The role of barley cell wall polysaccharides in host plant defence mechanisms against powdery mildew

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

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March 2016

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Abstract

- The cell wall is the first line of plant defence, presenting a barrier that protects cells from infectious pathogens in the surrounding environment. Plants respond dynamically to pathogen attack at the cell wall level by developing papillae at the infection site. In many plant/pathogen interactions, papillae formation is an important determinant of pre-invasion resistance by the host species. While many aspects of papillae are known, the cell wall components responsible for making papillae an effective barrier to fungal penetration are not fully understood. This project aimed to define the role of cell wall polysaccharides in the papillae-based penetration resistance mechanism of barley. Using the barley-powdery mildew host-pathogen system, papillae polysaccharide composition and the genetic factors responsible for their biosynthesis were examined.
- Here it is demonstrated that the major polysaccharides found in barley papillae are callose, arabinoxylan and cellulose. The papillae are layered with an inner core consisting of callose and arabinoxylan and an outer layer containing arabinoxylan and cellulose. A higher level of polysaccharide staining at non-penetrated papillae compared to the penetrated papillae was observed and this suggested that the polysaccharides are necessary components of the papillae-based penetration resistance mechanism of host plants.
- The members of the *Glucan synthase-like* (*Gsl*)gene family found in the barley genome have been characterised and identified and when *HvGsl6* was silenced this resulted in a loss of callose in the papillae and an increased rate of successful fungal penetration.
- A number of candidate genes from several glycosyltransferase families suspected to be associated with the biosynthesis of arabinoxylan in papillae have been identified. Transient down-regulation and up-regulation of the individual candidate genes using a biolistic DNA delivery system led to an altered level of susceptibility to powdery mildew. However, the highest levels of resistance were observed when GT43 (MLOC_54026) and the GT47

(MLOC_14407) genes were over-expressed together. These genes are putatively involved in arabinoxylan backbone biosynthesis.

- Furthermore, this PhD study also contributed to the characterisation of the role of the *HvCslD2* gene in non-host resistance, a project led by Dr. Patrick Schweizer, IPK, Germany. We showed that silencing of the *HvCslD2* gene in barley results in reduced cellulose accumulation in the papillae during powdery mildew infection, suggesting that *HvCslD2* is a key gene involved in cellulose biosynthesis in papillae.
- The association of high levels of arabinoxylan and cellulose deposition in papillae with the penetration resistance mechanism of the host plant, provides new targets for the improvement of papilla composition. The identification of the genes involved in the biosynthesis of each papilla component will provide new targets for the generation of novel crop lines with greater disease resistance.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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ACKNOWLEDGEMENTS

Completion of this doctoral dissertation was possible with the support of several people who so generously contributed to the work presented in this thesis. I would like to express my sincere gratitude to all of them.

First of all, I am extremely grateful to my PhD supervisors Professor Rachel Burton, and Professor Geoff Fincher for providing me all the supports and guidance throughout my PhD journey and for helping to complete the thesis. It has been an honour to work with you. Special mention goes to my enthusiastic supervisor Dr. Alan Little, who has been actively interested in my project, guided me to plan and execute the project, and helped me writing the manuscript. I am so grateful for his patience, motivation, enthusiasm, and guidance that, taken together, he has been a tremendous mentor for me.

I am thankful to our international project collaborator Dr. Patrick Schweizer, Pathogen genomics lab, IPK, Germany, and his group members Dr. Dimitar Douchkov and Stefanie Lück for giving me the opportunity to use their wonderful laboratory facilities and for contributing the RNAi experiments.

Special thanks to Dr Neil Shirley and Dr. Marilyn Henderson for their technical help and scientific advice in gene expression profiling analysis and electron microscopy, respectively. Similar, profound gratitude goes to Dr Matthew Tucker and Julian Schwerdt for many insightful discussion and scientific information.

I want to thank all the present and past members of the plant cell wall lab for creating such a fantastic working environment in the lab and for helping me in various times. Especially like to mention Natalie Betts, Yves, Kylie, Rohan, George, Ali, Ashley, Chao, Jelle, Wai Li and Kuok.

For the non-scientific side of my thesis, I am also hugely appreciative to David Mathew, Natalie Kibble and Emma Drew who went far beyond the call of duty for assisting me in numerous ways.

I express my sincere gratitude to the University of Adelaide for providing the ‘Adelaide Student International (ASI) scholarship’ to support tuition fees and living allowance and to ARC Centre of Excellence of Plant cell walls for the supplementary top-up scholarship.

I am also grateful to the Bangladesh Agricultural Research Institute (BARI), the Ministry of Agriculture, Bangladesh for providing me study leave to pursue this PhD program.

I am also very much indebted to my family, my mom, dad, brothers, mother-in-law and father-in-law, who always believe on me and supported me in every possible way to see the completion of this work.

Last but not least, I am greatly thankful to my devoted wife Jannat who has been by my side throughout my PhD, living every single minute of it. Without you, I would not have had the courage to board on this journey in the first place.

LIST OF PUBLICATIONS

1. **Chowdhury J**, Henderson M, Schweizer P, Burton RA, Fincher GB, Little A. 2014. Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated versus penetrated papillae on leaves of barley infected with Blumeria graminis f. sp. hordei. *New Phytologist* 204(3): 650-660.

2. **Chowdhury J**, Schober M, Shirley NJ, Jacobs A, Douchkov D, Schweizer P,. Fincher GB, Burton RA, Little A. Down-regulation of the *Glucan synthase-like 6* gene (*HvGsl6*) in barley leads to decreased callose accumulation and increased susceptibility to Blumeria graminis f. sp. hordei. Submitted to New Phytologist journal. Submission ID: NPH-MS-2016-21945.

