 22 min on *n*-hexacosanal or *n*-hexacosane coated glass slides was performed.

(a) *Synthesis of cDNA probes*

For the labelling reaction 3 µl of the unsubtracted SMART™-cDNA, 5 µl 10 x Biotherm PCR buffer (Genecraft, Köln), 2.5 µl 10 x DIG probe synthesis mix, 2.5 µl 10 x dNTP mix (Roche, Mannheim), PCR primer II A (Clontech; Saint-Germain-en-Laye, France) 1 µl BioThermBio™ Taq DNA Polymerase and 35 µl ddH_2O were incubated in a thermocycler (Mastercycler gradient, Eppendorf) for 2 min at 95°C followed by 21 cycles for 30s at 95°C, 20s at 65°C and 6 min at 72°C.

To equilibrate the *n*-hexacosanal and *n*-hexacosane probes, 5 µl were separated on a 1.2% (w/v) agarose gel and the relative quantities were determined with the Quantity one software suite (BioRad, München). The labelling efficiency was determined according to the *DIG Application Manual for Filter Hybridization* (2009) Chapter 3, pp. 23-25.

(b) *Immobilization of nucleic acids*

The PCR products were transferred with a vacuum dot-blot apparatus onto a positively charged nylon membrane (Roche, Mannheim). First the membrane was equilibrated in ddH_2O for 1 min. Then the membrane and the blotting paper (GB 002, Schleicher und Schuell, Dassel) were incubated in 20 x SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0) for 15 min. Each well of the blotting apparatus was rinsed twice with 20 x SSC. Four µl of each PCR product were diluted with 96 µl ddH2O and denatured for 10 min at 95°C. The samples were chilled on ice and spotted under vacuum in duplicates onto the nylon membrane. The nucleic acids were cross-linked with 120 mJ UV-light (Vilber Lourmat, BLX 254; La Vallee Cedeux, France).

(c) *Hybridization*

The membranes were sealed in hybridization bags (Roche, Mannheim) and incubated with 4 ml of Dig Easy Hyb-Buffer (Roche, Mannheim) at 40°C for 2 h in a water bath. Four µl of the *n*-hexacosanal probe and 2.8 µl of the *n*-hexacosane probe were denatured in a total volume of 50 µl in ddH_2O at 95° for 10 min and chilled immediately in an ice bath for 5 min. The denatured probes were diluted with 1 ml of preheated (40°C) Dig Easy Hyb-Buffer and transferred to the bags after discarding the prehybridization buffer. The hybridization was carried out over night at 40°C. The membranes were washed twice for 10 min in 2 x wash solution (2 × SSC, 0.1% SDS) and twice in 0.5 x wash solution at 68°C for 15 min. Prior to signal detection the washed membranes were equibrilated in detection buffer (0.1 M Tris-HCl; 0.1 M NaCl, pH 9.5)

(d) *Detection*

The hybridization signals were detected with a chemiluminescence assay (Roche, Mannheim) according to the manufacturers protocol (*Dig Application Manual for Filter Hybridization* (2009), Chapter 4 pp. 115-127; Roche, Mannheim). A complex of anti-digoxigenine antibodies and an alkaline phosphatase (anti-digoxigenine-AP; Roche, Mannheim) bind to DIG labelled hybridization probes. When the substrate Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate (CSPD) (Roche, Mannheim) was added (diluted 1:100 in detection buffer) a chemiluminescence signal is detectable on a chemiluminescence film (Roche, Mannheim). The films were developed for 1 min (developer diluted 1:5 in ddH_2O, Kodak, Stuttgart), rinsed in ddH_2O and were finally fixed for 1 min in a fixing bath (fixer diluted 1:5 in ddH_2O, Kodak, Stuttgart). According to the density of the chemiluminescence signal, the exposure time was adjusted in order to avoid saturated signal intensities and background signal.

Oligonucleotides

Gene specific primers were designed using Primer3 (version 0.4.0) (Rozen & Skaletsky, 2000). Primers used for quantitative reverse transcription PCR were designed according to Udvardi *et al.* (2008) using the following design criteria: T_m 60 ± 1°C, 40-60% GC content, length 18-25 bp and product length 60-150 bp.

Polymerase chain reaction (PCR)

The inserts of the plasmids were amplified by PCR prior to immobilization on the nylon membrane. A 20 µl reaction contained 5 ng of plasmid, 2.5 µl 10 x reaction buffer, 0.5 µl Nested primer 1, 0.5 µl Nested primer 2R; (Clontech, Saint-Germain-en-Laye, France) 0.15 µl BioTherm™ Taq DNA polymerase (Genecraft, Köln). The thermocycler (Mastercycler gradient, Eppendorf, Hamburg) was programmed for 2 min at 95°C followed by 36 cycles for 15 s at 95°C, 30 s at 66°C, 5 min at 72°C and 72°C at 5 min.

Quantitative reverse transcription PCR (RT-qPCR)

The expression patterns of differentially expressed sequences identified by reverse Northern blot analysis were confirmed by RT-qPCR. Total RNA was extracted from *B. graminis* conidia inoculated on four different surfaces as described in the section "RNA isolation" 22 min after inoculation: glass slides covered with Formvar®/*n*-hexacosane, Formvar®/*n*-hexacosanal, Formvar®/barley leaf wax and from barley leaves. For first strand synthesis of cDNA, 5 µg of total RNA in solution were mixed with 1 µl oligo$(dT)_{20}$ primer (50 µM) (Invitrogen, Karlsruhe), 1 µl dNTP mix (10 mM) (Genecraft, Köln) and filled up to 20 µl with DEPC treated ddH_2O. The mixture was incubated at 65°C for 5 minutes in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg) and chilled on ice for 1 min. After adding 4 µl 5 x first-strand buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT™ and 1 µl SuperScript™ III reverse transcriptase (Invitrogen, Karlsruhe), the reaction mixture was incubated at 50°C for 60 min. The enzyme was inactivated at 70°C for 15 min.

For a single RT-qPCR reaction, 10 µl KAPA™ FAST qPCR MasterMix (2x) Universal (Peqlab, Erlangen), 200 nM to 500 nM forward and reverse gene specific primer and 19 ng cDNA template were filled up to 20 µl with ddH_2O. The reaction was incubated in a real-time thermocycler (C1000™ with CFX96™ Real-Time System, BioRad, München) with the following program: 3 min at 95°C; 40 cycles: 10 s at 95°C, 30 s at 60°C.

For determination of the primer efficiency 1 µg of cDNA derived from conidia inoculated on all tested surfaces were pooled. A RT-qPCR reaction was performed with different amounts of template cDNA (20 ng, 10 ng, 2 ng and 0.2 ng) in a 20 µl reaction with different primer concentrations (150 nM – 500 nM). Additionally a no-template control reaction was performed. A standard curve was

generated with BioRad™ CFX Manager Software and the efficiencies were calculated. According to Bustin *et al.* (2009) reactions were optimized for efficiencies between 90-110% and R^2>0.980. Additionally, a melt curve analysis ranging from 60°C to 95°C (increment 0.5°C) and a gelelectrophoretic analysis (2% agarose gel) of the PCR products were performed, to ensure a single PCR product was amplified.

The RT-qPCR experiments were normalized using the histone H3 transcript as reference gene, since it is constantly expressed during different developmental stages (Both *et al.* 2005a). All RT-qPCR reactions were performed in three technical replicates and were repeated two times biologically to confirm the expression data. The expression levels were calculated according to the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001), whereas the expression levels of the non inductive *n*-hexacosane surface were set to 1. The expression levels of the *n*-hexacosanal surface, barley leaf wax surface and barley leaves were calculated as n-fold induction of the *n*-hexacosane expression levels

5’ and 3’ Rapid amplification of cDNA ends

In order to obtain cDNA from a mRNA template, from which only partially internal sequence information was available, a 5’ and 3’ RACE (5’ and 3’ Rapid amplification of cDNA ends) was performed (5’/3’ RACE Kit 2nd Generation, Roche, Mannheim). The cDNA amplification for 5’ and 3’ RACE was set up with total RNA from conidia incubated on Formvar®/*n*-hexacosanal coated surface for 22 min. For a 20 µl 5’ RACE reaction 4 µl of cDNA synthesis buffer, 2 µl dNTP mix, 1 µl gene specific primer Nes1a (5’-CATCCGTCCAAAATCGAAAT-3’; 12.5 µM), 2 µg total RNA and 1 µl Transcriptor reverse transcriptase were used. The reaction was incubated at 50°C for 60 min and terminated at 80°C for 5 min. The first strand cDNA was purified according to the manufacturers protocol with the High Pure PCR Purification Kit (Roche, Mannheim). For the homopolymeric A-tailing reaction, 19 µl of the purified cDNA sample, 2.5 µl reaction buffer (10 x), 2.5 µl dATP (2 mM) were incubated at 94°C for 3 min. After adding of 1 µl terminal transferase (80 U μl^{-1}) the reaction was incubated at 37°C for 30 min and terminated at 70°C for 10 min.

For the first nested PCR, 5 µl of dA-tailed cDNA, 1 µl Oligo dT-anchor primer, 1.25 µl gene specific primer LIIH1(1)C1_L (5’-CCTGCCCGTCTATCCAAATA-3’; 10 µM), 1 µl dNTP mix, 1 µl Advantage® 2 polymerase mix (Clontech, Saint-Germain-en-Laye, France), 5 µl 10 x Advantage® 2 PCR buffer were filled up to 50 µl with ddH_2O. The thermocycler program (Mastercycler gradient, Eppendorf, Hamburg) started at 94°C for 2 min followed by 10 cycles at 94°C for 15 s, 60°C for 30 s and 68°C for 40 s; 25 cycles: 94°C for 15 s, 60°C for 30 s and 68°C for 6 min; finally 68°C for 7 min. A second nested PCR was set up in a total volume of 50 µl with 1 µl of 1:20 diluted first PCR product, 1 µl PCR anchor primer, 1.25 µl gene specific primer Hexar_nes3

(5'-TTCACATACTGTGGGCGTGTA-3'; 10 µM), 1 µl dNTP mix, 1 µl Advantage® 2 polymerase mix (Clontech) and 5 x Advantage® 2 PCR buffer. A touchdown PCR from 68°C to 60°C annealing temperature was performed with the following cycling conditions: 95°C for 2 min; 5 cycles: 95°C for 30 s and 68°C for 3 min; 5 cycles: 95°C for 30 s, 66°C for 30 s and 68°C for 3 min; 5 cycles: 95°C for 30 s, 64°C for 30 s and 68°C for 3 min; 25 cycles: 95°C for 30 s, 60°C for 30 s, 68°C for 3 min and a final elongation step at 70°C for 10 min.

For the 3' RACE first strand cDNA synthesis 4 µl cDNA synthesis buffer, 2 µl dNTP mix, 1 µl oligo dT-anchor primer, 2 µg total RNA and 1 µl Transcriptor reverse transcriptase (Roche, Mannheim) were filled up to 20 µl with DEPC treated ddH_2O. The reaction was incubated at 55°C for 60 min and terminated at 85°C for 5 min. For PCR amplification 1 µl of first strand cDNA, 1 µl PCR anchor primer, 1.25 µl gene specific primer LIIH1(1)C1_R (5'-TTGCTGGAAGCAAACTGTTG-3'; 10 µM), 1 µl dNTP mix, 1 µl Advantage® 2 Polymerase and 5 µl Advantage® 2 PCR buffer (10 x) were filled up to 50 µl. For amplification a touchdown PCR was performed with the following cycling conditions: 95°C for 2 min; 5 cycles: 95°C for 30 s and 68°C for 3 min; 5 cycles: 95°C for 30 s, 66°C for 30 s and 68°C for 3 min; 5 cycles: 95°C for 30 s, 64°C for 30 s and 68°C for 3 min; 35 cycles: 95°C for 30 s, 60°C for 30 s, 68°C for 3 min and a final elongation step at 70°C for 10 min.

The DNA fragments were gel purified from a 1.2% agarose gel using NucleoSpin® Gel and PCR clean up kit (Machery Nagel, Düren) according to the manufacturers protocol. The purified PCR fragments were ligated to pGEM®-T vector (Promega, Madison, WI, USA) according to the manufacturers instructions. Heat competent *E. coli* NEB 5-alpha (New England Biolabs, Frankfurt a. M.) were transformed with the plasmids as described in the section "Establishment of cDNA library".

Sequence analysis

For identification and trimming of vector sequences, the data retrieved from sequencing was analyzed with VecSreen (Altschul *et al.*, 1997) using default settings. For determination of putative functional homologies, the sequences were subjected to a BLASTN search (database: nucleotide collection [nr/nt]; Zhang *et al*., 2000), using the discontiguous megablast program, which is optimized for cross species comparisons and a BLASTX search (database: non-redundant protein sequences [nr]; Altschul *et al.*, 1997). Additionally a BLASTN search was performed in the est_others database to identify homologous expressed sequence tags (ESTs) of *Blumeria graminis* during different developmental stages or growth conditions. Hits, which had an expectation value (E-value) larger than 10^{-10} were rejected.

The sequences obtained with 5'/3' RACE procedure were aligned using BioEdit (Version 7.0.9.0; Hall, 1999) and ORFs were detected with Vector NTI

(Advance version 9.0; Invitrogen, Karlsruhe). BLAST searches within the genome of *B. graminis* f.sp. *hordei* DH14 were performed using the megablast program of the BluGen sequencing project (www.blugen.org; Spanu *et al*., 2010).

Putative ORFs were analyzed with Prosite to identify functional domains (Sigrist *et al*., 2002, 2010) and, additionally, a BLASTP search (search set: non-redundant protein sequences) was performed to identify putative conserved sequences that are functionally annotated (Altschul *et al*., 1997, 2005). Putative signal sequences were identified with SignalP 4.0 (Nielsen *et al*., 1997; Petersen *et al*., 2011).

5.3 Results

5.3.1 Kinetics of *B. graminis* conidia development

Initially, *B. graminis* conidia development was determined on *n*-hexacosanal and *n*-hexacosane coated surfaces, since that process is individual for each substrate and the incubation conditions. The time point for RNA extraction was determined when conidia germination begins but differentiation has not started. For determination of the RNA sampling time point, the development of *B. graminis* conidia was determined on glass slides coated with inductive Formvar®/*n*-hexacosanal and non-inductive Formvar®/*n*-hexacosane during a period of 16 hours. On the inductive *n*-hexacosanal surface, within 30 min about 40% of the conidia developed a primary germ tube (pgt) and after approximately 6.5 h the maximum germination rate of 90% was achieved (Figure 6a). The first differentiated conidia occurred after 4.5 hpi (10% agt). The rate increased to approximately 20% after 8.5 hpi and reached, after a second phase of rapid increase (9 hpi), its plateau of 50%. The amount of conidia with mature appressoria increased up to about 20% after 10 hpi. In comparison to the non-inductive *n*-hexacosane coated surface, about 10% of the conidia developed a primary germ tube after 1 hour, whereas the maximum germination rate of 20% was reached after 90 min and did not further increase over the observed period of 16 h (Figure 6b). The overall differentiation rate on the *n*-hexacosane coated surface was below 10% and the rate of mature appressoria was under 2% after 16 hpi.

Since a significant increase of pgt stage conidia was visible at 30 min after inoculation 22 min post inoculation was selected as RNA extraction time point. The 22 min incubation time was a combination of 7 min in the settling tower, where the conidia were descending on the surfaces and 15 min of incubation on the surfaces. This procedure allowed the earliest possible sampling time point, at which successful surface recognition was assumed, supported by reports of increased cAMP levels and PKA activity 15 min after inoculation on an inductive substratum (Kinane *et al*, 2000).

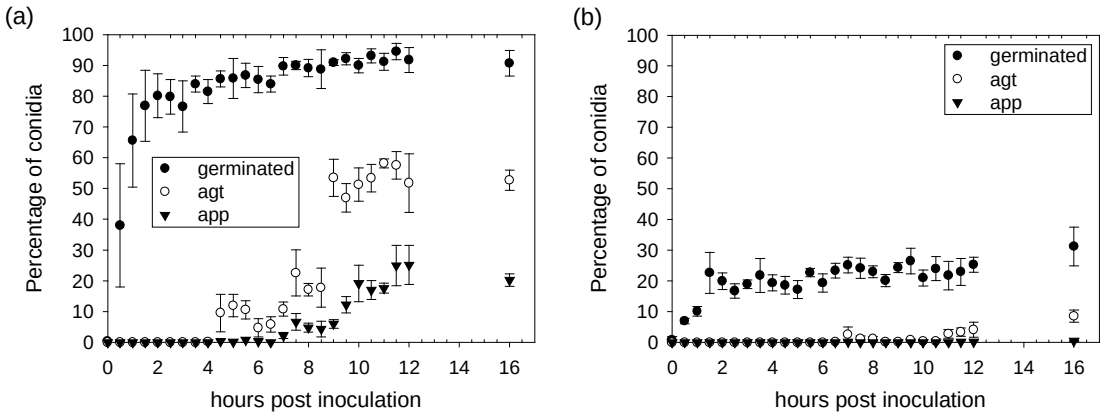


Figure 6 Development of *Blumeria graminis* conidia on Formvar®/*n*-hexacosanal (a) and Formvar®/*n*-hexacosane (b) coated glass slides. Percentage of germinated conidia (filled circles), conidia with appressorial germ tubes (open circles) and conidia with mature appressoria (filled triangles). Data are given as mean ± SD (*n*=3).

5.3.2 Library screening for differentially expressed transcripts by reverse Northern blot analysis

By forward and reverse subtraction, two SSH cDNA clone libraries were constructed. The forward cDNA clone library contains transcripts, which were putatively differentially expressed in *B. graminis* conidia in response to contact with the inductive Formvar®/*n*-hexacosanal coated surface and is subsequently referred as *n*-hexacosanal SSH cDNA clone library. The SSH cDNA clone library constructed by reverse subtraction harbours transcripts that were differentially expressed in conidia in response to contact with Formvar®/*n*-hexacosane coated glass slides and is referred as *n*-hexacosane SSH cDNA clone library. The *n*-hexacosanal SSH cDNA clone library contained 768 clones, whereas 136 clones were screened by reverse Northern blot analysis, which revealed 26 candidate sequences that were upregulated more than 2-fold. The *n*-hexacosane SSH cDNA clone library contained 576 clones, of which 104 were screened by reverse Northern blot and 21 candidates revealed to be upregulated more than 2-fold. The subtraction efficiencies were 21% for the *n*-hexacosanal and 20% for the *n*-hexacosane SSH cDNA clone library, respectively. All candidate clones were sequenced and a BLAST search analysis was performed (Table 1 & 2).