3. Douchkov D, Lück S, Hensel G, Jochen Kumlehn J, Rajaraman J, Johrde A, Aghnoum R, Rehman S, Kopischke M, Fuchs R, Lipka V, Niks R, **Chowdhury J**, Little A, Geoff Fincher G, Bacic T, and Schweizer P. The cellulose-synthase like *CslD2* gene mediates host- and nonhost resistance in barley. Accepted to publish in New Phytologist journal. Submission ID: NPH-MS-2016-21786.

CHAPTER 1

GENERAL INTRODUCTION

Schematic structure of the thesis

This thesis comprises seven chapters. Chaper 1 (this chapter) provides a context for the research described in the thesis, identifies the knowledge gaps in the field of study and sets out the main aims of the research. Chapter 2 is a literature review providing a detailed background on host-pathogen interactions. Chapters 3, 4 and 5 describe the four major experimental approaches and their outcomes. Chapter 3 contains a manuscript that has been published in a peer reviewed journal (New Phytologist). The work contained in Chapter 4 has been submitted to a peer reviewed journal (New Phytologist) and the submitted version of the manuscript has been presented in this thesis. Chapter 5 has been presented in a manuscript style. Chapter 6 presents only the portion of the work that was completed in this PhD to support an investigation into the role of the *HvCslD2* gene, as led by Dr. Patrick Schweizer at the IPK in Germany. It is important to note that my contribution to Chapter 6 was reduced due to the large number of collaborating authors/lab groups involved. However, my contribution was deemed to be essential by the other authors and its inclusion in the thesis completes the original aims of this project. Chapter 7 includes a general discussion of the thesis as a whole and covers the broader concept of work including the implications/significance of the results, the contribution to knowledge, limitations and future research directions.

Each experimental chapter is separated with a statement of authorship for the paper stating the contribution of each author to the relevant study and a short linking text to establish the relationship between one chapter and the next. This thesis format is in agreement with the specification of thesis guidelines by the Adelaide Graduate Centre, Higher Degree by Research, The University of Adelaide, Australia. Figure 1 demonstrates the schematic structure of the thesis.

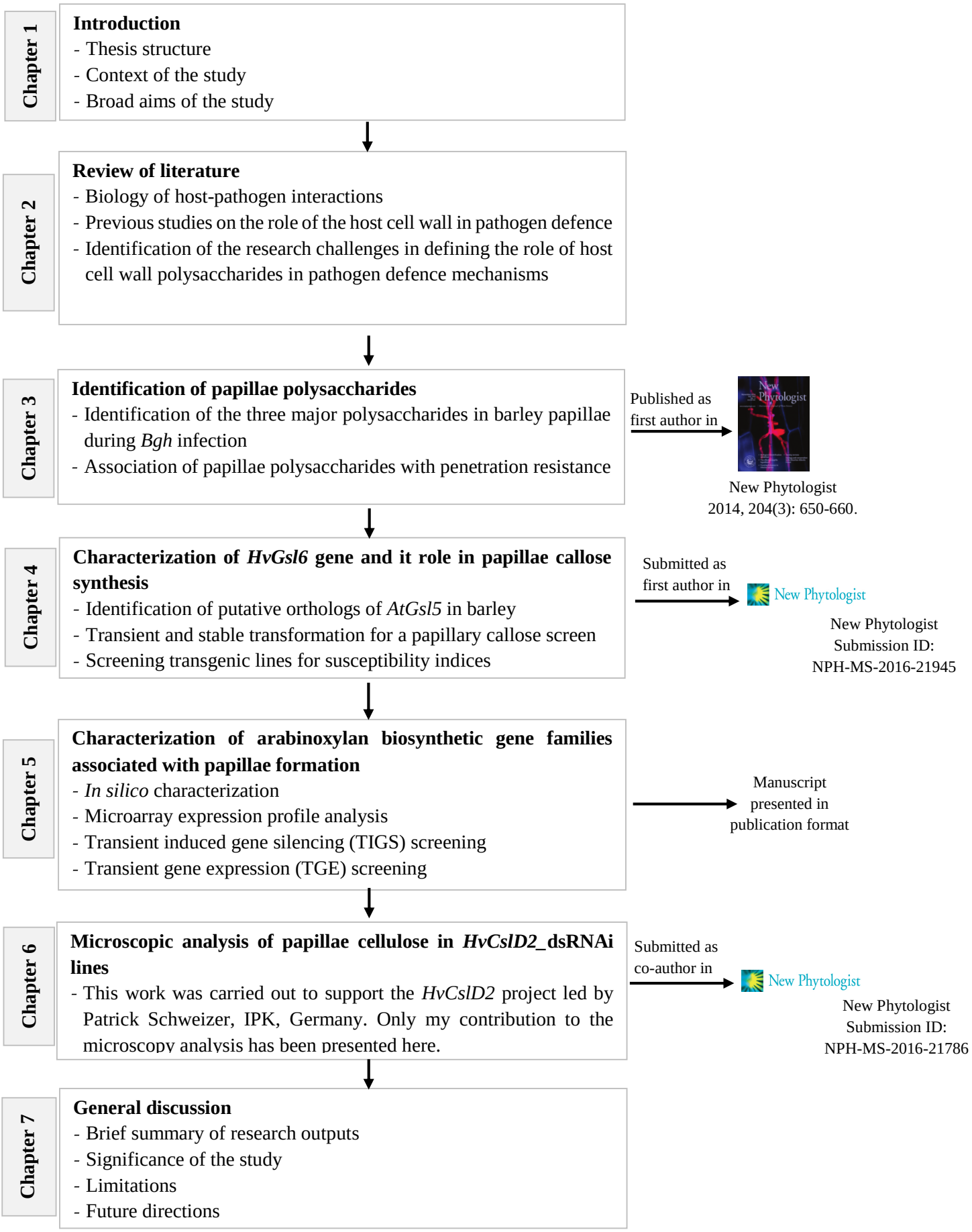

Figure 1. Schematic structure of the thesis

Context of the study

The cell wall is a complex structure composed of diverse polysaccharides, proteins, lignin and antimicrobial compounds (Caffall & Mohnen, 2009). Apart from its involvement in the normal growth and development of the plant, the cell wall has an essential role in plant defence. It is the first interface at which an interaction takes place between the plant and potentially pathogenic microbial organisms in surrounding environments. In the co-evolutionary battleground between plants and microbes over millions of years, plants have evolved a multi-layered defence system in which the cell wall serves multiple purposes.

The plant cell wall may serve as a pre-formed or passive structural barrier as well as an induced or active defence barrier. Microbes have to circumvent or penetrate the cell wall and other pre-formed barriers to establish the desired pathogenic relationship with host plants. The pathogenic microorganisms require appropriate host recognition strategies and the development of suitable infection structures and/or chemical weapons (Zentmyer, 1961; Turrà *et al.*, 2015). Failure to evolve appropriate infection structures and/or chemical weapons to breach the wall and other pre-formed structures, results in the microbe becoming non-pathogenic and/or non-adapted.

The host plant can also use the cell wall as an active defence barrier against those microbes that have evolved a mechanism of overcoming the pre-formed barriers. During infection, oligosaccharide elicitors are released from the wall of the host plant (damage-associated molecular patterns, DAMPs) or from the pathogen cell wall (pathogen-associated molecular patterns, PAMPs) as a result of partial hydrolysis of wall polysaccharides (Boller & Felix, 2009). Plants perceive these elicitors through plasma membrane immune receptors that trigger signalling cascades to activate numerous defence responses known as DAMP or PAMP triggered immunity (DTI or PTI) (Jones & Dangl, 2006). One common DTI or PTI-associated defence response is reinforcement of the cell wall in order to create more resistance to physical penetration and/or to enzymatic hydrolysis generated by the pathogens (Boller &

Felix, 2009; Ringli, 2010; Malinovsky *et al.*, 2014). Depending on the interaction type, the cell wall reinforcement process may occur in different ways, such as: rearrangement and cross-linking of pre-existing cell wall materials, incorporation of easily cross-linked polymerized materials into the existing cell wall and local apposition of new cell wall materials at the infection sites (Slusarenko *et al.*, 2012).

The local apposition of cell wall material to form structures known as papillae is an early defence response commonly used to impede penetration by potential biotrophic, hemi-biotrophic and bacterial pathogens (Bellincampi *et al.*, 2014). The micrometre-scale structure, formed at the site of infection, is often big enough to halt fungal penetration. Hence, in a number of non-host and host species, resistance is achieved at the pre-invasion stage due to the formation of papillae at the infection sites. However, the exact role of papillae is not well understood. They may act as a physical barrier that effectively halts pathogen penetration or slows down the penetration process so that other defence mechanisms can be activated before the infection takes hold (Stone & Clarke, 1992; Huckelhoven, 2005). Papillae also function as a chemical barrier that accommodates a variety of chemical weapons, such as antimicrobial toxins, phytoalexins and defensins, which are needed to directly attack the pathogens or to inhibit cell wall degrading enzymes produced by the pathogens (Albersheim *et al.*, 2011).

It has been hypothesized that papillae-mediated penetration resistance is the ability of a host genotype to develop effective papillae with the right composition at the right time. Therefore, understanding papillae composition and the factors involved in the development of an effective papillae, have been a major focus for researchers over a long period of time. While some of the physiochemical changes that occur during papillae-mediated cell wall reinforcement are now well described, many aspects are poorly understood. For example, papillary callose accumulation and lignification have received the most attention because of the availability of fluorescent stains for the detection of callose and because of the inherent autofluorescence of lignin compounds. However, the potential roles of other cell wall

components remain unknown. A formidable technical challenge in analysing cell wall modifications during infection is related to the fact that walls undergo complex and very rapid modifications in specific local cell wall micro-domains, which are difficult to detect and define using traditional *in situ* procedures (Malinovsky *et al.*, 2014). Even though conventional biochemical methods for the analysis of cell wall carbohydrates are very powerful, these techniques are often not suitable for analysing the local cell wall modifications made during pathogen infection, because they require a large amount of material in which local changes in composition are hidden by the greater amount of unaltered materials. These techniques also involve destruction of the cell wall into its individual constituents, so information about three dimensional architecture cannot be obtained.

The recent development of cell wall-specific antibodies, carbohydrate binding modules and small molecule stains provide a new opportunity to capture information about the three dimensional changes cell wall polysaccharides undergo at infection sites. It is now possible to apply these tools to test the hypothesis that several polysaccharides other than callose may be involved in papillae-associated penetration resistance in the host plant. Additionally, with the availability of the recently sequenced barley genome, scope exists to examine the deposition of host cell wall polysaccharides linked to the genetic control of this process during pathogenic interactions. For the current study, the barley-powdery mildew *(Bgh*) interaction system was used, for several reasons. Firstly, it is a model system for cereal crops, so the information could be useful for other economically important cereals and grasses. Secondly, the interaction is limited to the leaf epidermal layer, which can be relatively easily isolated for transcription profiling. Thirdly, the availability of the full genome sequence of barley allows the identification and manipulation of genes involved in the deposition of cell wall polysaccharides in the papillae. Using this plant-fungal interaction system, the current study was undertaken in order to more precisely define the role of cell wall polysaccharides in plant disease resistance mechanisms.