For clones of the *n*-hexacosanal SSH cDNA library showing the strongest induction in the screening [LibIIH1(1)C1, LibIH1(3)B2 and LibIIH1(1)H6] no homologies to functional genes or proteins were found. However, putatively homologous sequences for the clones LibIIH1(1)C1 and LibIIH1(3)B2 with highly supportive E values and query coverage are expressed 3 dpi and 5 dpi on barley leaves in epiphytic mycelium of *B. graminis* f.sp. *hordei* DH14. Clones with expression levels that were upregulated between 3 and 9-fold may encode putative

proteins of unknown function, whereas the sequence of clone LibIIH1(1)C3 might code for a pyruvate kinase. The expression levels of clones, which were induced about 2-fold, are possibly encoding for hypothetical proteins of unknown function. Homologous sequences of the clones LibIIH1(2)G2 and LibII H1(1)C10 were found to be expressed in infected barley tissue and in resting *B. graminis* conidia, respectively.

Clones of the *n*-hexacosane SSH cDNA library, which were upregulated between 14- and 22-fold showed no homologies to genes and proteins of known functions, but putatively homologous sequences were identified to be expressed in infected barley, wheat or *Lolium multiflorum* tissue. The sequences of the clones LibIIH2(2)E1 and LibIIH3(1)B8 match upstream a SNGH type hydrolase and are expressed on infected *Hordeum vulgare* leaf epidermis 6 and 24 hours post inoculation. The sequence of clone LibIIH3(1)D7 might encode for a putative virulence effector and clone LibIIH3(2) might harbour a transcript, which encodes for a GTPase activator-protein.

Table 1 BLAST search results of sequences from clones of the *n*-hexacosanal SSH cDNA clone library.

Clone	n-fold induction[1]	Homolgy (accession)	E value	Maximum identity	Query coverage	BLAST, search set
LibIIH1(1)C1	**12**	**No hits**				**blastn, nr; blastx, nr**
		3dpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000000254041_D17 3', mRNA sequence. (GT063810)	**3,00E-100**	**78%**	**70%**	**blastn, est others**
LibIH1(3)B2	**12**	**No hits**				**blastn, nr; blastx, nr**
		5dpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000000888650_L19 5', mRNA sequence (GT059371)	**4,00E-107**	**98%**	**85%**	**blastn, est others**
LibIIH1(1)H6	10	Hypothetical protein *Botryotinia fuckeliana* (XP_001552234)	5,00E-13	71%	24%	blastx, nr
LibIIH1(2)G9	9	*Sclerotinia sclerotiorum* 1980 hypothetical protein (SS1G_07000) partial mRNA (XM_001591504)	1,00E-37	73%	34%	blastn, nr
		20hpi *Blumeria graminis* f. sp. *horde*i DH14 cDNA clone 000000787942_A15 5', mRNA sequence (GT066210)	0	97%	47%	blastn, est others
LibIIH1(2)G3	**5**	**No hits**				**blastn, nr, est others; blastx**
LibIIH1(1)H9	**5**	**hypothetical protein *Botryotinia fuckeliana* (XP_001548142)**	**3,00E-60**	**67%**	**92%**	**blastx, nr**
		***Hordeum vulgare* cDNA clone HO10D05 3-PRIME, mRNA sequence (CK568491)**	**1,00E-159**	**94%**	**84%**	**blastn, est others**
LibIIH1(1)H4	4	*Blumeria graminis* bkr1 gene for PKA regulatory subunit (AJ304829)	2,00E-144	97%	86%	blastn, nr
		Hordeum vulgare cDNA clone HO22N13 5-PRIME, mRNA sequence (DN181946)	5,00E-101	93%	72%	blastn, est others
LibIIH1(1)C3	4	Pyruvate kinase *Sclerotinia sclerotiorum* 1980 (XP_001594760)	7,00E-122	87%	83%	blastx, nr
		Hordeum vulgare cDNA clone HO03H11 3-PRIME, mRNA sequence (CK566404)	0	100%	86%	blastn, est others
LibIH1(3)B11	**4**	***Blumeria graminis* f. sp. *tritici* clone Bgt_BACs_1f12_12c21; 27091 bp at 3' side flanking hypothetical protein (HQ437160)**	**8,00E-33**	**91%**	**87%**	**blastn, nr**
		***Hordeum vulgare* cDNA clone HO27D01 5-PRIME, mRNA sequence (DN180511)**	**1,00E-29**	**67%**	**58%**	**blastn, est others**
LibIH1(2)E9	4	No hits				blastn, nr, est others; blastx
LibIIH1(4)F7	3	No hits				blastn, nr, est others; blastx
LibIIH1(3)D7	**3**	**Ribosomal protein S3 *Phialocephala subalpina* (YP_004733050)**	**6,00E-24**	**54%**	**49%**	**blastx, nr**
		3dpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000048636641_H20 5', mRNA sequence (GT063670)	**0**	**99%**	**94%**	**blastn, est others**
LibIIH1(3)D6	**3**	**Hypothetical protein *Sclerotinia sclerotiorum* 1980 (XP_001590999)**	**3,00E-43**	**61%**	**73%**	**blastx, nr**
LibIIH1(3)B5	3	No hits				blastn, nr, est others; blastx
LibIIH1(3)B12	3	Conserved hypothetical protein *Talaromyces stipitatus* ATCC 10500 (XP_002483427)	2,00E-39	54%	60%	blastx, nr
		5dpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000001049568_B1 5', mRNA sequence (GT058184)	0	98%	93%	blastn, est others
LibIIH1(2)B9	3	No hits				
		Blumeria graminis f. sp. *hordei* cDNA clone C00736, mRNA sequence (AW788827)	6,00E-59	99%	20%	blastn, est others
LibIIH1(1)H8	3	No hits				blastn, nr, est others; blastx
LibIIH1(1)C2	**3**	**No hits**				**blastn, nr, est others; blastx**
LibIH1(2)E1	3	Chitinase *Hypocrea jecorina* (DAA05855)	2,00E-49	69%	63%	blastx, nr
		Triticum aestivum cDNA clone wlm96.pk0014.d6 5' end, mRNA sequence (CA683292)	1,00E-137	87%	68%	blastn, est others
LibIIH1(3)F2	2	tRNA (adenine-N(1)-)-methyltransferase *Aspergillus niger* CBS 513.88 (XP_001389248)	7,00E-33	41%	66%	blastx, nr
		Cleistothecia *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000001061105_I6 3', mRNA sequence (GT065953)	7,00E-122	95%	41%	blastn, est others
LibIIH1(3)B7	2	hypothetical protein BC1G_07095 *Botryotinia fuckeliana* (XP_001554507)	8,00E-71	71%	94%	blastx, nr
LibIIH1(2)G2	2	hypothetical protein SMAC_02535 *Sordaria macrospora* (XP_003352100)	1,00E-32	62%	47%	blastx, nr
		Hordeum vulgare cDNA clone HO16L09 3-PRIME, mRNA sequence (DN189803)	0	87%	76%	blastn, est others
LibIIH1(1)C10	2	60s Acidic ribosomal protein *Glomerella graminicola*	2,00E-27	75%	63%	blastx, nr
		Conidia *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000000910913_J9 5', mRNA sequence (GT061534)	0	97%	89%	blastn, est others
LibIH1(3)B9	2	No hits				blastn, nr; blastx, nr
		Blumeria graminis f. sp. *hordei* cDNA clone D01302, mRNA sequence (AW792633)	3,00E-171	97%	52%	blastn, est others
LibIH1(2)E8	2	No hits				blastn, nr; blastx, nr
		Appressorium stage EST library of *Blumeria graminis* f.sp. *hordei* mRNA sequence (BM361443)	6,00E-104	94%	34%	blastn, est others
LibIH1(2)E5	**2**	**hypothetical protein SS1G_08876 *Sclerotinia sclerotiorum* 1980 (XP_001590112)**	**9,00E-117**	**72%**	**96%**	**blastx, nr**
		***Hordeum vulgare* cDNA clone HO10J12 5-PRIME, mRNA sequence (CD055906)**	**3,00E-126**	**92%**	**45%**	**blastn, est others**

[1] Induction calculated according to the signal intensities of reverse Northern blot analysis. Clones depicted in bold were analyzed by RT-qPCR.

Table 2 BLAST search results of sequences from clones of the *n*-hexacosane SSH cDNA clone library.

Clone	n-fold induction[1]	Homolgy (accession)	E value	Maximum identity	Query coverage	BLAST, search set
LibIIH3(1)D8	22	*Blumeria graminis* genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA, isolate: MUMH1722 (AB273555)	7,00E-118	84%	99%	blastn, nr
		20hpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000000787942_N1 5', mRNA sequence (GT066191)	5,00E-119	99%	84%	blastn, est others
LibIIH3(2)A2	22	hypothetical protein SS1G_14306 *Sclerotinia sclerotiorum* 1980 (XP_001584693)	1,00E-92	86%	86%	blastx, nr
		Triticum aestivum cDNA clone infected with *Blumeria graminis* (CJ955117)	0	87%	88%	blastn, est others
LibIIH3(1)G3	18	No hits				blastn, nr; blastx, nr
		Lolium multiflorum cDNA clone SL010G03-5, mRNA sequence, Powdery mildew infected (AU250362)	2,00E-104	80%	55%	blastn, est others
LibIIH3(1)G2	18	No hits				blastn, nr; blastx, nr
		Blumeria graminis f. sp. hordei cDNA clone C00458, mRNA sequence (AW788454)	4,00E-113	99%	85%	blastn, est others
LibIIH3(2)E9	14	hypothetical protein *Sclerotinia sclerotiorum* 1980 (XP_001588310)	2,00E-30	70%	58%	blastx, nr
		Blumeria graminis f. sp. *hordei* cDNA clone C00065, mRNA sequence (AW787904)	4,00E-46	95%	26%	blastn, est others
LibIIH3(1)D7	9	*Blumeria graminis* f. sp. *hordei* , putative virulence effector-like mRNA (GQ470878)	3,00E-49	75%	60%	blastn, nr
		Avena barbata leaf, grown under high rainfall, mRNA (GR329431)	6,00E-51	78%	50%	blastn, est others
LibIIH3(1)D5	7	Hypothetical protein FG10871.1 *Gibberella zeae* PH-1 (XP_391047)	4,00E-14	34%	67%	blastx, nr
LibIIH3(2)E1	5	*Blumeria graminis* f. sp. *tritici* clone Bgt_BAC_2p10, 684bp upstream SNGH-hydrolase type esterase (HQ437159)	4,00E-124	86%	95%	blastn, nr
		Hordeum vulgare leaf epidermis, 6 and 24 hpi with *Blumeria graminis* (CK567626)	0	97%	95%	blastn, est others
LibIIH3(2)G9	4	*Blumeria graminis* f. sp. *Hordei,* ATPase assembly protein 11, trehalose phosphate synthase (EU098096)	2,00E-14	84%	41%	blastn, nr
		3dpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000000940014_I11 5', mRNA sequence (GT064571)	1,00E-54	99%	62%	blastn, est others
LibIIH3(2)A1	3	No hits				blastn, nr, est others; blastx, nr
LibIIH3(1)D4	3	No hits				blastn, nr; blastx, nr
		3dpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000048636641_G4 5', mRNA sequence (GT063798)	2,00E-107	97%	52%	blastn, est others
LibIIH3(2)E10	3	No hits				blastn, nr, est others; blastx, nr
LibIIH3(2)G7	3	GTPase-activator protein for Ras-like GTPase *Glomerella graminicola* (EFQ31703)	4,00E-51	89%	89%	blastx, nr
LibIIH3(3)C1	3	No hits				blastn, nr; blastx, nr
		Blumeria graminis f. sp. hordei cDNA clone C00621, mRNA sequence (AW788669)	7,00E-85	98%	70%	blastn, est others
LibIIH3(3)C3	3	hypothetical protein *Botryotinia fuckeliana* B05.10 (XP_001553189)	5,00E-72	97%	93%	blastx, nr
		Hordeum vulgare leaf epidermis, 6 and 24 hpi with *Blumeria graminis* (DN182876)	6,00E-177	94%	91%	blastn, est others
LibIIH3(2)A9	2	No hits				blastn, nr, est others; blastx, nr
LibIIH3(1)G1	2	No hits				blastn, nr; blastx, nr
		Conidia *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000000910613_B20 5', mRNA sequence (GT062570)	4,00E-111	99%	42%	blastn, est others
LibIIH3(1)G5	2	No hits				blastn, nr, est others; blastx, nr
LibIIH3(1)B8	2	*Blumeria graminis* f. sp. *tritici* clone Bgt_BAC_2p10, 741 bp upstream SNGH-hydrolase type esterase	2,00E-116	78%	87%	blastn, nr
		Hordeum vulgare leaf epidermis, 6 and 24 hpi with *Blumeria graminis* (CK567626)	0	93%	88%	blastn, est others
LibIIH3(1)D11	2	hypothetical protein *Sclerotinia sclerotiorum* 1980 (XP_001594435)	5,00E-78	82%	64%	blastx, nr
		Cleistothecia *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000001036805_M8 5', mRNA sequence (GT065927)	6,00E-155	99%	41%	blastn, est others
LibIIH3(1)B6	2	No hits				blastn, nr; blastx, nr
		Hordeum vulgare leaf epidermis, 6 and 24 hpi with *Blumeria graminis* (DN183660)	9,00E-62	91%	32%	blastn, est others

[1] Induction calculated according to the signal intensities of reverse Northern blot analysis.

5.3.3 Confirmation of differentially expressed transcripts by RT-qPCR

The expression profiles of ten transcripts identified in the *n*-hexacosanal SSH cDNA clone library were determined for conidia inoculated on barley leaves, on Formvar® coated surfaces supplemented with barley leaf wax extract, *n*-hexacosanal and *n*-hexacosane. The sampling time point for the RNA extraction was 22 min after inoculation, the same as for the SSH cDNA clone library construction. The PCR-efficiencies of all designed oligonucleotides ranged between 88% and 112% (Table 3).

Table 3 Oligonucleotides used for RT-qPCR expression analysis according to selected clones of the *n*-hexacosanal SSH cDNA library and reference gene.

Clone	Oligonucleotide name	Sequence 5' to 3'	Tm (°C)	Amplicon length (bp)	Efficiency	Slope	R^2	Final concentration (nM)
LibIIH1(1)C1	LIIH1(1)C1_L	CCTGCCCGTCTATCCAAATA	60	61	90%	-3.292	1.000	500
	LIIH1(1)C1_R	TTGCTGGAAGCAAACTGTTG	60					
LibIH1(3)B2	LIH1(3)B2_L	TGGAAATGTGCATCACGAAT	60	75	99%	-3.348	0.994	150
	LIH1(3)B2_R	TTCAGACTTTCCCAGCGTCT	60					
LibIIH1(1)H6	LIIH1(1)H6_L	GCGTGAAGCTGTCCGATAAT	60	115	112%	-3.049	0.998	150
	LIIH1(1)H6_R	CACTCTCAAGGGGCTACCAG	60					
LibIIH1(2)G3	LIIH1(2)G3_L	GTCGATGGAAACTCGCATTC	61	130	104%	-3.224	0.996	200
	LIIH1(2)G3_R	TTTGGTGTCGAGCCATACTG	60					
LibIIH1(1)H9	LIIH1(1)H9_L	CGCTGTTGATCCTGGTAGGT	60	150	107%	-3.148	0.998	150
	LIIH1(1)H9_R	CCGAATAATAATGGGCATGG	60					
LibIH1(3)B11	LIIH1(3)B11_L	AGTCGGAGCAACTGCTTGAT	60	146	103%	-3.263	0.996	200
	LIIH1(3)B11_R	CGAATCGAAATCGAGGGTAA	60					
LibIIH1(3)D7	LIIH1(3)D7_L	CTCAACCCCTGCCTCTTCTT	61	118	98%	-3.361	0.999	200
	LIIH1(3)D7_R	GTTGGCAAGTCAAGCCATCT	60					
LibIIH1(3)D6	LIIH1(3)D6_L	TGCTTCATCTAGCCTGCTCA	59	119	99%	-3.356	1.000	200
	LIIH1(3)D6_R	TAAAGCAGCAAAGTGCATGG	60					
LibIIH1(1)C12	LIIH1(1)C2_L	CGCGTGAAGGATGGTTAGTT	60	92	112%	-3.052	0.996	200
	LIIH1(1)C2_R	GCCAGCTATCGTTTCAAAGC	60					
LibIH1(2)E5	LI(H1(2)E5_L	GAGACTCCGCCTCAAAAGTG	60	68	88%	-3.661	0.986	200
	LI(H1(2)E5_R	TTGAATCGAAACCCAAGGTC	60					

Reference gene	Oligonucleotide name	Sequence 5' to 3'	Tm (°C)	Amplicon length (bp)	Efficiency	Slope	R^2	Final concentration (nM)
Histone H3	His_f	CTCGTCTCACTATTCGAGG	60	110	101%	-3.292	1.000	250
	His_r	TCCGTGTCGTCGTTACTA	60					

The relative expression levels from conidia inoculated on inductive surfaces (*n*-hexacosanal, barley leaf wax extract and barley leaves) are given as n-fold expression of the non-inductive *n*-hexacosane coated surface (Figure 7). From transcripts that showed the highest signal intensities in the reverse Northern blot analysis, only one clone was confirmed with a more than 2-fold relative transcript abundance in the RT-qPCR. LibIIH1(1)C1 had a 3-fold elevated relative expression on the *n*-hexacosanal surface, compared to the non-inductive *n*-hexacosane coated surface (Figure 7a). The expression levels were also increased on wax coated glass slides (30-fold induction) and on barley leaves (25-fold induction). On barley leaf wax coated glass slides, the expression levels of LibIIH1(1)H6 (Figure 7c) and LibIIH1(2)E5 (Figure 7 j) were 2-fold and 3-fold increased, respectively. However, no differences were detected between the *n*-hexacosanal and *n*-hexacosane coated surfaces. The transcript level of LibIIH1(3)D6 (Figure 7h) was not different between the *n*-hexacosane and *n*-hexacosanal coated surfaces. Nevertheless, the analysis revealed a 13- and 9-fold increase on slides coated with barley leaf wax extract and on leaves, respectively. The transcript levels according to six clones (LibIIH1(3)B2, LibIIH1(2)G3, LibIIH1(1)H9, LibIIH1(3)B11, LibIIH1(3)D7, LibIIH1(1)C2), which were identified in the reverse Northern blot analysis showed no significantly increased relative transcript abundances on the *n*-hexacosanal coated glass slides and the other tested inductive surfaces compared to the non-inductive *n*-hexacosane surface (Figure 7b, d, e, f, g, i).