The broad objectives of the current study were:

I. To investigate the composition, structure and dynamics of the polysaccharides in the papillae which confer altered penetration resistance in the barley-powdery mildew host-pathogen system.

II. To identify putative candidate genes involved in papillae polysaccharide biosynthesis.

III. To define the roles of individual papillae polysaccharides in penetration resistance mechanisms against pathogens.

References

Albersheim P, Darvill A, Roberts K, Sederoff R, Staehelin A. 2011. *Cell Walls and Plant-Microbe Interactions*: Garland Science, Taylor & Francis, 270 Madison Avenue, New York, Ny 10016 USA.

Bellincampi D, Cervone F, Lionetti V. 2014. Plant cell wall dynamics and wall-related susceptibility in plant–pathogen interactions. *Plant cell wall in pathogenesis, parasitism and symbiosis* **5**: 30-37.

Boller T, Felix G 2009. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annual Review of Plant Biology*, 379-406.

Caffall KH, Mohnen D. 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate Research* **344**(14): 1879-1900.

Huckelhoven R. 2005. Powdery mildew susceptibility and biotrophic infection strategies. *Fems Microbiology Letters* **245**(1): 9-17.

Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* **444**(7117): 323-329.

Malinovsky FG, Fangel JU, Willats WG. 2014. The role of the cell wall in plant immunity. *Plant cell wall in pathogenesis, parasitism and symbiosis* **5**: 38-49.

Ringli C. 2010. Monitoring the outside: cell wall-sensing mechanisms. *Plant Physiology* **153**(4): 1445-1452.

Slusarenko AJ, Fraser R, van Loon LC, eds. 2012. *Structural aspect of plant defence. In: Mechanisms of resistance to plant diseases*: Springer Science & Business Media.

Stone BA, Clarke AE. 1992. *Chemistry and Biology of (1→3)-β-D-Glucans*. Victoria, Australia: La Trobe University Press.

Turrà D, El Ghalid M, Rossi F, Di Pietro A. 2015. Fungal pathogen uses sex pheromone receptor for chemotropic sensing of host plant signals. *Nature* **527**(7579): 521-524.

Zentmyer GA. 1961. Chemotaxis of zoospores for root exudates. *Science* **133**(3464): 1595-1596.

CHAPTER 2

REVIEW OF THE LITERATURE

Powdery mildew, caused by the obligate biotrophic fungus *Blumeria graminis* (syn. *Erysiphe graminis*), is one of the most destructive foliar diseases of cereal crops, including barley and wheat (Abdel-Ghani *et al.*, 2008). Significant barley yield losses have been recorded due to powdery mildew infection in Europe, the United States, South America, Africa and Australia (Chan *et al.*, 1990). *B. graminis* is also representative of other mildews and obligate biotrophic fungal pathogens (Dean *et al.*, 2012). Although the host range of *B. graminis* is limited to barley and wheat, other powdery mildew species in the order 'Erysiphales' have diverse host ranges and affect vegetable and fruit crops with similar biology, aetiology and pathology (Glawe, 2008).

Events proceeding development of the fungal infection structure in a Bgh-barley interaction

Well-adapted pathogens overcome a range of basal and induced physical and chemical barriers in order to establish a pathogenic relationship with host cells. Infection cycles generally involve four common steps: adhesion to the host surface, penetration, colonisation and nutrient acquisition, and reproduction. However, strategies at each step may vary widely depending upon the fungal group. Barley powdery mildew is caused by the ascomycete fungus *B. graminis* f. sp. *hordei* (*Bgh),*which infects aerial parts of the plant (Gan *et al.*, 2012). Similarly with other powdery mildew pathogens, *Bgh* is an obligate biotroph that requires living host cells. Upon landing on the plant leaf surface, asexual conidia of *Bgh* release an exudate containing cutinases and esterases, which allow them to degrade the cutin layer and attach firmly to the leaf surface (Nicholson & Kunoh, 1995). Following adhesion, asexual conidia form two germ tubes. Growth of the primary germ tube is limited, but it helps the fungus attach more firmly to the surface, obtain water from the host, and perceive chemical and physical signals from the plant (Gan *et al.*, 2012). Perception of a signal by the primary germ tube initiates germination of a second germ tube that extends along the longitudinal grooves between adjacent epidermal cells and forms an appressorium at its tip on the outer periclinal wall of epidermal cells (Gan *et al.*, 2012). Penetration hyphae develop beneath the

appressorium and push directly against the host cell wall using mechanical pressure generated from high turgor pressure within the appressorium (Green *et al.*, 2002). The penetration process is supported by cell wall degrading enzymes (CWDEs). Cellobiohydrolase isoform I has been found at the tip of appressorial penetration hyphae whereas isoform II has been found at the tip of the primary germ tube (Pryce-Jones *et al.*, 1999). Once through the wall, the infection hyphae quickly form nutrient feeding structures called 'haustoria' that have finger-like outgrowths that invaginate the plasma membrane of the infected host cells and keep the invaded cell intact. The interaction of *Bgh* with barley remains limited to a single epidermal cell and the haustoria provide the only interface with the host tissue. Through this the pathogen can acquire enough nutrition to produce abundant mycelia on the leaf surface to complete its asexual life cycle (Aist & Bushnell, 1991; Green *et al.*, 2002). Table 1 summarizes the important events preceding fungal infection structure development in a *Bgh*-barley host system.

Table 1. Important events proceeding fungal infection structural development & localized plant responses in *B. graminis*-barley system

Events	Timing (hpi)	Structure, shape & length	Possible function	References
Attachment of asexual conidia	-	Dumbbell-like swelling at apex and base, 50 µm	Contain water, fungal units of multiplication	-
Release of liquid exudate	0.05	N/A	Non-specific esterase & cutinase, adhesion of conidia and cutin layer degradation	(Nicholson and Kunoh, 1995)
Release of matrix material (elicitors)	0.05	N/A	Glucosyl residues glucose, xylose-,& mannose, elicit plant defence response	(Schweizer et al., 2000)
Formation of primary germtube (PGT)	0.5-2	5-10 µm	Conidia attachment, initial water & nutrition uptake, signal perception, suppression of host induced resistance	(Green et al., 2002; Yamaoka et al., 2007)
Small cytoplasmic aggregation beneath the conidia	4	-	Mitochondria, rough endoplasmic reticulum, Golgi bodies, & polyribosomes, role in deposition of papillae materials	(Bushnell and Zeyen, 1976)
Sub-cellular localization of ROS under primary germtube	6	N/A	Defence signaling, cross-linking of cell wall polymers, SAR, cell death, anti-microbial activity, and phytoalexin production	(Ride and Pearce, 1979), (Ride and Barber, 1987)
Formation of appressorial germtube	9-10	30-40 µm	Tip swells to form appressoria	(Green et al., 2002)
Appressorial differentiation	10	Hook shaped, Lobed,	Penetration of cuticle-cell wall barrier by enzymatic hydrolysis followed by mechanical pressure	(Green et al., 2002)
Large cytoplasmic aggregates beneath the appressoria	8-10	-	Mitochondria, rough endoplasmic reticulum, Golgi bodies, & polyribosomes, role in deposition of papillae materials	(Bushnell and Zeyen, 1976)
Development of penetration hyphae under appressoria	10-15	Cylindrical, 1-2 µm	Fungal infection structures enter the host cell and later develop into haustoria	(Green et al., 2002)
Deposition of papillae beneath the appressoria	12-14	Dome shaped, length 4-6 µm, Depth 1.5-3 µm	Mechanical and chemical barrier of pathogen penetration	(Bushnell and Bergquist, 1975; Stein and Somerville, 2002)
Sub-cellular localization of ROS under appressoria	15	N/A	Defence signaling, cross-linking of cell wall polymers, SAR, cell death, anti-microbial activity, and phytoalexin production	(Ride and Pearce, 1979), (Ride and Barber, 1987)
Development of haustoria from penetration hyphae	13-48	Multi-lobed digitate, 6-32 µm wide	Nutrition acquisition, release of effectors, biosynthesis of metabolites	(Bushnell and Bergquist, 1975)
Formation of full haustorial encasement	23-24	2.6-4 µm thick	Physical barrier against haustoria for nutrient uptake, protection of effector molecules	(Zeyen et al., 2002)

hpi= hours post inoculation, N/A= not applicable, ROS= Reactive oxygen species, SAR= Systemic acquired resistance

* Plant's responses are shown in the shaded lines

Host defence mechanisms against powdery mildew

All plants exclude most microbes with preformed physical and constitutive chemical defence barriers present at their surfaces; these include the waxy cuticle, the cell wall, antimicrobial compounds, a differential pH environment and defensins. Microbes might not overcome these barriers if they have inappropriate infection structures and/or surface degrading enzymes. Such interactions could be termed as 'Basic incompatibility'. Many powdery mildew pathogens, despite having the appropriate infection tools, cannot invade non-hosts because of the plant's ability to recognize the pathogen-released and highly conserved elicitor molecules known as pathogen associated molecular patterns (PAMPs) (Boller & Felix, 2009). For example, a carbohydrate elicitor released from the wheat pathogen *Blumeria graminis* f.sp. *tritici* (*Bgt)* is recognized by barley and other non-host cereal crops (Schweizer *et al.*, 2000). Barley may also recognize the molecules released from the host cell wall itself, which are known as damage associated molecular patterns (DAMPs) (Lotze *et al.*, 2007; Boller & Felix, 2009), due to the cell wall degrading activity of *B. graminis* (Green *et al.*, 2002). These PAMPs and DAMPs are recognised through specific plasma membrane-bound proteins called pattern recognition receptors (PRRs), which induce defence responses called PAMP triggered immunity (PTI) or DAMP triggered immunity (DTI) through activating mitogen-activated protein kinase (MAPK) cascades (Jones & Dangl, 2006). In barley, some of the early common responses associated with PTI/DTI include increased ion influx across the membrane, generation of reactive oxygen species (ROS), altered expression of some defence related genes, and cell wall reinforcement, including papillae formation at the site of infection (Huckelhoven *et al.*, 1999). These defence responses are often efficient enough to halt penetration leading to incompatible interactions. For example, the wheat pathogen *Bgt* in non-host barley cultivars and the barley pathogen *Bgh* in recessive *mlo* (*Mildew Locus O*) based resistance cultivars (Jørgensen, 1992).