Due to the *Rsa*I digest of the cDNA during the SSH cDNA library construction, the clones harbour only fragments of the corresponding transcripts. To obtain further sequence information of the fragment cloned in LibIIH1(1)C1, which was differentially expressed on *n*-hexacosanal coated glass slides, a 3’ and 5’ RACE reaction was performed and the cloned cDNA was named transcript A1.

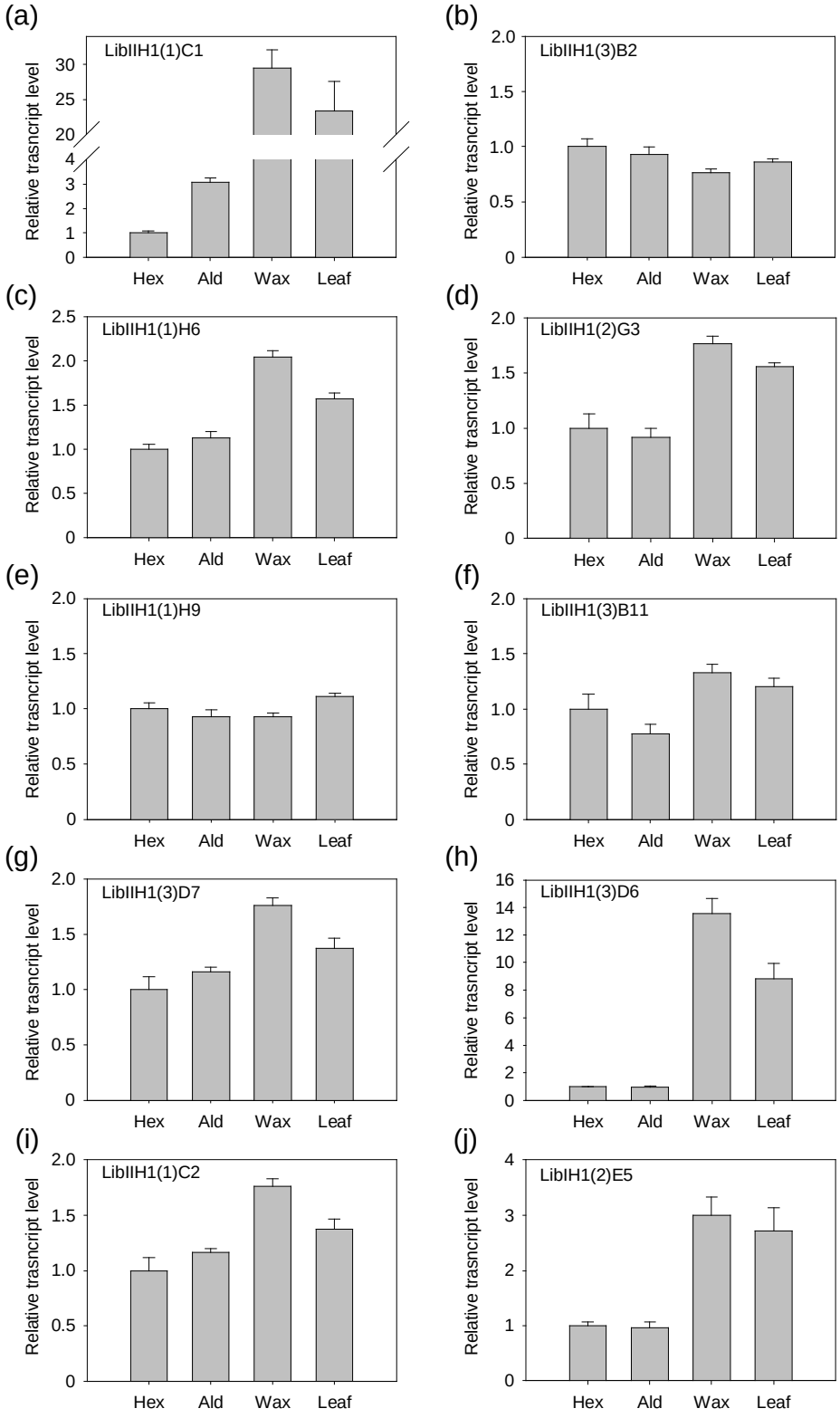


Figure 7 Relative expression levels of selected transcripts identified in the *n*-hexacosanal SSH cDNA clone library. Relative expression levels ($2^{-\Delta\Delta Cq}$) were determined by RT-qPCR. Values are given as n-fold expression of conidia inoculated on Formvar® coated glass slides supplemented with either *n*-hexacosanal (Ald), total barley leaf wax extract (Wax) or on barley leaves (Leaf), compared to the expression levels on non-inductive Formvar®/*n*-hexacosane (Hex) coated glass slides. The values are given as mean ± SE of three technical replications. The RT-qPCR was repeated twice with independent biologically samples with similar results.

5.3.4 Cloning of transcript A1

To obtain a full length cDNA sequence of LibIIH1(1)C1, 5’ and 3’ RACE were performed. For the 5’ RACE, gene specific primer Hexar_nes1b, LIIH1(1)C1_L and Hexar_nes3 were designed according to the sequence information of LibIIH1(1)C1. These primers were used in three consecutive nested PCR reactions in order to amplify the full length 5’ end of transcript A1 from total RNA of conidia incubated for 22 min on Formvar®/*n*-hexacosanal coated glass slides. For amplification of the full length 3’ end of transcript A1, the gene specific oligonucleotide LibIIH1(1)C1_R was used in combination with an oligo(dT) anchor primer. According to the overlapping regions of the 5’ and 3’ RACE sequences the cDNA sequence of transcript A1 was deduced to have a length of 1495 bp (Figure 8; Appendix Figure 9-1). The 3’ end of transcript A1 is 100% identical with the 661 bp long sequence obtained for LibIIH1(1)C1.

The sequence of transcript A1 is highly conserved and was identified in the genome of the sequenced *B. graminis* f.sp. *hordei* strain DH14 on contig 00320 on the minus strand (megablast, E-value 4.59E-124; Appendix Figure 9-2). According to the alignment with the genomic sequence of *B. graminis* f.sp. *hordei* strain DH14, transcript A1 of *B. graminis* f.sp. *hordei* strain CC1 has an insertion of 19 bp length located between 869 bp and 888 bp (Figure 8). When the cDNA sequence of transcript A1 was compared with *B. graminis* f.sp. *hordei* DH14 genomic sequence information, it does not possess an intron, which has to be confirmed by cloning the corresponding genomic sequence of *B. graminis* f.sp. *hordei* strain CC1. A BLASTN and BLASTX search using transcript A1 as query did not reveal homologous sequences that are functionally annotated. In the sequence of transcript A1 four putative open reading frames (ORFs) with a start- and stop codon were identified and the depicted amino acid sequences were subjected to further analyses in order to obtain possible biochemical functions (Figure 9). The BLASTP analysis of all four ORFs did not reveal any hits of putative functional proteins (E-value > 1). Further analyses with Prosite on the ExPAsy bioinformatics resource portal for identification of putative functional regions or domains in the sequences of the four putative ORFs did not reveal any hits. The putative proteins also seem not be secreted peptides, since SignalP analyses were negative and hydrophobic domains at the N-terminal site were not identified.

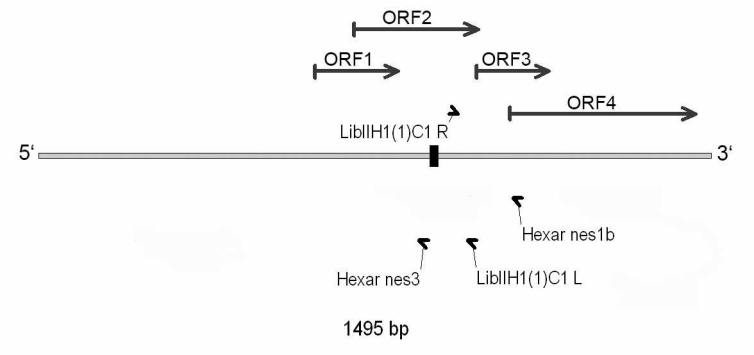


Figure 8 cDNA sequence of transcript A1 cloned by 5' and 3' RACE from *B. graminis* f.sp. *hordei* strain CC1. Position of primers and putative open reading frames (ORF) are indicated. The black box indicates a 19 bp insertion compared to the genomic sequence of *B. graminis* f.sp. *hordei* DH14.

>ORF1 (614-799 bp)
vsftnlggiilpvpstvkfrntkriglrandpllptnsishhqqgqfwpslnksasttllq

>ORF2 (702-799 bp)
mirysqliqyltinkdnsgqasislqvqpyfndhssrqvslwpqmactytptvceytpqyvntrpqyvntiagskllrenladeyldrrag

>ORF3 (974-1135 bp)
vmqtmhtileevifrfldgcgqveqcalhdlvceragdridavmagwcgyvss

>ORF4 (1048-1461 bp)
mcatrlglrtcrrpyrrrhgwmvrlcivlaglpsrvwiartpiplemrvrrlefgktplhfsrrsytrmtkarsdnqrflnfplvgargggqtiprrqt
ylvvafaneiqsitvsddqgviqyvytslitphsqiae

Figure 9 Amino acid sequences of predicted open reading frames (ORF) in the cDNA of transcript A1.

The amino acid sequences of the four putative ORFs were used as BLAST queries searching in the genome of *B. graminis* f.sp. *hordei* DH14 and the nucleotide database of NCBI (Tables 4 & 5). For ORF 1-3 only one hit was indentified in the genome in the same region where transcript A1 was matching to (E value <1.00E-25). In contrast, for ORF4 110 hits in diverse regions in the genome were identified (E value <1.00E-25). The matching regions in the genome are annotated as repetitive elements. Additionally, a homologous sequence was identified in the genome of *B. graminis* f.sp. *tritici* (E value 8.00E-24). Homologous sequences of transcript A1 are expressed during all different developmental stages (Table 6 & 7). Considering only data of the BluGen sequencing project, sequences related to transcript A1 may not be expressed in conidia and 20 hpi, but related transcripts appear quite frequently in cleistothecia (E value 1.26E-110), in *B.graminis* 3 dpi (E value 3.60E-111) and in infected epidermis (E value 8.48E-94). However, homologous transcripts of A1 were identified in conidia, which were directly collected from infected barley (E value <3E-94;Table 7) when the BLAST search was performed on the NCBI platform.

Table 4 BLAST search results in the genome of *B. graminis* f.sp. *hordei* DH14 using the open reading frames of transcript A1 as query.

ORF	Hits	Contig	Annotation	E value[1]	Max Score	Total Score	BLAST, search set
ORF1	1 hit	contig 000320	repetetive	3.83E-29	124	124	tblastn, Bgh DH14 Genome_v3
ORF2	1 hit	contig 000320	repetetive	1.54E-33	138	138	tblastn, Bgh DH14 Genome_v3
ORF3	1 hit	contig 000320	repetetive	1.00E-25	276	276	tblastn, Bgh DH14 Genome_v3
ORF4	110 hits	various contigs	repetetive	<1.00E-25	645-206	1162-747	tblastn, Bgh DH14 Genome_v3

[1]E value cut off >1.00E-25

Table 5 BLAST search results on NCBI using the open reading frames (ORF) of transcript A1 as query.

ORF	Accession	Description	E value[1]	Maximum identity	Qery coverage	BLAST, search set
ORF1	-	-	>1E-10	-	-	tblastn, nr
ORF2	-	-	>1E-10	-	-	tblastn, nr
ORF3	-	-	>1E-10	-	-	tblastn, nr
ORF4	HQ437160.1	*Blumeria graminis* f. sp. *tritici* clone Bgt_BACs	8.00E-24	62%	100%	tblastn, nr

[1]E value cut off >1.00E-10

Table 6 ESTs identified by BLAST search in the BluGen sequencing project database using transcript A1 as query according to developmental stages.

Developmental stage BluGen	Hit ID of best hit	Annotation	E value	Max Score	Total Score	BLAST, search set
Cleistothecia	000000761712L09	*B.graminis hordeii* EST (Cleistothecia library)	1.26E-110	440	440	blastn, ESTs
Conidia	no hits					blastn, ESTs
20 hpi	no hits					blastn, ESTs
3 dpi	000001702902F20	*B.graminis hordeii* EST (3 dpi library)	3.60E-111	442	442	blastn, ESTs
6 dpi	no hits					blastn, ESTs
Infected Epidermis	000000837572D11	*B.graminis hordeii* EST (Infected barley epidermis library)	8.48E-94	378	378	blastn, ESTs

Table 7 ESTs identified by BLAST search in the NCBI database using A1 as query according to developmental stages.

Developmental stage	Accession	Description	E value	Maximum Identity	Query Coverage	BLAST, search set
Cleistothecia	GT065902	Cleistothecia *Blumeria graminis* f. sp. hordei DH14 cDNA clone 000001061805_F23 3', mRNA sequence	1.00E-92	78%	30%	blastn, est others
Conidia	GT062479	Conidia *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000000761512_G16 3', mRNA sequence	3.00E-94	78%	30%	blastn, est others
3 dpi	GT063810	AGENCOURT_103666662 DH14 3dpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000000254041_D17 3', mRNA sequence	1.00E-92	78%	30%	blastn, est others
5 dpi	GT059401	AGENCOURT_103765786 DH14 5dpi *Blumeria graminis* f. sp. *hordei* DH14 cDNA clone 000001049568_K3 5', mRNA sequence	1.00E-91	76%	34%	blastn, est others
Infected Epidermis	CK565684	HO01C08w HO *Hordeum vulgare* cDNA clone HO01C08 3-PRIME, mRNA sequence	5.00E-84	77%	29%	blastn, est others

5.4 Discussion

The development of *B. graminis* conidia and the early differentiation processes have been characterized in detail on the morphological and cytological level on inductive artificial and native host and non-host surfaces (Chapter 2-4). However, molecular biological data about genes, which are induced upon contact with the most

inductive wax component, the very-long-chain aldehyde *n*-hexacosanal, were not available until now. Therefore, two SSH cDNA clone libraries were constructed with cDNA from conidia that were inoculated on Formvar® resin coated glass slides supplemented with the inductive *n*-hexacosanal or with the non-inductive *n*-hexacosane. The *n*-hexacosanal SSH cDNA clone library comprised 768 clones, from which 26 clones were differentially expressed in an initial screening, bearing putative *n*-hexacosanal dependent induced transcripts. The *n*-hexacosane SSH cDNA clone library contained 576 clones and the screening revealed 21 candidate cDNA clones. The subtraction efficiencies according to the initial screening were about 20% for the *n*-hexacosanal and the *n*-hexacosane SSH cDNA clone library. The subtraction efficiencies range from 5% to 95%, whereas the efficiencies are at lower levels, the less differentially expressed sequences exist in both RNA populations (Diatchenko *et al*., 1996, 1998). The candidates of both libraries were sequenced and the relative expression levels of ten selected clones of the *n*-hexacosanal SSH cDNA library were confirmed by RT-qPCR on barley leaves and *in vitro* on Formvar® resin coated glass slides supplemented with *n*-hexacosane, *n*-hexacosanal or barley leaf wax extract 22 min after inoculation. The expression patterns of the *n*-hexacosane SSH cDNA clone library were not investigated by RT-qPCR, since the key aspect at this stage of the study was the identification of *n*-hexacosanal dependent regulated genes.

The time point of the library construction was 22 min after inoculation on *n*-hexacosanal coated glass slides, when the early differentiation has not started, but an initial surface contact took place. About 15 min after inoculation, prior to a visible pgt emergence, *B. graminis* conidia respond with increased cAMP levels and protein kinase A activity, which trigger downstream signal transduction pathways during further development (Kinane *et al*, 2000). On *n*-hexacosanal coated glass slides the first primary germ tubes were visible after 30 min, similar as on barley leaves (Both *et al*., 2005a). The onset for early conidia differentiation on the Formvar® *in vitro* system was after 4-5 hours, when the first appressorial germ tubes emerged, which is comparable with the developmental progress on barley leaves (Both *et al*., 2005a). The first fully differentiated *B. graminis* conidia in the agt stage were observed 9-10 hpi, as on barley epidermal strips or barley leaves, where all conidia are fully differentiated at this time point (Chapter 4; Both *et al*., 2005a). In general, the obtained overall differentiation rates were lower on the Formvar® resin based *in vitro* systems than on barley leaves as substratum (Chapter 2 & 3). Similar differentiation rates of *B. graminis* conidia were achieved with the Formvar® resin based wax membranes on water agar and on barley epidermal strips (Chapter 4). However, whether the RNA extraction in comparable quantities and quality is possible with this *in vitro* system still has to be elucidated.