Some successful pathogens suppress PTI/DTI and overcome papillae barriers by delivering into host plant cells avirulence effector proteins (Avr) that target specific molecules involved in PTI/DTI pathways, resulting in effector-triggered susceptibility (ETS) (Jones & Dangl, 2006). Barley has evolved systems that can recognize the Avr proteins through proteins encoded by the so-called resistance R genes, which mostly encode coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR)-type proteins. This results in effector-triggered immunity (ETI), which was formerly called gene-for-gene resistance or vertical resistance (Jones & Dangl, 2006). At least 85 R genes including the members of the *Mlk* (*Mildew Locus K*) and *Mla* (*Mildew Locus a*) gene families have been characterized in the barley genome (Stergiopoulos & de Wit, 2009). The ETI, also known as race-specific resistance, is usually characterized by hypersensitive cell death through release of ROS, accompanied by production of phytoalexins and expression of so-called pathogenesis-related (PR) proteins (Glazebrook, 2005). Although the mechanism is largely unknown, some *Bgh* isolates establish 'compatible interaction' with host barley plants through evolving new kinds of effectors, possibly through horizontal gene transfer or by selection pressure, to avoid R protein recognition and become established as virulent pathogens. Figure 1 summarizes the barley defence mechanisms deployed against *B. graminis* at different stages of development.

It is hard to differentiate PTI from ETI upon perception of microbes by the host plants, because in many cases they share the same downstream signalling machinery (Tsuda *et al.*, 2009; Katagiri & Tsuda, 2010). Although it is generally considered that ETI responses are more prolonged and robust than those of PTI, longevity and robustness of immune responses are increasingly energy expensive and sometimes deleterious to the plant (Bolton, 2009). Plants need to balance the effort required for immunity and its negative impact on fitness (Katagiri & Tsuda, 2010).

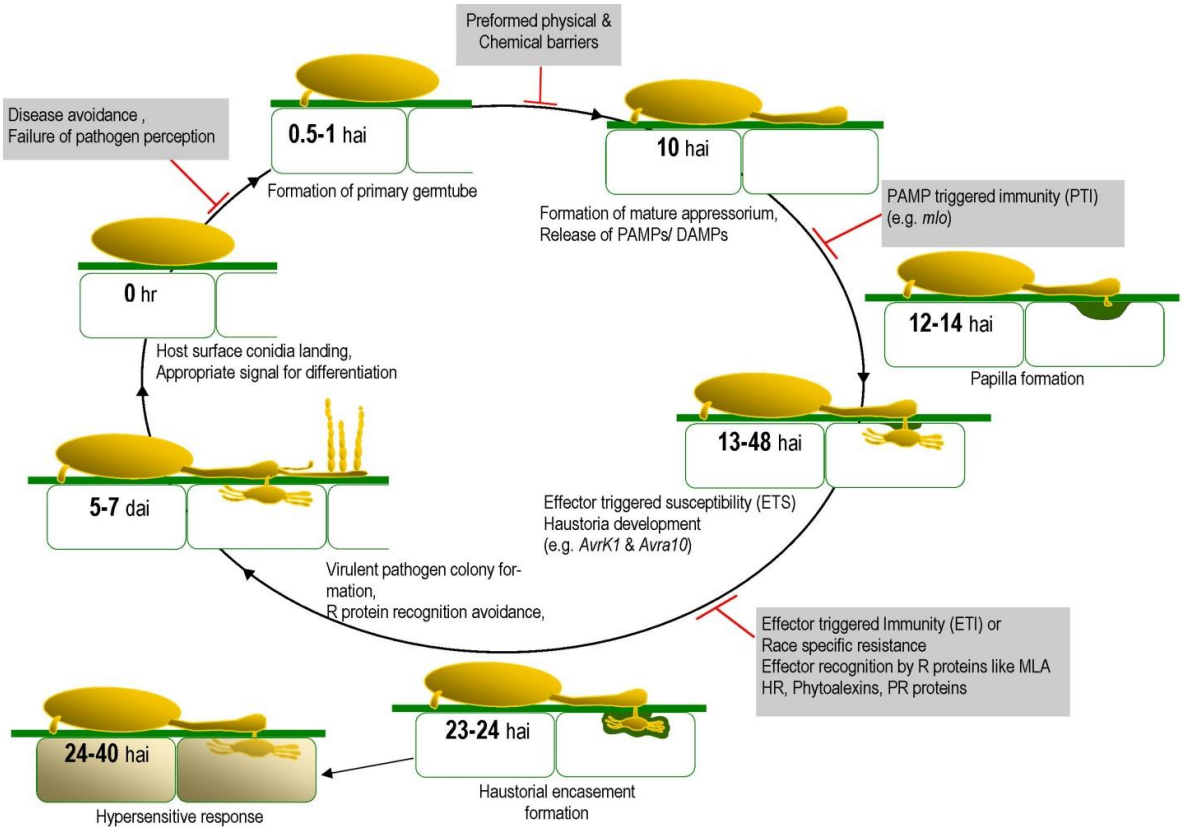

Figure 1. Overcoming the host defence barrier in *Bgh*-barley interaction. Plant defence responses are shown in grey boxes outside the circle, pathogen activities are noted inside the circle.