The classification of the sequenced clones according to the BLAST analyses was similar in both libraries. About 42% of the clones did not reveal functionally putative homologous hits in genomic and proteomic databases and roughly 20% of these differentially expressed cDNA sequences were not present in *B. graminis* EST databases, which were constructed during different developmental stages of this fungus. Generally, most of the transcripts of the *n*-hexacosanal (65%) and *n*-hexacosane (66%) SSH cDNA library revealed hits of putatively homologous transcripts with high sequence similarities (E value <1.00E-50), expressed during different phases of *B. graminis* asexual life cycle. These sequences might encode for essential conserved proteins, which are necessary for establishment and perpetuation of the biotrophic lifestyle of *B. graminis* and are expressed after contact with the host surface. About one fifth of the cloned sequences were annotated as hypothetical fungal proteins. Transcripts that are assigned to the primary metabolism, like glycolysis or the pentose phosphate pathway were very rare in the *n*-hexacosanal SSH cDNA clone library, which indicates an efficient subtraction of ubiquitously expressed sequences. The relative expression of enzymes, which belong to primary metabolism are initially low expressed in ungerminated conidia, increases when the early differentiation of *B. graminis* conidia is completed and peaks between 4 and 8 hpi (Both *et al*., 2005a). A single clone of the *n*-hexacosanal SSH cDNA library, LibIIH1(1)C3, putatively carries a transcript encoding for a glycolytic enzyme assigned as a pyruvate kinase. The hybridization signal of the putative pyruvate kinase is 4-fold upregulated on the inductive *n*-hexacosanal coated surface. The expression of pyruvate kinase peaks in *B. graminis* conidia on barley leaves 8 hpi (Both *et al*., 2005a), but finding this transcript in the *n*-hexacosanal SSH cDNA library might indicate that the increased synthesis of specific glycolytic enzymes is induced immediately after contact with the inductive very-long-chain aldehyde *n*-hexacosanal. However, this result has to be confirmed by RT-qPCR, but the focus in this study was drawn towards putative novel genes, which might show a correlation with the early surface recognition process.

The expression levels of ten out of 21 differentially expressed transcripts of the *n*-hexacosanal SSH cDNA library were monitored by RT-qPCR, where the transcript of clone LibIIH1(1)C1 was verified with a 3-fold higher relative transcript abundance on *n*-hexacosanal coated glass slides. The relative expression levels on wax coated glass slides and on barley leaves were about 10-fold higher in comparison to *n*-hexacosanal coated slides. Since a dramatically increased expression level of LibIIH1(1)C1 occurs on barley leaves and *in vitro* on barley wax extract, it is very likely that an additional compound in the leaf cuticular wax might be responsible for this effect. The primary alcohol *n*-hexacosanol could be responsible for this effect, since it was able to significantly enhance conidia

germination and differentiation *in vitro* and is the most prevalent primary alcohol in barley cuticular wax (Chapter 2; Zabka *et al*., 2008). The impact of *n*-hexacosanol on the expression of LibIIH1(1)C1 still has to be elucidated by RT-qPCR. This effect could also be a response to unidentified wax constituents or a cumulative effect based on the whole wax blend. The surface micromorphology is considered to have no impact on the morphogenesis of *B. graminis* conidia (Carver & Thomas, 1990; Rubiales; *et al*., 2001; Zabka *et al*., 2008; Ringelmann *et al*., 2009). However, it cannot be excluded that the surface structure and topology has an impact on gene expression. The epicuticular wax of barley leaves typically possess crystalline platelets. The Formvar®/wax coated slides have also plate like protrusions, even though they are larger and less densely distributed than barley wax crystals are. In contrast, Formvar®/*n*-hexacosanal coated slides have a smoother surface with less pronounced protrusions. The enhanced humidity or increased water availability on barley leaves can be excluded as a stimulating factor, since the relative transcript levels on leaves and barley wax extract are generally the same. Nevertheless, the water availability might have an impact on the transcription levels at later stages, during secondary germ tube swelling and appressorium formation.

The cDNA was cloned by 5' and 3' RACE and named hexacosanal responsive transcript A1. Transcript A1 is a 1495 bp long cDNA with four putative open reading frames (ORFs). Bioinformatic analyses could not identify possible functional homologies according to the cDNA sequence information. However, a homologous sequence of ORF4 on transcript A1 was identified in BAC libraries of *B. graminis* f.sp. *tritici* (E value 8.00E-24). For ORF4 about 110 hits of related sequences were identified in the genome of *B. graminis* f.sp. *hordei* DH14 (E value <1.00E-25), whereas only one hit was found for the other three putative ORFs (E value <1.00E-25). The corresponding regions in the genome of *B. graminis* f.sp. *hordei* DH14 are annotated as repetitive elements. More than 64% of the genome of *B. graminis* is annotated as transposable elements (Spanu *et al*., 2010), but possibly these regions do contain genes, which are very specific for *B. graminis* and encode for proteins that are necessary during conidial development. Possibly, transcript A1 is one potential transcript of a larger gene family, which is expressed according to different developmental stages, external plant derived signals or environmental conditions. This idea could explain the finding of a multitude of homologous ESTs at all developmental stages in *B. graminis* EST databases.

B. graminis is an obligate biotrophic fungus and methods for a stable targeted mutagenesis of single genes and appropriate selection markers are not available yet. Hence, other experimental strategies were developed, which circumvent these limitations. Recently, a novel reverse genetic approach was developed, based on the expression of fungal antisense RNA by the host plant, which led to decreased transcript abundance in *B. graminis* (Nowara *et al*., 2010).

Barley and wheat epidermal cells were transformed by biolistic bombardment with an RNAi silencing vector carrying an antisense construct in order to reduce the overall expression of the fungal effector protein *Avr10* (Avirulence 10), which led to decreased infestation with *B. graminis*. Another successfully applied strategy for studying the impact of *B. graminis* genes on pathogenic development was the overexpression of *B. graminis* f.sp. *tritici CYP51*, encoding a lanosterol 14 α-demethylase, in the closely related necrotrophic fungus *Botrytis cinerea* (Yan *et al*., 2012). The expression of *Bgh CYP51* tagged with GFP was successfully translated and the transformants showed a reduced sensitivity towards 14 α-demethylase inhibitor based fungicides, suggesting that this heterologus expression system is generally suitable for functional studies of *B. graminis* genes. A further strategy for investigating the impact of obligate biotrophic fungi genes is based on the complementation of deletion strains, obtained in non-obligate biotrophic fungi with the corresponding homologous *B. graminis* sequence information. The *Magnaporthe grisea ΔcpkA* null mutant is non-pathogenic due to a delayed and incomplete appressorium formation (Bindslev *et al*., 2001). PKA-c encodes for subunit of protein kinase A signalling pathway. The differentiation of germinating *B. graminis* conidia relies on PKA dependent signalling cascades. A homologous sequence of PKA-c (*Bgh Bka1*) was identified in an EST library of germinating conidia (Hall *et al*., 1999). The complementation of *Magnaporthe grisea ΔcpkA* with *Bgh Bka1* could fully restore the pathogenicity of *ΔcpkA Magnaporthe* conidia (Bindslev *et al*, 2001), which implicates that functional studies are possible with this experimental approach in the case of conserved virulence pathways. In the case of transcript A1 an overexpression approach would be eventually the most promising strategy to study a putative phenotype, since no homologous genes of transcript A1 were identified in other fungi.

Well described specific *B. graminis* gene families that are expressed in affected barley tissue and were associated with pathogenicity are *Egh7* and *Egh16*, from which several homologous were identified in the genome of *B. graminis* (*Egh16H*) (Justesen *et al*., 1996; Thomas *et al*., 2002; Grell *et al*., 2003). The roles of *Egh16* are not clear, but the expression levels of *Egh16H1* and *Egh16H4* are upregulated in germinating conidia *in vitro*. These genes also showed a high expression level during appressorium and haustorium formation on the host plant, compared to later stages, when colony formation was active (Grell *et al*., 2003). Variants of *Egh16* exist in a variety of pathogenic fungi with necrotrophic, hemi-biotrophic and biotrophic lifestyles. In *Magnaporthe grisea* knock-out mutants of *GAS1* and *GAS2*, which are *Egh16* orthologous showed reduced penetration and lesion rates (Xue *et al*., 2002). Neither sequences were identified in the *n*-hexacosanal nor in the *n*-hexacosane SSH cDNA library that credit to the expression of *Egh16H* 22 minutes after inoculation. This is in accordance with the finding that *Egh16* plays a role

during later stages of conidia development and haustorium formation. In contrast to a previously constructed SSH cDNA library 4 hpi, where sequences were enriched in response to inductive barley leaf wax extract, *Egh16H1* and other isoforms were identified (Liebrich, 2008). The expression of putative *Egh16H1* precursor was monitored by semi quantitative RT-PCR over a period of 8 hpi on glass slides covered with barley leaf wax extract (Reisberg, 2009). The expression of *Egh16H1* was hardly detectable in conidia of conidiophores and in those that had three minutes contact with barley leaf wax *in vitro*. After 2 h of incubation the expression of the putative *Egh16H1* was dramatically increased and maintained constant over 8 hpi.

The SSH technique is a fast and efficient method for the identification of differentially expressed sequences and any information about the putative differentially expressed sequences is not necessary (Diatchenko *et al*., 1998). An essential prerequisite for obtaining a SSH cDNA clone library are several micrograms of high quality total or poly(A) RNA from two RNA populations. In the case of *Blumeria graminis* the Formvar® based *in vitro* system served as an ideal platform providing different chemical surface characteristics, for the generation of total RNA from conidia in sufficient amounts. The homogeneity of the surface coating, reproducible contact angles and layer quantities largely exclude experimental variabilities, in contrast to glass slides sprayed with cuticular waxes or single wax constituents (Zabka *et al*., 2008). This system and the presented experimental strategy turned out to be a suitable approach for the identification of putative genes that are expressed upon contact with *n*-hexacosanal or other cuticular wax constituents or mixtures of them. Pursuing detailed expression kinetics and construction of additional SSH cDNA libraries at later developmental stages might amend the understanding of surface dependent expressed genes during early pathogenesis of *B. graminis*.

6 Summarizing discussion

6.1 The Formvar® resin based *in vitro* system in comparison with other *in vitro* systems and native leaf surfaces

6.1.1 Contact angles

The coating of artificial surfaces with lipophilic compounds and total cuticular wax mixtures for *in vitro* bioassays is associated with several technical difficulties. Cuticular waxes are usually extracted in organic solvents (Jetter *et al*., 2006). The application of single droplets of a wax extract and the subsequent evaporation of the solvent, results in an irregular distribution of the wax compounds and in uncoated regions. This leads to variable results in bioassays investigating the germination and early developmental processes of phytopathogenic fungi. Coating of glass slides with lipophilic constituents and wax extracts for fungal bioassays using the Langmuir Schaefer technique results in a single, homogenous layer of chemical compounds, at which the polar head groups point to the air when the lipophilic compounds are transferred to glass slides (Reisige *et al*., 2006). However, the need of sophisticated technical equipment is disadvantageous for high throughput assays. When blends of waxes and single wax compounds dissolved in chloroform are sprayed with a glass chromatographic sprayer, distinctly reproducible experimental data are obtained (Zabka *et al*., 2008). Yet, the varying amounts deposited on the glass slides made it impossible to clearly distinguish between the influence of cuticular wax chemistry, absolute quantity and surface hydrophobicity on *B. graminis* conidia development (Zabka *et al*., 2008; Ringelmann *et al*., 2009).

The wettability of leaf surfaces is determined by the chemical constitution, the orientation of constituent molecules in the solid state and the surface roughness (Holloway, 1970). The wettability of leaf and artificial surfaces is measured as contact angle between the surface and the plane of a tangent to the surface of a water droplet (Shepherd & Griffiths, 2006). Hydrophilic surfaces typically have contact angles ≤ 90°, when the contact angle ranges between > 90° and ≤ 120° the surface is termed hydrophobic. Contact angles between > 120° and < 180° are typical for superhydrophobic surfaces. The higher the contact angle of a surface is the lesser is the wettability. The wettability of surfaces may also be influenced by the acidity of carboxylic acids present at the superficial interface (Knoll & Schreiber, 1998).

Spraying glass slides with *n*–hexacosane exclusively, resulted in contact angles of approximately 90° – 110°, whereas the amounts deposited on the glass slides varied strongly from 2 – 15 µg cm^{-2} (Appendix Figure 9-3a). This effect was

less pronounced when barley cuticular leaf wax extract, which is a complex mixture of lipophilic constituents, was sprayed on the glass slides. The contact angles were consistently about 108°, whereas the surface coating was ranging between 1 – 7 µg cm^{-2} (Appendix Figure 9-3b). When spraying dilutions of very-long-chain aldehydes, which were adjusted to a final concentration of 7×10^{-5} mol l^{-1} with *n*-hexacosane in the range of 0.01% (n/n) to 10% (n/n), it became obvious that the contact angle depends on different parameters like quality and quantity of the deposited compounds (Appendix Figure 9-4). At concentrations between 0.01% (n/n) – 0.1% (n/n) the contact angles were below 100° when the surface coatings were less than 5 µg cm^{-2}. Increased aldehyde concentrations up to 1% (n/n) and 10% (n/n) led to contact angles mainly above 110°, even when the surface coatings were below 5 µg cm^{-2}. When the very-long-chain aldehyde *n*-hexacosanal was sprayed on glass slides only, a contact angle above 80° was necessary to promote the germination and differentiation processes of *B. graminis* (Zabka *et al*., 2008). This critical contact angle may mainly be attributed to the fact that spraying lipophilic constituents on glass slides results in an inhomogeneous or patchy coating on the surface, leading to decreased contact angles. An increased absolute amount above 1 µg cm^{-2} deposited on the glass surfaces led to an increased overall surface hydrophobicity, which was not further raised by elevated amounts of *n*-hexacosanol or *n*-hexacosanal (Zabka *et al*., 2008). When mixtures of very-long-chain aldehydes and *n*-hexacosane were sprayed on glass slides the contact angles remained static at surface coatings that were larger than 5 µg cm^{-2} (Appendix Figure 9-4), which is accordance with Zabka *et al*. (2008).

To avoid the previously described variabilities in surface quality, a Formvar® resin based *in vitro* system was developed that provides a homogenous layer with consistent quantities deposited on a glass surface, and thus highly reproducible contact angles. The Formvar® coated surfaces supplemented with barley or wheat leaf wax extracts had contact angles in the range between 108° and 110°. This is in accordance with the properties of a smooth wax surface that has a contact angle of approximately 110° (Holloway, 1970). Glass slides coated with Formvar® containing maize leaf wax extracts had slightly decreased contact angles between 91° and 99°. It is known that hydrophobic properties of wax layers depend on the chemical constitution, which determines the micromorphology (Holloway, 1970). Hence, the slight reduction of surface hydrophobicity might be explained by quantitative alterations in *n*-alkanols, alkanes, fatty acids and alkyl esters of maize leaf wax compared to that of wheat and barley. The overall contact angles were decreased significantly, when glass slides were coated with polar wax constituents solely, such as primary alcohols or very-long-chain fatty acids. The addition of different wax constituents to the *n*-hexacosane, like very-long-chain aldehydes, primary alcohols,

very-long-chain fatty acids and wax esters up to a molar ratio of 10% did not further influence the contact angles.

Glass slides coated with Formvar® resin in combination with complex cuticular waxes or single wax constituents showed a typical surface microstructure. When extracted wheat or barley leaf waxes were added to the 0.5% Formvar® resin solution, a regular pattern of small protrusions with a plate-like appearance were visible on scanning electron micrographs. The protrusions were much less pronounced when the slides were coated with Formvar® resin and *n*-hexacosane or with mixtures of *n*-hexacosane and *n*-hexacosanal. *In vitro* primary alcohols form horizontally orientated platelets on non-polar surfaces (Koch *et al*., 2006). Thus, the cuticular waxes of barley and wheat may form horizontal platelets on a non-polar glass slide by mainly consisting of polar primary alcohols. In contrast, when glass slides were coated with a polyvinylpyrrolidone (PVP) layer prior to dipping into the Formvar® solution supplemented with extracted barley leaf wax, a layer without regularly distributed plate-like shaped protrusions was formed (Appendix Figure 9-6a). The PVP polymer film provides a more polar substratum for the Formvar® coating than glass, since PVP is partially loaded due to the resonance effect between the nitrogen and oxygen residue of the functional vinylpyrrolidone moiety (Prause, 2003). Hence, here the waxes are embedded into a more polar environment compared to the *in vitro* system consisting of glass and Formvar® resin only. This may lead to an altered microstructure due to modified spatial orientation of the wax constituents.

6.1.2 Effects on *B. graminis* conidia

When *B. graminis* conidia were allowed to germinate on a thin membranous film of Formvar® resin on water agar, the emergence and growth of germ tubes was not inhibited (Kobayashi *et al*., 1991). The same behaviour of *B. graminis* conidia was observed on glass slides coated with a thin Formvar® film only, but 90% of the differentiated conidia (agt + app) were desiccated after 16 hours of incubation (Chapter 2). Desiccated conidia were not turgid and the surfaces appeared wrinkled by visual inspection under the light microscope. The surface hydrophobicity of the Formvar® coated glass slides was relatively low (contact angle 70°), which is below the critical value reported to be necessary for successful differentiation of *B. graminis* conidia on an inductive surface (Zabka *et al*., 2008). Likewise, the absence of water supply in terms of an agarose block beneath the Formvar® might lead to desiccation of the conidia. Additionally, the low surface hydrophobicity of the substratum could be disruptive for germ tube growth, since barely leaves usually provide a hydrophobic surface (Zabka *et al*., 2008; Chapter 2). The surface of *B. graminis* conidia itself converts from a hydrophobic to hydrophilic state by release

of the conidial exudate during germination and differentiation (Nicholson *et al*., 1993). The hydrophilicity of the substratum at the infection site is increased by the conidial exudate in order to convert the naturally hydrophobic leaf surface, which is considered as an important event for successful conidia maturation (Nicholsen *et al*., 1993). Therefore, after increasing the surface hydrophobicity by addition of cuticular waxes or the alkane *n*-hexacosane to the 0.5% Formvar® solution (contact angles > 100°), conidia in the agt and app stage were never desiccated in great quantities. This experimental setup finally allowed the establishment of a stable bioassay for studying the impact of single wax components and foliar waxes on the prepenetration processes of *B. graminis*.