Role of papillae and papillae related structures during Bgh-barley interactions

Formation of cell wall appositions commonly known as 'epidermal cell papillae' at penetration sites is a common plant defence response against most biotrophs, irrespective of whether the interactions are compatible or incompatible. The papillae are believed to provide mechanical and chemical barriers to halt penetration of pathogens or to delay the infection process so that the plant's other defences become active (Huckelhoven, 2005; Albersheim *et al.*, 2011). Outcomes of interactions are often differentiated by the timing of papillae formation. It has been reported that in *Bgh*-barley interactions, more rapid papillae formation occurs in induced resistant lines compared with wild type susceptible cultivars (Aist & Israel, 1977; Inoue *et al.*, 1994). Papillae size is probably another factor linked to compatibility in *Bgh*-barley interactions. Some studies report that bigger papillae form under unsuccessful penetration sites in chemically induced resistant lines while papillae formed under successful penetration sites in wild type lines are much smaller in size (Gold *et al.*, 1986; Aist *et al.*, 1988; Ebrahim-Nesbat & Bohl, 1993).

The importance of papillae in host penetration resistance mechanism can also be explained from the structural point of view. Deposition of defence components often leads to the formation of distinct layers in papillae as observed by electron microscopy (Zeyen & Bushnell, 1979). Analysis across the micrographs suggests that materials are deposited in four sequential stages to form a mature papilla: (1) electron dense osmophilic materials are deposited first and are believed to be lipidic or phenolic, (2) non-osmiophilic amorphous materials start appearing outside the osmiophilic materials and are thought to be insoluble polysaccharides, (3) the non-osmiophilic amorphous material hardens and forms a compact inner layer and (4) osmiophilic materials are incorporated into the wall and into compact non-osmiophilic amorphous material. Mature papillae with distinct layers are more efficient in trapping the fungal penetration pegs than the papillae with less distinct layers, suggesting that failure of papillae in halting pathogen penetration might be attributable to the inability of the

host plant to complete the entire sequence of events of papillae development before penetration (Zeyen & Bushnell, 1979).

Hydrogen peroxide (H_2O_2) is likely to play a role in papillae-based resistance against *Bgh* because it accumulates near unpenetrated papilla (effective papillae) developed in *mlo*-based resistant lines and it is not observed in penetrated papillae (ineffective papillae) (Huckelhoven *et al.*, 1999). Despite these findings, it remains unclear what plant and/or pathogen derived factors are actually responsible for making the papillae more effective.

In the event of a successful penetration, the fungus forms haustoria while papillae wall materials form a band around the neck of the haustorium (Micali *et al.*, 2011; Underwood, 2012). In a second attempt at defence, the host plant gradually deposits cell wall polymers outside the extra-haustorial matrix and extra-haustorial membrane to entrap the haustoria (Bracker, 1964; Micali *et al.*, 2011). These cell wall associated structures are called 'haustorial encasements' and they are likely to form from the physical obstruction of nutrient uptake by the pathogen from the host tissues and/or from protecting the host tissue from the pathogen effector molecules (Wang *et al.*, 2009). Well adapted pathogens are likely to have the ability to suppress the formation of such encasements (Underwood, 2012).

Papillae composition observed using histochemical and microanalytical analyses

In the last three decades, research groups have attempted to identify the components of papillae formed against different biotrophic pathogens in various crops. Papillae have been studied using histochemical analysis, immunohistochemistry, polarising microscopy, inhibitory compounds and X-ray microanalysis (Zeyen *et al.*, 2002). Recently, a group has compared candidate gene expression during papillae formation against known papillae components and their likely functions in defence (Bhuiyan *et al.*, 2009).

Although variation is present between species, some common components have been reported to be present in epidermal cell papillae, such as callose, phenolics (lignin and phenolic conjugates), cell wall structural glycoproteins (eg. arabinogalactan proteins, hydroxyproline-

rich glycoproteins), inorganic elements and ROS (Zeyen *et al.*, 2002; Underwood, 2012). The (1,3)-β-glucan polymer, callose, is abundant in papillae formed in several plant-microbe interactions and is easily visible using fluorescence when stained with the aniline blue fluorochrome (Smart *et al.*, 1986; Micali *et al.*, 2011). However, aniline blue fluorescence overlaps the autofluorescence spectrum of lignin-like phenolics and this often obscures the result, unless the data can be confirmed with transmission electron microscopy (TEM) using callose-specific polyclonal antibodies.

Lignin is reported to be present abundantly in papillae and may be present either as simple phenolics or crosslinked with callose, other cell wall polysaccharides or proteins (von Ropenack *et al.*, 1998). Complex lignin-like polymers have been detected in wheat epidermal cell papillae by histochemical staining and autofluorescence (Ride & Barber, 1987; Nicholson & Hammerschmidt, 1992). Gene expression profiling and silencing of some genes involved in the monolignol biosynthesis pathway revealed that lignin is an important constituent of wheat papillae formed during *Bgt* infection (Bhuiyan *et al.*, 2009). Surprisingly, lignins have never been detected in papillae of barley (Zeyen *et al.*, 2002). Instead, a phenolic-polyamine conjugate, *p*-coumaroyl-hydroxyagmatine (*p*-CHA) was observed in epidermal cell papillae of *mlo* barley lines (von Ropenack *et al.*, 1998). Two important cell wall polymers, cellulose and pectin, have never been found in papillae in any plant species, but are found in wound plugs (Smart *et al.*, 1986).

Oligomers of N-acetylglucosamine and chitobiose have been found by histochemical staining in the papillae of mature barley leaves, but are believed to be derived from degradation of the fungal cell wall (Ebrahim-Nesbat *et al.*, 1986). A few studies have identified antimicrobial components in the papillae of barley leaves. For instance, thionin proteins (Ebrahim-Nesbat & Bohl, 1993), guanidine- and arginine-rich proteins (Wei *et al.*, 1994) and hydrolytic enzymes (Takahashi *et al.*, 1985) are consistently accumulated in barley papillae. Some of the inorganic chemical elements are also reported to be present at an increased level

at appressorium contact sites during papilla deposition. Using X-ray microanalysis coupled with scanning electron microscopy, elevated levels of soluble calcium, magnesium, manganese, and silicon were detected near the infected cell wall before papillae maturation (Zeyen *et al.*, 1993).