In comparison with native leaf surfaces, glass slides coated with Formvar® resin supplemented with barley or wheat leaf wax extracts, showed decreased germination and differentiation rates. While on Formvar®/wax coated glass slides six times more conidia remained in the appressorial germ tube stage, the amount of conidia that formed mature appressoria was four times smaller than on native leaf surfaces. The germination and differentiation rates of conidia inoculated on glass slides sprayed with barley leaf wax or on Formvar®/barley leaf wax coated glass slides did not differ significantly. Advantageously, the amount of desiccated and broken conidia was significantly reduced on the Formvar®/wax coated slides. This might be a consequence of the uniform coating, even though very low amounts of wax was deposited on the surface (0.5 µg cm^{-2}) Hence, the Formvar® resin based coating provides highly reproducible surface qualities and thus supplies a reliable experimental setup for bioassays compared to the spraying method. The surface topology and the physical microstructure are not essential cues and play a minor role in the surface recognition process of *B. graminis* conidia for germination and the development of mature infection structures (Carver & Thomas, 1990; Rubiales; *et al*., 2001; Zabka *et al*., 2008; Ringelmann *et al*., 2009) With improvements of the Formvar® resin based *in vitro* system further increased appressorium formation rates of *B. graminis* conidia were achieved. A Formvar® membrane containing the inducing barley leaf wax in combination with a layer of water agar underneath led to appressorium formation rates up to 70%, which was almost as high as on barley epidermal strips, where appressorium formation rates of >70% – 80% were achieved. In comparison with epidermal strips, the appressorium formation rates of *B. graminis* conidia were larger than 80% on native leaves, 16 h after incubation. The appressorium formation rates obtained with the Formvar®/wax membrane/agar system were higher than reported for similar setups based on inductive cellulose membranes, which might be a consequence of providing additionally inducing cuticular waxes (Kobayashi *et al*., 1991; Kinane *et al*., 2000). The primary germ tube of *B. graminis* conidia may take up water from the host plants apoplast during the prepenetration phase (Carver & Bushnell, 1983). The

Formvar®/wax membrane is estimated to be about 200 nm in thickness (Appendix Figure 9-6b), possibly thin enough to be penetrated by the primary germ tube, which could lead to an improved water supply. The putative increased water uptake of *B. graminis* conidia might be beneficial for the establishment of an increased turgor in the appressorium cell, which is a prerequisite for mature appressoria (Pryce-Jones *et al*., 1999).

The Formvar®/wax membrane system also allows the application of hydrophilic compounds or bioactive pharmaceuticals, but without immersion of conidia in aqueous solutions. Free water impedes the germination process of *B. graminis* conidia and leads to conidial burst when aqueous solutions are applied during the early differentiation stages (Manners & Hossian, 1963; Sivapalan, 1994, Bindslev *et al*., 2001). The advantage of an *in vitro* system that permits the additional application of water soluble compounds makes bioassays possible, which exclude possible interferences of the plants metabolism. The integration of additional cuticle derived factors could further increase the appressorium formation rates of *B. graminis* conidia in the Formvar® wax membrane/agar system of maximal 70%, similarly high as on barley leaves (>80%). This could be achieved by supplementing cutin monomers to the wax membrane that were reported to induce appressorium formation *in vitro* and are presumably released by secreted cutinases (Francis *et al*., 1996) or by addition of apoplastic organic compounds like carbohydrates. According to the experiments of this study 70% of *B. graminis* conidia differentiation (50% agt + 20% app) is triggered by leaf cuticular waxes. The appressorium formation rate is further increased to 60-70% when water is available, e.g. from an agarose block or from the leaf.

6.2 The impact of cuticular wax constituents on *B. graminis* prepenetration processes

6.2.1 Very-long-chain aldehydes *in vitro*

Very-long-chain aldehydes are common constituents of epi- and intracuticular waxes in many plant species (Post-Beittenmiller, 1996). The chain length distributions of very-long-chain aldehydes differed in the analyzed Poaceae species, which served as an ideal basis for *in vivo* and *in vitro* assays for studying prepenetration processes of *B. graminis* f.sp. *hordei* and f.sp. *tritici*. The very-long-chain aldehyde *n*-hexacosanal is the most prevalent aldehyde component of cuticular barley leaf wax (von Wettstein-Knowles, 1971; Giese, 1976; Baum *et al*., 1989; Tsuba *et al*., 2002), whereas the main aldehyde component of wheat leaf wax is *n*-octacosanal (Tulloch & Hoffmann, 1973; Bianchi *et al*., 1980). Quantitative analyses of barley and wheat leaf waxes revealed a relative amount of very-long-chain aldehydes of approximately 2%. The absolute amounts of cuticular leaf wax

coverage were between 8–11 µg cm^{-2}, which is consistent with previously published data (von Wettstein-Knowles, 1971; Bianchi *et al*., 1980; Zabka *et al*., 2008). Beside *n*-hexacosanal and *n*-octacosanal, the cuticular leaf waxes of barley and wheat contain *n*-tetracosanal (C_{24}), *n*-triacontanal (C_{30}) and *n*-dotriacontanal (C_{32}). Studies, where authors had the theory that very-long-chain aldehydes in general might have a substantial impact on the prepenetration processes of *B. graminis* were previously published (Tsuba *et al*., 2002; Zabka *et al*., 2008; Ringelmann *et al*., 2009)

In order to asses the impact of cuticular aldehydes on the prepenetration processes of *B. graminis* f.sp. *hordei*, glass slides were covered with a Formvar®/*n*-hexacosane mixture, which was spiked with different quantities of single, chemically synthesized very-long-chain aldehydes (C_{22}-C_{30}). Thereby, *n*-hexacosanal (C_{26}) and *n*-octacosanal (C_{28}) were most effective in promoting germination and differentiation of *B. graminis* conidia. *N*-hexacosanal significantly increased the appressorium formation rates at a very low concentration of 0.01% (n/n) in the dipping solution. Shorter and longer chain aldehydes, like *n*-docosanal (C_{22}), *n*-tetracosanal (C_{24}) and *n*-triacontanal (C_{30}) significantly enhanced the appressorium formation rates only at concentrations, which were two orders of magnitude higher. The appressorium formation rate at 10% (n/n) of *n*-triacontanal in the dipping solution was 65% compared to the value for *n*-hexacosanal at the same concentration. When pure *n*-hexacosanal was offered as substratum in previous *in vitro* studies, similar appressorium formation rates were observed (68%), but a detailed quantification was not provided (Tsuba *et al*., 2002). At concentrations of 10% (n/n) and 1% (n/n) of very-long-chain aldehydes in the dipping solutions, the appressorium formation rates were increasing in a chain-length dependent manner compared to *n*-hexacosanal (C_{26}) according to the following succession: $C_{22}<<C_{24}<C_{26}>C_{28}>>C_{30}$. When the proportions of aldehydes were further decreased in the dipping solutions to 0.1% (n/n) and 0.01% (n/n), only *n*-hexacosanal and *n*-octacosanal had a significant impact on appressoria formation. Lower differentiation rates of *B. graminis* conidia were also observed when *n*-triacontanal was offered as substratum compared to the rates obtained with *n*-hexacosanal (Tsuba *et al*., 2002).

The minimum proportion of very-long-chain aldehydes that has to be present in surface waxes, in order to significantly increase *in vitro* appressoria differentiation compared to the control, was determined at 1% (n/n) for the chain lengths C_{22}, C_{24} and C_{30}. The very-long-chain aldehydes *n*-hexacosanal and *n*-octacosanal are already capable to significantly enhance appressoria formation at proportions of 0.1% (n/n) in the wax coverage. Only *n*-hexacosanal is as potent that it can induce condida differentiation at proportions of 0.01% (n/n). At proportions between 1% (n/n) and 10% (n/n) very-long-chain aldehydes are able to induce differentiation rates as they are observed on barley wax extracts, irrespective of their chain lengths. This range reflects the amount of very-long-chain aldehydes, as

they occur in cuticular waxes on leaves of Poaceae. These findings also apply in the same manner to *B. graminis* f.sp. *tritici*.

6.2.2 Host-pathogen co-evolution

The host plants barley and wheat are infested by host-specific *formae specialis* of *B. graminis*. The waxes of barley and wheat have a species specific very-long-chain aldehyde distribution. *Hordeum vulgare* and *Triticum aestivum* have diverged from their last common ancestor 12 million years ago (Chalupska *et al*., 2008; SanMiguel *et al*., 2002). A comparative sequence analysis of *B. graminis* f.sp. *hordei* and *B. graminis* f.sp. *tritici* based on conserved non-gene coding regions of two unlinked loci revealed that both fungi diverged approximately 10 million years ago, which supports the idea of host-pathogen co-evolution (Oberhaensli *et al*., 2011). To asses, whether *B. graminis* f.sp. *hordei* and *B. graminis* f.sp. *tritici* have adapted to the aldehyde spectrum of their corresponding hosts, the germination and differentiation inducing capabilities of chemically synthesized very-long-chain aldehydes (C_{22}-C_{30}) were also tested for *B.graminis* f.sp. *tritici in vitro*. However, the most inducing very-long-chain aldehydes were *n*-hexacosanal (C_{26}) followed by *n*-octacosanal for both fungi. Furthermore, the shorter and longer chain aldehydes (C_{22}, C_{24} and C_{30}) were able to enhance germination rates and appressoria formation of *B. graminis* f.sp. *tritici* conidia in the same concentration and chain-length dependent manner, as observed for conidia of *B. graminis* f.sp. *hordei*. These findings indicate that certain adaptations to specific aldehyde profiles of barley and wheat wax as inducing signals during the prepenetration processes is very unlikely. These results seem coherent in the light of other phylogenetic analyses of *B. graminis* f.sp. *hordei* and f.sp. *tritici*, which do not support a co-evolution between the hosts and its powdery mildews. Based on ITS sequence comparisons the mildews *B. graminis* f.sp. *hordei* and *B. graminis* f.sp. *tritici* diverged about 4.6 million years ago (Inuma *et al*., 2007). Wyand & Brown (2003) included rye and oat powdery mildews in the phylogenetic tree calculation based on rDNA ITS and β-tubulin genes, suggesting a recent divergence of about 14,000 years for cereal mildews, which is too short for a host-pathogen co-evolution.

6.2.3 Primary alcohols, alkyl esters and fatty acids *in vitro*

Very-long-chain aldehydes are minor constituents of barley and wheat leaf wax and represent only 2% - 4% of total cuticular waxes (Tulloch & Hoffmann, 1973; Zabka *et al*., 2008). The most prevalent compound class are primary alcohols, which make up to 80% of the total cuticular wax. About 10% of the wax consists of alkyl esters, 2% *n*-alkanes and 1% very-long-chain fatty acids. The non-aldehyde wax constituents

were assayed at two different concentrations. Glass slides were covered with Formvar® dipping solutions supplemented with primary alcohols (C_{20}-C_{30}), alkyl esters (C_{40}-C_{44}) and fatty acids (C_{20}-C_{28}), respectively. Additionally, 10% (n/n) dilutions of the same compound classes were adjusted with *n*-hexacosane in the dipping solutions in equimolar proportions. Glass slides covered with alkanes exclusively (C_{24}-C_{33}) were prepared with dipping solutions of 6.8×10^{-4} mol l^{-1}, only. In all experiments, solely the C_{26} primary alcohol *n*-hexacosanol was able to induce significantly increased germination and differentiation rates (27% ng; 17% agt; 5% app) of *B.graminis* f.sp. *hordei* conidia, when the substrate was offered purely. The differentiation inducing effect of *n*-hexacosanol of about 20% is fully in accordance with Zabka *et al*. (2008), who applied *n*-hexacosanol by spraying onto glass slides. The inducing effect on the agt formation rates of *B. graminis* f.sp. *hordei* conidia were reduced to 9%, when *n*-hexacosanol was assayed in a mixture with *n*-hexacosane, but the rate of appressorium formation was not significantly increased, compared to the control. The impact of primary alcohols was also determined for *B. graminis* f.sp. *tritici* conidia, where a similar behaviour was observed. Only pure *n*-hexacosanol was capable of increasing germination and appressorium germ tube formation of *B. graminis* f.sp. *tritici* conidia (Appendix, Table 9-2) and not the most prominent primary alcohol *n*-octacosanol of wheat wax. The differentiation rates were generally larger for all tested substance classes compared to the rates of *B. graminis* f.sp. *hordei* conidia, which might be a strain specific effect. The assayed alkanes were not capable of promoting germination and differentiation of *B. graminis* f.sp. *hordei* conidia, too. These results are contradictory to the findings of Feng *et al*. (2009), who reported that the alkanes *n*-tetracosane (C_{24}) and *n*-pentacosane (C_{25}), presumably released by a secreted cuticular wax liquefying esterase, stimulate germination and differentiation of *B.graminis* f.sp. *tritici* conidia. Conidia of *B. graminis* f.sp. *tritici* did not respond with elevated germination and differentiation rates on Formvar®/alkane (C_{24}-C_{33}) covered glass slides (Appendix, Table 9-2).

6.2.4 Very-long-chain fatty acids do not inhibit germination and differentiation

The differentiation rate of *B. graminis* f.sp. *hordei* conidia was decreased to 70% on leaves of the barley mutant *cer*-yj.667 after epicuticular waxes were mechanically removed. The mutant has an increased level of very-long-chain fatty acids to 4% in its cuticular wax compared to less than 0.5% in the wildtype (Zabka *et al*., 2008). Furthermore, the alkyl ester content of the maize mutant 215B was distinctly increased to approximately 24% compared to 12% in the wild type. Therefore, the question arose, whether the elevated amount of very-long-chain fatty acids or alkyl

esters in the cuticular waxes inhibits the differentiation of *B. graminis* conidia. Glass slides were covered with dipping solutions containing either inducing barley leaf wax, or 1% *n*-hexacosanal and additionally very-long-chain fatty acids at a concentration of 10% (C_{20}-C_{28}). The tested fatty acids did neither inhibit germination, nor influence the differentiation rates of *B.graminis* conidia *in vitro*, which were induced by barley leaf wax or *n*-hexacosanal. The alkyl esters are mainly esterified C_{16}, C_{18}, C_{20}, C_{22} or C_{24} very-long-chain fatty acids and *n*-hexacosanol in barley or *n*-octacosanol in wheat, respectively (Tulloch & Weenik, 1969; Giese, 1975). The tested alkyl esters were esters of C_{20} fatty acid and C_{20} alcohol, of C_{20} fatty acid and C_{22} alcohol or C_{22} fatty acid and alcohol, which occur only in traces in the cuticular waxes of barley and wheat. *In vitro*, none of the applied alkyl esters were capable of significantly inducing germination and differentiation of *B. graminis* f.sp. *hordei* conidia. When wildtype maize leaves were sprayed with alkyl esters, the differentiation rates of *B. graminis* conidia were below 10%, similar as *in vitro,* on surfaces coated with alkyl esters. By addition of 2% *n*-hexacosanal to the alkyl ester spraying solution, the appressorium formation rates increased to 50%, which is comparable to those on barley leaves. Therefore, an inhibitory effect on *B. graminis* conidia germination and differentiation, due to fatty acids or alkyl esters, which are present in cuticular waxes, is not the case. The observed decreased differentiation rates on leaves of barley mutant *cer*-yj.667 are a result of the non-inducing properties of fatty acids and alkyl esters and not due to an inhibition. The data suggest that the identified compound classes of cuticular waxes on Poaceae leaves generally do not contain inhibitory compounds. Nevertheless, the existence of cuticular derived compounds that inhibit *B. graminis* conidia development cannot be excluded, but so far experimental evidence that supports this assumption, is not available.

6.2.5 Very-long-chain aldehydes *in vivo*

In order to asses the impact of very-long-chain aldehydes on the prepenetration processes of *B. graminis* on living plant leaves, inoculation experiments were performed with *glossy* mutants of maize. The non-host *Zea mays* was used for the experiments, since no barley mutants were available completely lacking very-long-chain aldehydes. The aldehyde fraction of maize leaf wax consists mainly of *n*-dotriacontanal (C_{32}) (Bianchi & Salamini, 1975; Bianchi *et al*., 1978; Avato *et al*., 1985). The absolute amount of cuticular waxes for the wildtype *Zea mays* lines Lambada and C836B, used in this study were about 8 µg cm^{-2} and the relative amounts of very-long-chain aldehydes accounted to 8%. The assayed maize *glossy* mutants *gl11* (215B) and *gl5* (428A) had a reduced cuticular wax coverage of 1.3 µg $cm^{-2}$and 4 µg cm^{-2}, respectively. The mutant line 215B (*gl11*) has a reduced

pool of acyl precursors, which leads to reduced flux into the decarbonylation pathway. Thus, the saturation level of the aldehyde forming reductase might not be achieved, which leads to a lack of aldehydes in the cuticular waxes (Avato *et al*., 1985). The glossy mutant line 428A (*gl5*) has a block in the fatty acyl reduction, which results in an increased aldehyde content and a decreased primary alcohol content (Bianchi *et al*., 1978). The total cuticular wax of the 428A (*gl5*) line used in this study, had an increased amount of about 20% of very-long-chain aldehydes, compared to the wild type.

The germination and differentiation rates of *B. graminis* conidia were drastically reduced on leaves of the very-long-chain aldehyde deficient *gl11* mutant line 215B, compared to the wildtype lines and the *gl5* mutant line 428A, which all contain very-long-chain aldehydes in their cuticular waxes. Nevertheless, on leaf surfaces as well as on wax extracts applied by the *in vitro* Formvar® system, the differentiation rates caused by 215B wax were about 30% and therefore much higher than the expected value for a very-long-chain aldehyde deficient surface according to the *in vitro* studies with non-inducing wax compounds, such as alkanes or primary alcohols (about 10% agt+app; Chapter 2). Probably, unidentified cuticular constituents of the maize leaf may induce the increased differentiation rate, which still has to be elucidated. Furthermore, *n*-hexacosanol in maize cuticular leaf wax might be responsible for the increased differentiation rates as it was observed *in vitro*. However, the amounts of *n*-hexacosanol in maize leaf waxes are very low. Conidia inoculated on native leaf surfaces of the *gl5* mutant line 428A or on glass slides coated with Formvar®/428A wax extract, which consists of about 60% of very-long-chain aldehydes, showed only slightly increased germination and differentiation rates compared to wildtype maize leaves or glass slides covered with wildtype maize leaf wax extracts. This indicates that a certain proportion of very-long-chain aldehydes is sufficient to trigger the maximum amount of conidia differentiation. When the proportion is further increased above 10%, no further conidial differentiation is achieved. This behaviour was also observed, when chemically synthesized very-long-chain aldehydes were offered in a mixture with *n*-hexacosane (Chapter 2) or when pure *n*-hexacosanal was used as substratum (Tsuba *et al*., 2002; Zabka *et al*., 2008; Chapter 3).

6.2.6 Mimicking of leaf wax phenotypes

To demonstrate that the wax chemistry is mainly responsible for the observed decreased germination and differentiation rates of *B. graminis* conidia on the *gl11* mutant line 215B, leaves were sprayed with wax from wildtype line C836B or with pure *n*-hexacosanal. Generally, coating leaves with film forming substances led to a reduction of *B. graminis* infestation (Sutherland & Walters, 2002; Walters,

2006). Spraying the *gl11* mutant line 215B with wildtype C836B leaf wax led to germination and differentiation rates as they were observed for untreated C836B leaves (40% agt and 40% app). Hence, the presence of very-long-chain aldehydes in the leaf wax was sufficient to fully restore conidia differentiation. Spraying wild-type C836B leaves with pure chemically synthesized *n*-hexacosanal even led to a significantly enhanced appressoria formation of 70%, which is almost the same value that was obtained when the conidia were inoculated on barley leaves (70%-80% app). Adding 2% (w/w) of *n*-hexacosanal to extracted *gl11* mutant line 215B wax extract *in vitro*, led to the same differentiation rates of *B. graminis* conidia as in experiments with barley leaf wax extract as substratum. This clearly demonstrates that providing *n*-hexacosanal or generally very-long-chain aldehydes in the cuticular wax *in vivo* is fully sufficient to promote conidia differentiation at high levels.

The non-inductive effects of primary alcohols, very-long-chain fatty acids and *n*-alkanes that were observed *in vitro* were also tested in experiments *in vivo.* Wildtype leaves of *Zea mays* cv Lambada were sprayed with the alkane *n*-docosane, the primary alcohol *n*-docosanol or the C_{44} alkyl ester behenic acid behenyl ester in order mask the inductive effect of very-long-chain aldehydes in the cuticular wax. All tested substances were able to reduce the germination and differentiation rates significantly. This effect was fully reverted by addition 2% of the very-long-chain aldehyde *n*-hexacosanal to the spraying solution of C_{44} alkyl ester.

These experiments strongly suggest that modified cuticular waxes of leaves, where very-long-chain aldehydes of the native leaf wax are masked, do not stimulate the prepenetration processes of *B. graminis* conidia. The findings about the behaviour of *B. graminis* conidia on waxes that provide very-long-chain aldehydes and those that provide other cuticular wax compound classes, clearly demonstrated that very-long-chain aldehydes are necessary and absolutely sufficient to induce the prepenetration processes *in vitro* and *in vivo*.

Growing of crop plants, which are devoid of very-long-chain aldehydes, could offer a new possibility of a durable resistance mechanism towards *B. graminis*. Recently it was demonstrated that cuticular leaf wax of *Medicago truncatula*, which had a drastically reduced amount of primary alcohols, lead to decreased appressoria formation in the rust fungi *Phakospora pachyrhizi* and *Puccina emaculata* (Uppalapati *et al*., 2012). A similar non-specific resistance mechanism towards *B. graminis* based on altered cuticular wax composition could be achieved by introduction of the *Gl11* phenotype into barley and wheat. However, wax biosynthesis of maize is not fully understood. Furthermore, the gene product of *Gl11* is not yet identified and cloned, but the locus is mapped in maize to chromosome 2 (Krakowsky *et al*., 2006).

6.3 Cuticular waxes and cutin provide chemical signals for phytopathogenic fungi

Phytopathogenic fungi need to penetrate the host plant tissue for successful parasitism. For directly penetrating the plant, phytopathogenic fungi have evolved specialized infection structures, the appressoria (Mendgen *et al*., 1996; Tucker & Talbot *et al*., 2001). The morphogenetic development of fungal spores on the plant surface, which finally leads to the appressorium formation, is triggered by physical and chemical signals provided by the plant cuticle (Table 6-1).

Considering own work and previous studies on the impact of cuticular waxes on prepenetration processes, three chemical compound classes trigger germination and differentiation of phytopathogenic fungal spores: very-long-chain aldehydes, primary alcohols and *n*-alkanes. Conidia of the obligate biotrophic ascomycete *Blumeria graminis* form significantly increased rates of appressoria in response to very-long-chain aldehydes in a dose and chain length dependent manner *in vitro* (Chapter 2). The very-long-chain aldehydes are also responsible for promoting the development of appressoria *in vivo* on leaf surfaces. When cuticular waxes are completely devoid of very-long-chain aldehydes, the germination and differentiation rates are significantly decreased compared to leaves, which contain very-long-chain aldehydes (Chapter 3). The urediniospores of the wheat stem rust fungus *Puccinia graminis* f.sp. *tritici* are able to form appressoria on glass slides covered with the very-long-chain aldehyde *n*-octacosanal, the main aldehyde present in wheat leaf wax (Reisige *et al*., 2006). Alkanes were also reported to significantly induce conidia differentiation of *B. graminis* f.sp. *tritici* (Feng *et al*., 2009). The primary alcohol *n*-hexacosanol is also capable of triggering germination and appressorium formation in *B. graminis* and the pea powdery mildew *Erysiphe pisi* (Chapter 2; Gniwotta *et al*., 2005). The primary alcohols *n*-octacosanol and *n*-triacontanol induce appressoria in the hemi-biotrophic rice blast fungus *Magnaporthe oryzae,* the rust fungi *Phakospora pachyrhizi* and *Puccina emaculata* (Uppalapati *et al*., 2012). Beside cuticular wax constituents also physical properties alone trigger conidia differentiation in *M. oryzae*, like high surface hydrophobicity and surface hardness (Jelitto *et al*., 1994; Lee & Dean 1994; Xiao *et al*., 1994). Surface hydrophobicity and a physical contact stimulus are also important for *B. graminis conidia*, since these cues determine the emergence site of the primary germ tube (Carver & Ingerson 1987). Several *n*-alkanols with chain lengths between C_{22} and C_{32} trigger the appressorium formation of the anthracnose fungus *Colletotrichum gloeosporioides* (Podila *et al*, 1993). Conidia of the necrotrophic grey mould ascomycete *Botrytis cinerea* may invade the plant tissue by direct penetration of cuticle and cell wall, but also through wounds and natural openings (Doehlemann *et al*., 2006). Generally, conidial germination is stimulated by fructose. However, in absence of nutrients conidia of *C. gloeosporioides* germinate

on glass slides covered with tomato or apple fruit wax (Doehlemann *et al*., 2006). Cutin monomers like hydroxy fatty acids, presumably released by fungal enzymatic activities also stimulate conidia differentiation in *B. graminis* (Francis et al., 1996), *M. oryzae* (Gilbert *et al*., 1996) and in the basidiomycete *Ustilago maydis* (Mendoza-Mendoza *et al*., 2009).

An attempt, to identify fungal sensors that may recognize plant cuticular surface signals was published in a comprehensive study by Liu *et al*. (2011) using the *M. oryzae* – rice pathosystem. Two proteins were identified that are involved in surface sensing during conidia germination and differentiation. MoMsb2 is involved in sensing surface hydrophobicity and cutin monomers, whereas MoSho1 is involved in sensing rice leaf waxes. MoMsb2 shares homologies to yeast Msb2, which is a surface mucin protein that is necessary for filamentous growth and MgMsho1 shares homologies with a yeast membrane sensor that is involved in osmoregulation. Double mutants of *Mosho1* and *Momsb2* were less efficiently able to form appressoria *in vitro* on glass surfaces coated with either *n*-octacosanol or *n*-triacontanol, the main alcohols present in rice leaf cuticular wax. When the cutin monomers 1,16 hexadecanediol or *cis*-9-octadecen-1-ol were added to the conidia suspension incubated on glass slides, *Mosho1* mutants formed germ tubes and appressoria efficiently, but not in the *Momsb2* and *Mosho1/Momsb2* double mutants. Another transmembrane protein family, Mopth11 is also involved in surface sensing (DeZwaan *et al*., 1999). Pth11 is needed for the activation of appressoria differentiation in response to inductive surface cues, such as the cutin monomer 1,16-hexadecanediol in combination with surface hydrophobicity. The application of exogenous cAMP is able to restore the defects of *Mopth11* mutants, such as appressorium formation and establishment of pathogenicity *in planta* due to the activation of downstream protein kinase C (DeZwaan *et al*., 1999). The involvement of several proteins, which respond to the same inductive cues, leads to the assumption that surface sensing mechanisms are redundant in *M. oryzae*. Downstream cAMP dependent protein kinase A (PKA) signalling is also of importance for germination and differentiation in *B. graminis* conidia in response to exogenous inducing surfaces, like cellulose membranes and barley epidermis (Hall *et al.*, 1999). Measurements of cAMP contents in *B.graminis* conidia revealed a biphasic increase of endogenous cAMP 14 min and 4 h after inoculation (Hall & Gurr, 2000; Kinane *et al*., 2000).

Table 6-1 Overview of plant pathogenic fungi developing infection structures in response to chemical properties of the plant cuticle.

Pathogen		Host plant	Cuticular origin	Chemical compound classes	Identified inducing constituents	References
Powdery mildew	*Blumeria graminis* f.sp. *hordei*	Barley (*Hordeum vulgare*)	Wax	Very-long-chain aldehyde	*n*- docosanal (C_{22}); *n*-tetracosanal (C_{24}); *n*-hexacosanal (C_{26}); *n*-octacosanal (C_{28}); *n*-triacontanal (C_{30}); *n*-dotriacontanal (C_{32})	this work; Tsuba *et al*., 2002; Zabka *et al*., 2008; Ringelmann *et al*., 2009
			Wax	Primary alcohol	*n*-hexacosanol (C_{26})	
			Cutin	Hydroxy fatty acid	*cis* -9,10-epoxy-18-hydroxyoctadecanoic acid 8,16-dihydroxyhexadecanoic acid	Francis *et al*., 1996
Powdery mildew	*Blumeria graminis* f.sp. *tritici*	Wheat (*Triticum aestivum*)	Wax	Very-long-chain aldehyde	*n*-docosanal (C_{22}); *n*-tetracosanal (C_{24}); *n*-hexacosanal (C_{26}); *n*-octacosanal (C_{28}); *n*-triacontanal (C_{30}); *n*-dotriacontanal (C_{32})	this work
			Wax	Primary alcohol	*n*-hexacosanol (C_{26})	this work
Powdery mildew	*Blumeria graminis* f.sp. *tritici*	Wheat (*Triticum aestivum*)	Wax	Alkane	*n*-docosane (C_{22}); *n*-tetracosane (C_{24}); *n*-pentacosane (C_{25})	Feng *et al*., 2009
Powdery mildew	*Erisyphe pisi*	Pea (*Pisum sativum*)	Wax	Primary alcohol	*n*-hexacosanol (C_{26}) *n*-octacosanol (C_{28})	Gniwotta *et al*., 2005
Rice blast	*Magnaporthe oryzae*	Rice (*Oryza sativa*)	Wax	Alcohols, alkyl esters, alkyl aldehydes	unknown	Uchiyama & Okuyama, 1989
			Wax	Primary alcohol	*n*-docosanol (C_{22})	Hedge & Kolattukudy, 1997
				Fatty acid	*n*-docosanoic acid (C_{22})	
				Alkane	docosane (C_{22})	
			Wax	Primary alcohol	*n*-octacosanol (C_{28}); *n*-triacontanol (C_{30})	Liu *et al*., 2011
			Cutin	Hydroxy fatty acid	*cis* -9,10-epoxy-18-hydroxyoctadecanoic acid 9,10,18-trihydroxyoctadecanoic acid	Gilbert *et al*., 1996
			Cutin	Unsaturated fatty alcohol	*cis* -9-octadecen-1-ol	
			Cutin	Alkanediol	1,16-hexadecanediol 1,18-octadecanediol 2,15-hexadecanediol	
			Cutin	α,ω-dialdehyde	1,16-hexadecanedial	
Stem rust	*Puccinia graminis* f. sp. *tritici*	Wheat (*Triticum vulgare*)	Wax	Very-long-chain aldehye	*n*-octacosanal (C_{28})	Reisige *et al*., 2006
Rust	*Phakopsora pachyrhizi*	Soybean (*Glycine max*)	Wax	Primary alcohol	*n*-triacontanol (C_{30})	Uppalapati *et al*., 2012
Rust	*Puccinia emaculata*	Switchgrass (*Panicum virgatum*)	Wax	Primary alcohol	*n*-triacontanol (C_{30})	Uppalapati *et al*., 2012
Anthracnose	*Colletotrichum gloeosporioides*	Avocado (*Persea americana*)	Wax	Primary alcohol	*n*-docosanol (C_{22}); *n*-tetracosanol (C_{24}); *n*-hexacosanol (C_{26}); *n*-octacosanol (C_{28}); *n*-triacontanol (C_{30}); *n*-dotriacontanol (C_{32})	Podlla *et al*., 1993
Grey mould	*Botrytis cinera*	Broad host range: vegetables, fruit crops	Waxes of tomato unknown and apple		unknown	Doehlemann *et al*., 2006
Corn smut	*Ustilago maydis*	Matze (*Zea mays*)	Cutin	Hydroxy fatty acid	12-hydroxyoctadecanoic acid 16-hydroxyhexadecanoic acid	Mendoza-Mendoza *et al*., 2009

6.4 Morphogenesis and cell cycle are linked during *B. graminis* prepenetration processes

6.4.1 Mitosis and the prepenetration processes

Fungal conidia are resting reproductive structures, which only germinate under appropriate inductive conditions (Tucker & Talbot, 2001). In the case of plant pathogenic fungi, germination and early differentiation are stimulated by plant derived physical and chemical signals. Chemical signals that trigger *B. graminis* conidia germination and further differentiation are very-long-chain aldehydes of the host plant cuticular wax (Tsuba *et al*., 2002; Zabka *et al*., 2008; Ringelmann *et al*., 2009; Chapter 2 & 3) and cutin monomers (Francis *et al*., 1996). Prior to host plant

penetration, conidia have to undergo sequential events on the plant surface during the prepenetration phase, like activation of cellular metabolism, a period of polar hyphal growth associated with cell divisions and subsequently a switch from polar growth to appressorium differentiation. The successful coordination of replication, mitosis and finally cytokinesis is known for being a prerequisite during pathogenic development in the hemi-biotrophic plant pathogenic rice blast *Magnaporthe oryzae* and the anthracnose fungus *Colletotrichum gloeosporioides* (Nesher *et al.* 2008; Saunders *et al.* 2010ab).

Chapter 4 provides a detailed study about the temporal development of *B. graminis* during the prepenetration phase *in vitro*, on glass slides sprayed with barley leaf wax extract and *in vivo*, on barley leaf epidermal strips. Furthermore it was investigated, to which extent mitosis and morphogenesis is synchronized during appressoria formation in *B. graminis.* Resting *B. graminis* conidia exhibited a single nucleus in the centre of the cell. First, the nucleus was always translocated to the emergence site of the primary germ tube and later to the emergence site of the secondary germ tube. After swelling of the secondary germ tube, mitosis occurred between 5 and 5.5 hours post inoculation. On epidermal strips mitosis was never observed prior to appressorial germ tube formation, whereas on wax covered glass slides, mitosis was observed before and after swelling of the secondary germ tube. After six to seven hours one of the daughter nuclei migrated into the appressorium germ tube and a septum was formed. The separation of the two nuclei always occurred in the primordial conidium, prior to cytokinesis. Nuclear translocations of developing *B. graminis* conidia were already reported by Mc Keen *et al*. (1972a), but without describing the preceding mitosis. The spatial uncoupling of nuclear division and cytokinesis was also reported for *M. oryzae* (Saunders *et al*., 2010b), whereas in the fungus *C. gloeosporioides,* nuclear division and cytokinesis are coupled and occurs after primary hypha formation during the appressorium formation (Nesher *et al*., 2008). Conidia of *C. gloeosporioides* germinate in response to primary alcohols of cuticular waxes and hard hydrophobic surfaces (Podila *et al*., 1993). The uninucleate conidium undergoes a first round of mitosis followed by germ tube growth (Nesher *et al*., 2008). During germ tube elongation, two rounds of mitotic divisions occur, prior to appressorium formation. The detailed observations of nuclei during the prepenetration phase of *B. graminis*, suggest a synchronisation between morphogenesis and mitosis. On barley leaves the coordination between mitosis and morphogenesis was much higher, which may lead to the assumption that leaf derived signals or more favourable growth conditions may be responsible for maintenance of synchrony.

6.4.2 Inhibition of cell cycle

The eukaryotic cell cycle is highly regulated and entry in different phases is controlled by cell cycle checkpoints (Elledge, 1996). The coupling of infection structure development and cell cycle progression was investigated in detail for the rice blast fungus *M. oryzae* (Saunders *et al*., 2010a). Mitosis is necessary for appressorium formation and subsequent autophagic cell death in the primordial conidium is a prerequisite for disease establishment (Venault-Fourrey *et al*., 2006). Studies with DNA replication deficient mutants of *M. oryzae* demonstrated that the checkpoint for entry into S-phase is critical for initiation of appressorium formation (Saunders *et al*., 2010a). Hence, the recognition of inductive cues in *M. oryzae*, such as surface hydrophobicity, surface hardness, cutin monomers, cuticular waxes and absence of nutrients must occur early after contact with the plant surface, which leads to a rapid S-phase entry (Saunders *et al*, 2010a). Furthermore, the transition of G1/S-phase checkpoint is sufficient for appressorium development at later stages. Conidia of *M. oryzae* defective in formation of anaphase promoting complex (*bim1*^{F1763*}), which results in unseparated nuclei, developed fully melanized mature appressoria, but were unable to cause rice blast disease (Saunders *et al*., 2010a).

To investigate, whether mitosis is necessary for the appressorium differentiation in *B. graminis*, different chemical cell cycle inhibitors were applied, since generating conditional mutants of *B. graminis* is not yet possible. In this study the DNA synthesis inhibitor hydroxyurea (HU) was applied in order to interrupt S-phase, which inhibits the enzyme ribonucleotid reductase. Blocking DNA synthesis in *M. oryzae* with HU resulted in germ tube growth, but no further differentiation occurred and mitosis was inhibited (Saunders *et al*., 2010a). HU treated *B. graminis* conidia developed to the appressorium germ tube stage, possessing a single nucleus in the conidium. Blocking the cell cycle of *C. gloeosporioides* with HU prevented the first mitosis, but germ tube growth was not inhibited, similarly as it was observed for *B. graminis* conidia. Chemically induced cell cycle arrest during mitosis leads to continued growth of hyphae, although cellular division is impossible (Bachewich *et al*. 2005; Berman, 2006). However, HU treated conidia of *C. gloeosporioides* were able to form fully mature appressoria on plant onion epidermal cells (Nesher *et al*., 2008), which was never observed for *B. graminis* conidia incubated on barley epidermal strips.

In order to block M-phase benomyl was applied, which causes disassembly of microtubule cytoskeleton and prevents the separation of daughter nuclei (Bergen and Morris 1983; Spector *et al.* 1983; Li & Murray 1991). In this case, conidia of *B. graminis* developed until to the appressorial germ tube stage, whereas the undivided nucleus moved into the appressorial germ tube, approximately to the position where the septum usually is formed. When *C. gloeosporioides* conidia were treated with benomyl, only a short germ tube emerged and no further development

was observed (Nesher *et al*., 2008). In order to chemically inhibit the hyphal polar growth, conidia were treated with latrunculin A (LatA), which disrupts actin filament polymerization that finally prevents entry into M-phase (Rupeš *et al*. 2001). *B. graminis* conidia treated with LatA developed a primary germ tube, ceased further development of hyphae and remained uninucleate. Conidia of *C. gloeosporioides* did not enter in mitosis, too and developed no germ tube (Nesher *et al*., 2008). *B. graminis* conidia were still able to develop their primary germ tubes, maybe because the water soluble inhibitors are taken up over the primary germ tube after contact with the surface. The primary germ tube remains in the apoplast and may possibly be able to take up water and small molecules (Carver & Bushnell, 1983; Nielsen *et al*., 2000). The pgt emerges relatively fast after contact with an inductive surface (<30 min, Chapter 5), probably before LatA reaches its target in the cell.

Generally, the chemical approach applied for *B. graminis* does not allow to determine clearly, whether the cell cycle transition points are connected to the pathogenetic developmental program during the prepenetration processes, as it is possible with specific mutants (Saunders *et al*, 2010ab). Furthermore, the time point when the chemicals enter the cell cannot be determined exactly, because the contact with the inhibitors takes place after inoculation on the Formvar® wax membrane or the epidermal strips, since they are not directly applied in aqueous solutions. Therefore, it is not clear when the chemicals start to affect the biochemical process within the cell and whether cell cycle checkpoints are successfully passed or not. But nevertheless, the data suggest that complete DNA replication and mitosis are necessary prerequisites for appressorium formation, but not for the appressorium germ tube elongation and swelling. Hence, the morphogenetic development is independent of DNA replication and mitosis. However, as soon as inducing cuticular waxes or *n*-hexacosanal are sensed by the conidium, the morphogenetic development is triggered. As a consequence morphogenesis, replication, mitosis and cytokinesis follow, which are necessary for appressorium formation. Whether cuticular waxes are able to influence the cell cycle directly as a signal remains to be elucidated, but seems unlikely according to the present data, obtained so far. However, the presence of *n*-hexacosanal and barley cuticular wax induced the nuclear movement to the site of primary germ tube emergence, indicating cytological responses to cuticular waxes. Later during further development the nucleus migrated to the secondary germ tube. Nuclear migration during conidial development to the emergence site of the germ tube prior to mitosis was also described for the entomopathogenic ascomycete *Metarhizium anisopliae* (St. Leger *et al*., 1989).

6.5 Identification of genes expressed in response to *n*-hexacosanal during the prepenetration processes of *B. graminis*

Plant surfaces are covered with a membranous layer - the cuticle, which is composed of cutin, intra- and extracuticular waxes (Jetter *et al*., 2000). Conidia of *B. graminis* start to germinate rapidly upon contact with plant surfaces and follow a sequential morphogenesis, which results in formation of mature appressoria that are a prerequisite for host plant infestation (Tucker & Talbot 2001). Hence, the first contact of the conidium with the host plant surface takes place in an environment mainly dominated by cuticular waxes. Cuticular wax constituents that mainly trigger germination and differentiation of *B. graminis* conidia, in a dose and chain length dependent manner, are very-long-chain aldehydes (Chapter 2 & 3). The initial surface recognition may lead to the expression of genes, which are involved in regulating morphogenesis and the establishment of appressoria. Chapter 5 presented a strategy to identify potential candidate genes, which are specifically induced by *B. graminis* conidia after contact with inducing wax constituents.

Very-long-chain aldehydes occur with 2-4% in the cuticular wax of barley and wheat leaves, whereas the most inductive aldehyde is *n*-hexacosanal (1% in barley leaf wax). In order to identify genes that are induced by *n*-hexacosanal, an experimental approach based on suppression subtractive hybridization (SSH) was conducted. As non-inductive surface glass slides were coated with the alkane *n*-hexacosane (Chapter 2). In order to provide an inductive surface *n*-hexaocsane was supplemented with the very-long-chain aldehyde *n*-hexacosanal. The release of single conidia from the conidiophores, the transition to the stimulated airborne state and subsequently landing on a surface are critical events, which occur prior to substratum recognition (Carver *et al*., 1996). Generally, comparisons of gene expression, and also proteomics, were usually done with conidia of different developmental stages, in comparison with conidia that were directly harvested from the conidiophores of barley leaves (Both *et al*., 2005ab; Bindschedler *et al*., 2009, 2011). The inoculation process on the non-inductive surface is a crucial step in the experimental procedure, because these conidia are also released from the conidiophores and have contact to a hydrophobic surface. This treatment enhanced the chance to exclude an enrichment of transcripts in the *n*-hexacosanal cDNA library that are induced by the process of conidia release or contact with a hydrophobic surface. Consequently, neither the *n*-hexacosanal, nor the *n*-hexacosane cDNA library contained an appreciable number of clones that carried transcripts related to cellular primary metabolism, although metabolic genes quantitatively constitute most of the transcripts in *B. graminis* conidia (Both *et al*., 2005a).

In the *n*-hexacosanal SSH cDNA library a transcript was confirmed by RT-qPCR that was 3-fold increased on Formvar® *n*-hexacosanal coated surfaces and 20-fold

induced on waxed coated surfaces and on barley leaves in comparison with *n*-hexacosane coated surfaces 22 min after inoculation. The cDNA sequence was cloned by 3' and 5' RACE (random amplification of cDNA ends) and named aldehyde responsive transcript A1. The cloned cDNA is 1495 bp in length and four putative open reading frames (ORFs) were identified. Bioinformatic analyses did not reveal any putative functions or functional domains within the sequences. Nevertheless, 110 putatively homologous sequences of transcript A1 were found in the genome of *B. graminis* f.sp*. hordei* DH14 (E value <1.00E-25; Max score 625-206; Total Score 1162-747) and a multitude of homologous ESTs are expressed during all developmental stages (E value ≤5.00E-84; Maximum Identity ≥77%; Query coverage ≥29%). The corresponding genomic regions of the hits in *B. graminis* f.sp. *hordei* DH14 are annotated as repetitive elements. Additionally, a related sequence of transcript A1 was found in the genome of *B. graminis* f.sp. *tritici* (Accession HQ437160.1; E value 8E-24; Maximum identity 62%; Query coverage 100%). These findings lead to the hypothesis that transcript A1 might be a member of a group of genes that is expressed during the life cycle of *B. graminis*. However, the initial attempt of this part of the work, the identification of transcripts that are upregulated in expression in response to the most inductive very-long-chain aldehyde *n*-hexacosanal, was attained successfully. So far, the question about the function of the cloned transcript and whether a novel gene was identified, which is critical for *B. graminis* differentiation during the prepenetration, still has to be elucidated. However, it was demonstrated that indeed transcripts or putative genes do exist, which are stronger expressed in response to cuticular wax components.

It was demonstrated that the relative expression of transcript A1 is increased on barley leaves and on barley leaf wax extract, indicating that *in vitro* systems which provide the wax fraction are suitable tools for the identification of candidate genes that are responding to cuticular waxes. To address the question, whether the identified putative gene is characteristic for certain developmental stages, detailed expression analyses of transcript A1 on different inducing and non-inducing substrata could provide more information concerning the relevance during the prepenetration processes. Critical time points for further RT-qPCR analyses are after 2 hours post inoculation when conidia are germinated, after 6 hours when the appressoria maturation begins and after 9 hours when mature appressoria are formed (Chapter 5). Additionally, single cell expression analyses of single conidia of different developmental stages separated by laser dissection could give more detailed insights into the expression levels of individual cells (Chapter 4). By cellulose acetate stripping conidia are harvested of heterogeneous developmental stages. Single cells obtained by laser microdissection may provide sufficient amounts of RNA for RT-qPCR experiments (Kehr, 2003). The knockout of a homologous *n*-hexacosanal responsive transcript A1 related gene in pathogenic

fungi is not possible, since candidate genes of transcript A1 in any other fungi, beside *B. graminis* were not detected (Chapter 5). The knockout of the putative A1 gene in *B. graminis* is not yet possible due to the obligate biotrophy of the fungus and the lack of a stable transformation system. The overexpression of *B. graminis* genes in the related phytopathogenic fungus *Botrytis cinerea* might be a possibility to study a potential phenotype of the putative A1 gene (Yan *et al*., 2012). However, further screening of the SSH cDNA library will be necessary to identify further candidate cDNA sequences that have to be confirmed by RT-qPCR.

In this work a strategy identifying differentially expressed genes was followed. However, an approach for identification of different proteomic profiles of conidia inoculated on different substrata could lead to the identification of proteins that are involved in substratum recognition or in the prepenetration processes. Previous studies could obtain sufficient amounts of protein from the extracellular matrix of conidia that were inoculated on cellulose membranes (Nicholsen *et al*., 1988). The proteins were still enzymatic active. Protein preparations of fungal mycelium can be obtained by the cellulose acetate stripping method (Bindschdler *et al*., 2009). B. graminis conidia can also be harvested in sufficient amounts from artificial surfaces and from barley leaves (Chapter 5). The amount of protein that can be isolated from *B. graminis* conidia is sufficient for proteomic analyses (Noir *et al*., 2009).

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8 Abbreviations

aberr	aberrant
agt	appressorial germ tube
app	appressorium
Bgh	*Blumeria graminis* f.sp. *hordei*
Bgt	*Blumeria graminis* f.sp. *tritici*
BLAST	basic local alignment search tool
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
cDNA	copy DNA
CoA	Coenzyme A
CoASH	free Coenzyme A
cv	cultivar
DAPI	4',6-diamidino-2-phenylindole
deg	degree
DIG	digoxigenine
DMF	dimethylformamide
DMSO	dimethylsufoxide
DNA	desoxyribonucleic acid
DTT	dithiothreitol
EST	expressed sequence tag
f.sp.	*forma specialis*
G1/S-phase	gap1/DNA synthesis phase
g	gravity
h	hour
hpi	hours post inoculation
HU	hydroxyurea
kPa	Kilo Pascal
LatA	Lantrunculin A
LB	lysogeny broth
Mb	megabases
M-pahse	mitosis phase
min	minute
mJ	millijoule
pg	primary germ tube
RACE	random amplification of cDNA ends
RNA	riobonucleic acid
RNAi	RNA interference
RT	reverse transcription
s	second
SD	standard deviation
sgt	seconary germ tube
SMART	switching mechanism at 5' end of RNA template
S-phase	DNA synthesis phase
SSH	suppression subtractive hybridization
VLC	very-long-chain
X-Gal	bromo-chloro-indolyl-galactopyranoside

9 Appendix

```
>Bgh aldehyde responsive (direct) 1495bp
AACTCATCCTTTCGCTCCCAAGGGAGATCAGAATTTGTCACTTGGGTGATGTGACCATAGAGGAAATGGG
GGAGAAGCATGGCATCTGGAACACAAGTAAAGCTCCTCTAATTTTGATTTTAGGTGTGCTATTTCCTATT
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AGGATATCAGAGACAGGCAGAGACTATATGCTGACGTGCACGTACCATATGTACGCACGTCATCTTTCCT
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CATTGCTGGAAGCAAACTGTTGCGCGAAAATCTTGCAGACGAATATTTGGATAGACGGGCAGGGTGATGC
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```

Figure 9-1 cDNA sequence of aldehyde responsive transcript A1, cloned by 5’ and 3’ RACE**.**

```
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 Identities = 866/870 (99%), Gaps = 2/870 (0%)
 Strand=Plus/Minus

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Hexar1 61     AGGAAATGGGGGAGAAGCATGGCATCTGGAACACAAGTAAAGCTCCTCTAATTTTGATTT  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bgh14  12804  AGGAAATGGGGGAGAAGCATGGCATCTGGAACACAAGTAAAGCTCCTCTAATTTTGATTT  12745

Hexar1 121    TAGGTGTGCTATTTCCTATTCATGAGAAGAGAAGGTAGTGTGAAGGTGATCTAATGTAGA  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bgh14  12744  TAGGTGTGCTATTTCCTATTCATGAGAAGAGAAGGTAGTGTGAAGGTGATCTAATGTAGA  12685

Hexar1 181    GATGTAAGGAGTGGATTGGCCGCGAAGAGGAGGATATCAGAGACAGGCAGAGACTATATG  240
              ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Hexar1 241    CTGACGTGCACGTACCATATGTACGCACGTCATCTTTCCTAGGGTCAGGGTTAGGCGAGG  300
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Bgh14  12624  CTGACGTGCACGTACCATATGTACGCACGTCATCTTTCCTAGGGTCAGGGTTAGGCGAGG  12565

Hexar1 301    CCGTAATCTGTTCCCTTTTTGTCGTAACATGCGGATAGAGTGAGTTGGGCTGTGGGATAA  360
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Hexar1 361    ATCACCGGACCCCCCGGCTGGCTCCATCAGTCCTGTGCCTAAAGCGGTCATGGAGCTTTA  420
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Hexar1 421    GATAGGACTTTAACAAATACAAACAAACGCCTTAACCAGCCTAACACCCTTACTTGTCTT  480
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Hexar1 481    GTCTTTGTGGAGTGGTAACCTACTGTCCATAGAATACACTAGTGTTATGAAAGAGCTCGA  540
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continued on next page

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 Identities = 603/609 (99%), Gaps = 2/609 (0%)
 Strand=Plus/Minus

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Bgh14  11418  AATAATCTC  11410
```

Figure 9-2 Alignment of the aldehyde responisve transcript A1 cloned from *B. graminis* f.sp. *hordei* strain CC1 conidia with the genome sequence of Blumeria graminis f.sp. hordei strain DH14.

Sequences of *n*-hexacosanal SSH cDNA library clones

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Quantification of cuticular waxes and single wax constituents sprayed on glass slides

Single wax constituents or mixtures of wax constituents and barley leaf wax extracts were sprayed with a glass chromatographic sprayer onto Histobond® (Marienfeld, Lauda-Königshofen) glass slides. Of each batch five glass slides were washed off with chloroform and the amount was quantified by gas chromatography. Additionally the surface hydrophobicity was measured (Figure 9-3 and Figure 9-4). For the experiments with *B. graminis* conidia only glass slides with a contact angle larger than 90° were used.

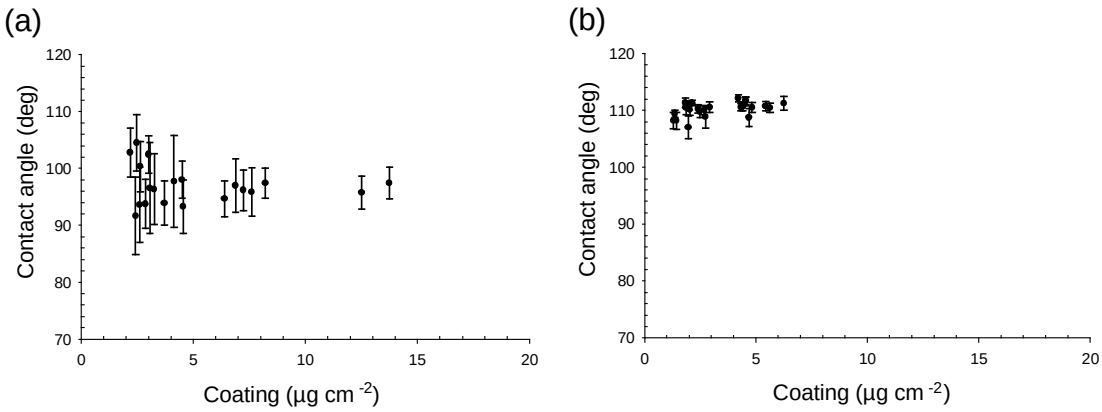

Figure 9-3 Quantifications of coatings and contact angles of glass slides sprayed with *n*-hexacosane and (a) barley leaf wax extract (b). Each data point corresponds to the quantification of one glass slide. Value of the contact angle for each glass slide is given as the mean ± SD (n=20 measurements).

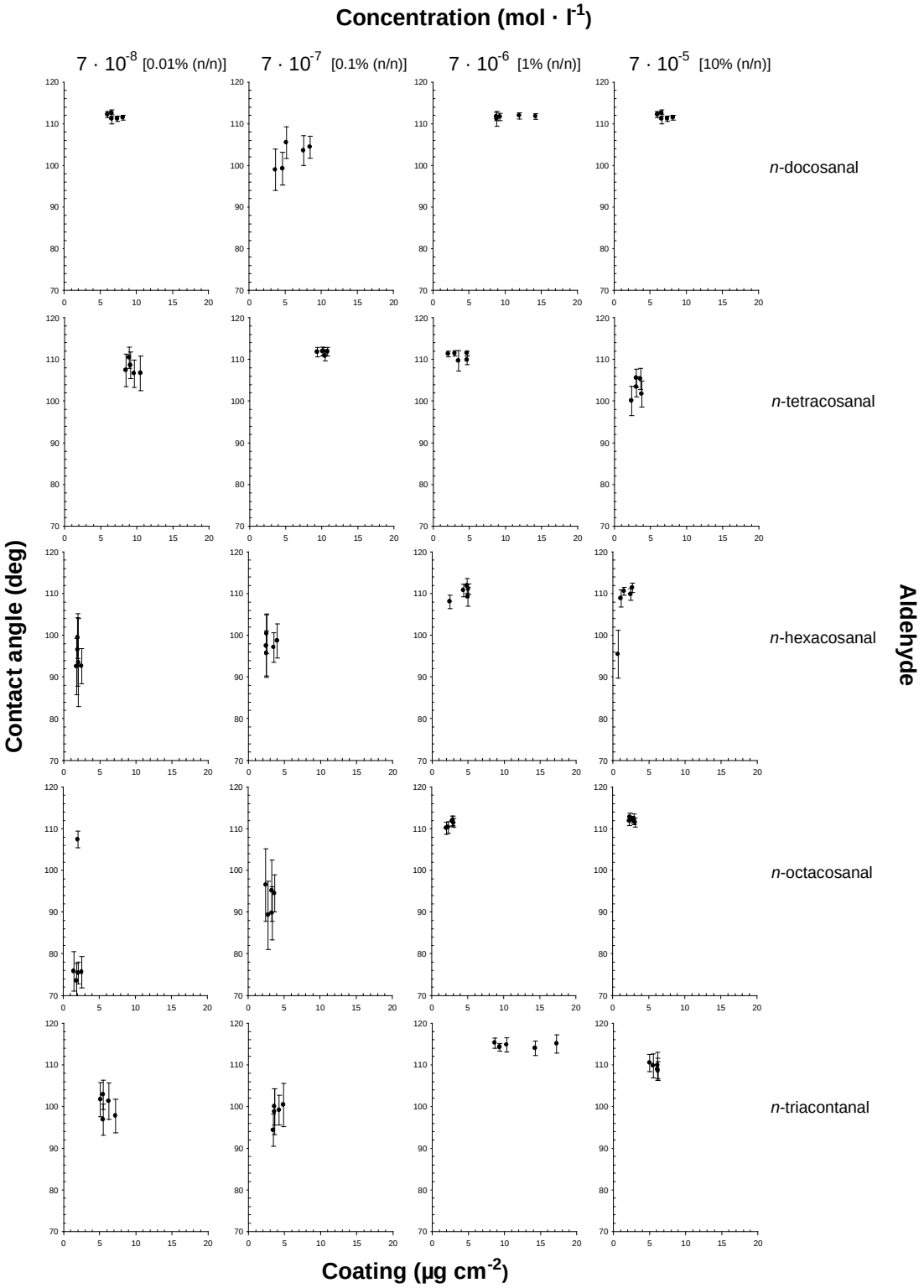

Figure 9-4 Coating of glass slides sprayed with different concentrations of aldehyde/*n*-hexacosane mixtures and the corresponding contact angles. Each data point corresponds to the quantification of one glass slide. Value of the contact angle for each glass slide is given as mean ± SD (n=20 measurements).

Quantification of cuticular waxes and wax constituents transferred by Formvar® resin based dipping solutions to glass slides

For estimation of the amount transferred to one glass slide, dipping solutions with 0.5% Formvar® resin (w/v) were prepared with different concentrations of the chloroform soluble reference dye Sudan III (M=278.32 g mol^{-1}; Sigma Aldrich, Steinheim). The absorption of Sudan III was measured photometrically at the wavelength of maximum absorption of 511 nm (Nanodrop, Peqlab, Erlangen) in a quartz cuvette. First a standardization curve with Sudan III only was generated (Figure 9-5a). Then different dipping solutions with different Sudan III concentrations were prepared. The total concentrations in each dipping solution were 6.8×10^{-4} mol l^{-1} (=190 µg ml^{-1}) and were adjusted with *n*-hexacosane when 5.1×10^{-4} mol l^{-1} (=142 µg ml^{-1}), 3.4×10^{-4} mol l^{-1} (=95 µg ml^{-1}), 1.7×10^{-4} mol l^{-1} (=47 µg ml^{-1}) and 6.8×10^{-5} mol l^{-1} (=19 µg ml^{-1}) of Sudan III were used in a dilution series (Figure 9-5b, x-axis). Ten glass slides were covered with each dipping solution of the dilution series and were subsequently washed off in 2.5 ml chloroform. To estimate the amount of Sudan III transferred by one dip onto the slide in ng cm^{-2}, the concentrations of Sudan III in the dipping solutions were plotted against the coatings calculated according to equitation (1). According to equitation (2) the amounts transferred by one dip were calculated for all chemical compound classes and dilutions used in this study. Equitation (3) and (4) indicate the calculations for preparation of equimolar dilutions. Table 9-1 gives a detailed overview of the concentrations and absolute amounts of all dipping solutions according to their chemical compound class that were prepared in this study.

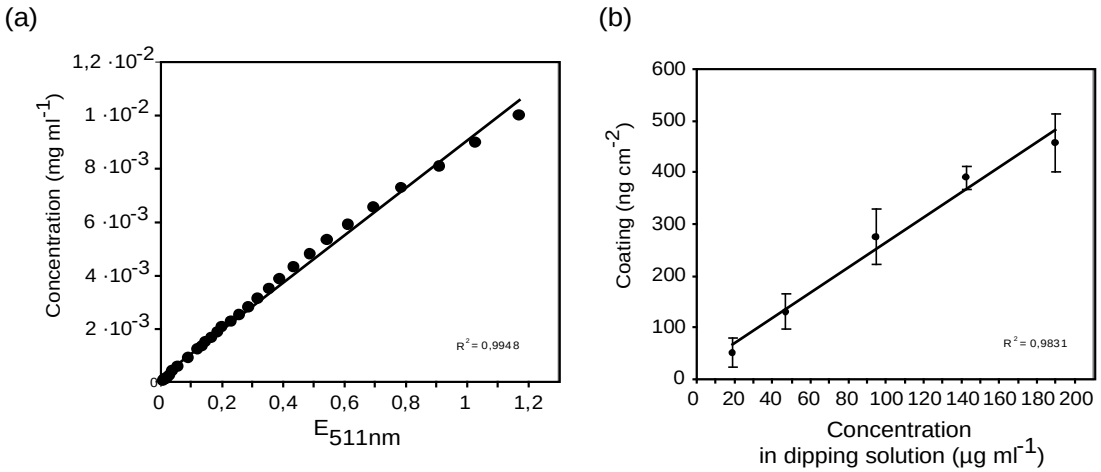

Figure 9-5 Standardization curve for Sudan III (a) and calculated amount of Sudan III transferred by a single dip onto a glass slide (b). The maximum Sudan III concentration in the dipping solution was 6.8 10^{-4} mol l^{-1} (= 190 µg ml^{-1}).

Equitation for calculation of transferred amount of Sudan III per square centimeter in dependence of Sudan III concentration in the dipping solution

$$B_{Sudan} = \frac{(0.0089 \cdot E_{511nm} + 0.0002) \cdot V_W}{A} \quad (1)$$

B_{Sudan}: Coating (mg cm^{-2})
E_{511nm}: Extinction at 511 nm
V_w: Volume of Solvent = 2.5 ml
A: Surface of glass slide, both sides = 27.5 cm^{-2}

Equitation for calculation of transferred amount per square centimeter of a single compound in the dipping solution.

$$B = 0.0024 \cdot c + 0.0198 \quad (2)$$

B: Coating µg cm^{-2}
c: Concentration of compound in the dipping solution (µg ml^{-1})

Equitation for calculation of dipping solutions-dilutions.

$$m_{comp} = 6.8 \cdot 10^{-4} \frac{mol}{l} \cdot P_{comp} \cdot M_{comp} \cdot V_{total} \quad (3)$$

$$m_{Hex} = 6.8 \cdot 10^{-4} \frac{mol}{l} - (6.8 \cdot 10^{-4} \frac{mol}{l} \cdot P_{comp}) \cdot M_{Hex} \cdot V_{total} \quad (4)$$

m_{comp}: Mass of single compound (g)
m_{Hex}: Mass of *n*-hexacosane in the mixture (g)
P_{comp}: Molar ratio of the compound
M_{comp}: Molecular weight of compound (g mol^{-1})
M_{Hex}: Molecular weight of *n*-hexacosane (g mol^{-1})
V_{total}: total volume of dipping solution (ml)

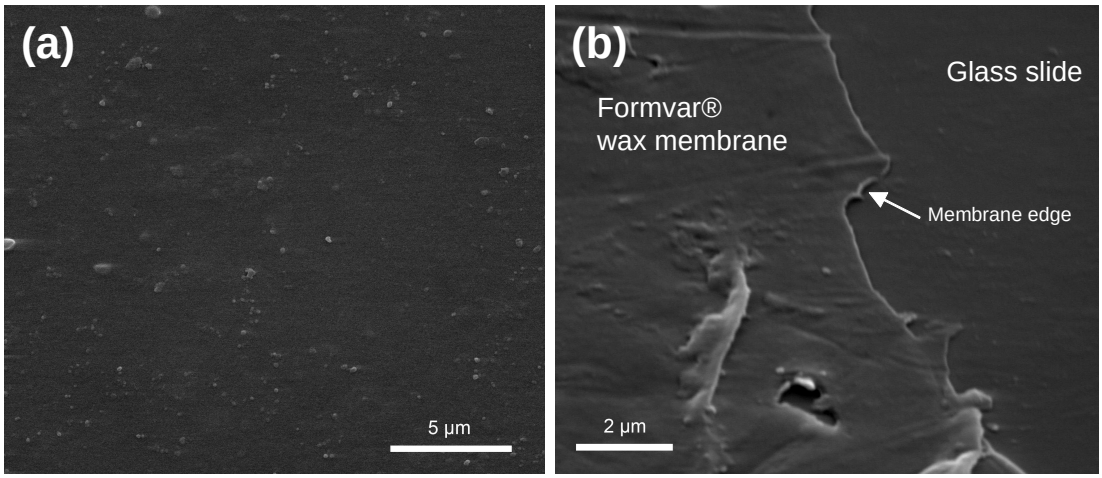

Figure 9-6 Scanning electron micrograph pictures of Formvar® wax membrane. A microscopic glass slide (Elka, Sondheim) was dipped in 1% (w/v) PVP40 solution and was subsequently dipped after drying in a 0.5% Formvar® resin solution in chloroform supplemented with total barley leaf wax extract (500 µg ml^{-1}). The membrane was floated on a water surface, transferred onto 1% Bacto water agar and was incubated for 16 hours at 20°C in darkness. The contact angle of the membrane on the water agar was 102 ± 7°. After incubation the membrane was transferred onto a positively charged glass slide (Menzel Superfrost Plus, Brauschweig) was freeze dried and subjected to SEM. The specimens were photographed at a rotation angle of 0° (a) 22° (b) to visualize the edge of the Formvar® wax membrane. The membrane is about 200 nm in thickness.

Table 9-1 Concentrations of the dipping solutions used for preparation of Formvar® coated glass slides with equimolar amounts of single wax constituents.

Compound class	Chain length	Concentration		Concentration *n*-hexacosane		Total concentration	
		(µg ml^{-1})	(mol l^{-1})	(µg ml^{-1})	(mol l^{-1})	(µg ml^{-1})	(mol l^{-1})
Alkanes	24	230	6.8×10^{-4}	0	0	230	6.8×10^{-4}
	25	240				240	
	26	250				250	
	27	260				260	
	28	270				270	
	29	280				280	
	30	290				290	
	31	300				300	
	33	310				310	
Primary alcohols	20	20	6.8×10^{-5}	225	6.1×10^{-4}	245	6.8×10^{-4}
	22	22		225		247	
	24	24		225		249	
	26	26		225		251	
	27	27		225		252	
	28	28		225		253	
	30	30		225		255	
	20	203	6.8×10^{-4}	0	0	203	6.8×10^{-4}
	22	222				222	
	24	242				242	
	26	261				261	
	27	271				271	
	28	280				280	
	30	300				300	
Alkyl esters	40	40	6.8×10^{-5}	225	6.1×10^{-4}	265	6.8×10^{-4}
	42	42		225		267	
	44	44		225		269	
	40	404	6.8×10^{-4}	0	0	404	6.8×10^{-4}
	42	424				424	
	44	443				443	
Fatty acids	20	22	6.8×10^{-5}	225	6.1×10^{-4}	247	6.8×10^{-4}
	22	23		225		248	
	24	25		225		250	
	26	27		225		252	
	28	29		225		254	
	20	215	6.8×10^{-4}	0	0	215	6.8×10^{-4}
	22	232				232	
	24	251				251	
	26	271				271	
	28	290				290	
Aldehydes	22	0,02	$6,8 \times 10^{-8}$	250	6.1×10^{-4}	250	6.8×10^{-4}
	24	0,02		250		250	
	26	0,03		250		250	
	28	0,03		250		250	
	30	0,03		250		250	
	22	0,22	6.8×10^{-7}	250	6.1×10^{-4}	250	6.8×10^{-4}
	24	0,24		250		250	
	26	0,3		250		250	
	28	0,3		250		250	
	30	0,3		250		250	
	22	2,2	6.8×10^{-6}	248	6.1×10^{-4}	250	6.8×10^{-4}
	24	2,4		247		250	
	26	2,6		248		250	
	28	2,8		248		250	
	30	3		248		250	
	22	22	6.8×10^{-5}	225	6.1×10^{-4}	247	6.8×10^{-4}
	24	24		225		249	
	26	26		225		251	
	28	28		225		253	
	30	30		225		255	

Table 9-2 Development of *Blumeria graminis* f.sp. *tritici* conidia on Formvar® coated glass slides supplemented with alkanes or primary alcohols

Substratum		Percentage of conidia					Contact angle (deg)
		ng	pgt	sgt	agt	app	
Wax (*Triticum aestivum*)		7 ± 3	4 ± 2	9 ± 1	56 ± 3	23 ± 3	110 ± 1
7×10^{-4} mol l^{-1} *n*-hexacosane		62 ± 5	25 ± 4	3 ± 1	9 ± 3	1 ± 1	114 ± 1
Concentration mol l^{-1}	**Chain length**						
Alkanes							
7×10^{-4}	24	59 ± 3^{a}	30 ± 4^{a}	3 ± 1^{a}	7 ± 2^{a}	1 ± 1^{a}	114 ± 1
	26	62 ± 5^{a}	25 ± 4^{a}	3 ± 1^{a}	9 ± 3^{a}	1 ± 1^{a}	113 ± 1
	28	52 ± 5^{a}	28 ± 4^{a}	4 ± 1^{a}	13 ± 5^{a}	2 ± 2^{a}	113 ± 1
	30	61 ± 8^{a}	23 ± 4^{a}	4 ± 2^{a}	11 ± 5^{a}	1 ± 1^{a}	113 ± 1
Primary alcohols							
7×10^{-5} †	20	58 ± 4^{a}	30 ± 3^{a}	2 ± 1^{a}	2 ± 1^{a}	2 ± 2^{a}	114 ± 1
	22	58 ± 5^{a}	29 ± 4^{a}	2 ± 1^{a}	4 ± 2^{ab}	3 ± 3^{a}	113 ± 1
	24	58 ± 4^{a}	31 ± 5^{a}	1 ± 1^{a}	3 ± 1^{a}	2 ± 1^{a}	113 ± 1
	26	46 ± 10^{a}	29 ± 2^{a}	4 ± 2^{a}	8 ± 3^{b}	8 ± 6^{a}	113 ± 1
	27	55 ± 3^{a}	30 ± 3^{a}	2 ± 1^{a}	5 ± 2^{ab}	4 ± 2^{a}	113 ± 1
	28	57 ± 6^{a}	28 ± 3^{a}	2 ± 1^{a}	4 ± 2^{ab}	3 ± 1^{a}	114 ± 1
	30	58 ± 5^{a}	27 ± 5^{a}	2 ± 1^{a}	5 ± 2^{ab}	3 ± 1^{a}	113 ± 1
7×10^{-4}	20	59 ± 3^{ab}	34 ± 2^{ab}	1 ± 1^{ab}	2 ± 2^{ab}	1 ± 1^{a}	49 ± 3
	22	56 ± 8^{ab}	35 ± 6^{ab}	1 ± 1^{ab}	2 ± 1^{ab}	2 ± 1^{a}	31 ± 6
	24	61 ± 2^{ab}	32 ± 3^{ab}	1 ± 1^{a}	1 ± 1^{a}	1 ± 1^{a}	38 ± 3
	26	34 ± 12^{c}	47 ± 8^{c}	3 ± 1^{b}	5 ± 2^{b}	3 ± 2^{a}	39 ± 4
	27	45 ± 4^{bc}	41 ± 2^{bc}	2 ± 1^{ab}	3 ± 2^{ab}	2 ± 3^{a}	51 ± 4
	28	56 ± 5^{ab}	35 ± 4^{ab}	1 ± 1^{ab}	1 ± 1^{ab}	1 ± 1^{a}	61 ± 2
	30	57 ± 5^{a}	28 ± 5^{a}	2 ± 1^{ab}	4 ± 2^{ab}	2 ± 1^{a}	86 ± 1

† Supplemented with *n*-hexacosane to a final concentration of 7×10^{-4} mol l^{-1}.

Values are means ±SD of five independent experiments. Different letters within a column, with the same concentration and same compound class, indicate significant differences ($P < 0.05$) determined in a one-way ANOVA followed by a Tukey *post hoc* test.

Publications and presentations

Publications

Hansjakob A, Riederer M, Hildebrandt U. 2011. Wax matters: Absence of very-long-chain aldehydes from the leaf cuticular wax of the *glossy11* mutant of maize compromises the prepenetration processes of *Blumeria graminis*. *Plant Pathology* **60:** 1151-1161.

Hansjakob A, Bischof S, Bringmann G, Riederer M, Hildebrandt U. 2010. Very-long-chain aldehydes promote *in vitro* prepenetration processes of *Blumeria graminis* in a dose- and chain length-dependent manner. *New Phytologist* **188:** 1039-1054.

Presentations

Congress "Botanikertagung": 18.–23.09.2011, Berlin, Germany. Poster " Absence of very-long-chain aldehydes from cuticular wax of *glossy11* mutant of maize compromises *Blumeria graminis* prepenetration processes"

9th International Mycological Congress (IMC9): 01.–06.08.2010, Edinburgh, Scotland. Poster "The impact of cuticular wax components on the prepenetration processes of the barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei*"

Congress "Botanikertagung": 06.–11.09.2009, Leipzig, Germany. Poster "Very-long chain aldehydes promote pre-penetration process of *Blumeria graminis* in a concentration and chain-length dependent manner"

Curriculum vitae

Affidavit

I hereby confirm that my thesis entitled “The role of cuticular waxes in the prepenetration processes of *Blumeria graminis* f.sp. *hordei*” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 22.05.2012
Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Der Einfluss kutikulärer Wachse auf die Präpenetrationsprozesse von *Blumeria graminis* f.sp. *hordei*“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 22.05.2012
Ort, Datum Unterschrift