Localized release of ROS at the site of infection is a common event that is likely to be associated with synthesis, deposition, assembly and cross-linking of the organic and inorganic molecules in papillae. Diaminobenzidine (DAB) stained hydrogen peroxide (H_2O_2) is readily visible beneath the primary germ tube and the appressoria at 6 hours post inoculation (hpi) and 15 hpi, respectively, during barley-powdery mildew interactions (ThordalChristensen *et al.*, 1997). In the barley-*Bgh* interaction, the presence of H_2O_2 in papillae has been considered as a biochemical marker to separate non-penetrated from penetrated cells (Hueckelhoven, 2007).

Possible functions of papillae constituents

The role of individual papillae constituents in halting fungal penetration is not well understood. While callose has long been thought to act as a mechanical barrier to penetration, its role has recently been questioned in several plant-pathogen interactions. In *Arabidopsis*, the callose synthase gene *GSL5*/*PMR4* is responsible for callose deposition in papillae and wound plugs (Jacobs *et al.*, 2003). The *gsl5*/*pmr4* mutant lines, which do not produce callose, show marginally increased penetration resistance in the *Arabidopsis*-*Golovinomyces cichoracearum* interaction (Jacobs *et al.*, 2003) and in the *Arabidopsis*-*Erysiphe cichoracearum* interaction (Nishimura *et al.*, 2003). It is suggested that the enhanced resistance was due to up regulation of genes involved in the salicylic acid (SA) defence signaling pathway, implying that callose may not act as a direct physical barrier at least during powdery mildew pathogen infection. In contrast, enhanced susceptibility has been reported in callose synthase mutant lines of lemon, when leaves were inoculated with the bacterial pathogen *Xanthomonas citri* (Enrique et al., 2011). It is not clear if the absence of callose in lemon leaves has an effect on papillae structure or on other plant defence responses

(Underwood, 2012). Two possible hypotheses have been made so far about the putative role of callose: it may serve as a protective barrier to separate underlying plant tissues from toxic metabolites accumulated in papillae and the haustorial encasement or callose deposition may limit pathogen or plant derived defence elicitors that reduce the level of SA activation pathways (Jacobs *et al.*, 2003; Underwood, 2012).

Given its mechanical and chemical properties, lignin should form an excellent physical barrier against pathogen invasion (Garcion *et al.*, 2007; Bhuiyan *et al.*, 2009). Lignification provides mechanical resistance to the cell wall against the pressure generated by the fungal appressoria, through cross-linking with cell wall polysaccharides (Bechinger *et al.*, 1999). Moreover, lignified walls are less accessible to cell wall degrading enzymes (von Ropenack *et al.*, 1998). However, it is not clear that lignin has an antimicrobial activity or has any impact on the SA signalling pathway (Underwood, 2012).

ROS can be produced upon the host plant's perception of danger via elicitors or wounding through the action of NADPH oxidase or peroxidases, which are also believed to be key molecules in plant defence (Lamb & Dixon, 1997). ROS serves multiple functions in the plant defence mechanism: They are involved in further downstream defence signalling (Torres *et al.*, 2006), SA-dependent Systemic Acquired Resistance (SAR) pathways (Neuenschwander *et al.*, 1995) and the hypersensitive response (Lamb & Dixon, 1997). ROS also contribute to strengthening of the cell wall by stimulating the cross-linking of phenolics to the other cell wall polymers (Mellersh *et al.*, 2002) and proline-rich proteins (Brisson *et al.*, 1994). Table 2 summarizes papillae constituents and their putative functions in several plant-pathogen interactions.

Table 2. Papillae constituents and their functions observed in different plant-pathogen interactions

Constituents	Interactions	Detection methods	Putative functions	References
Callose	*Bgh*-barley *Fusarium graminearum*-wheat *G. orontii- Arabidopsis* *A. candida- Arabidopsis* *Uromyces vignae-Vicia faba*	1. aniline blue 2. monoclonal antibodies 3. polyclonal antibodies	Mechanical barrier against penetration, Protect protoplast from toxins accumulated in papillae	Smart et al., (1986), Micali et al., (2011), Soylu et al., (2003),
Lignin	*Bgt*- wheat *F. graminearum*- wheat	1. chlorine-sulphite stain for syringyl lignin 2. phloroglucinol-HCL test 3. gene expression profiling 4. Loss-of-function	Mechanical & chemical resistance against appressorial and cell wall degrading enzymes, respectively.	Ride and Barber, (1987), Nicholson and Hammerschmi (1992), Bhuiyan et al., (2009)
Phenolic conjugates (*p*-CHA)	*Bgh*-barley (*mlo*)	HPLC profile	Resistance papillae to saponification in *mlo* lines	von Ropenack et al., (1998)
Xyloglucans	Poinsettia- *Oidium sp*	Immunogold labelling with specific monoclonal antibodies	Unknown	(Celio *et al.*, 2004)
Arabinogalactan/ Arabinogalactan proteins	*F. oxysporum*-cotton *Oidium sp*. - Poinsettia	Immunogold labelling with specific monoclonal antibodies	Unknown	(Rodriguezgalvez & Mendgen, 1995; Celio *et al.*, 2004)
Polygalacturonic acid	*F. oxysporum*-cotton *U. vignae-Vicia faba*	Immunogold labelling with specific monoclonal antibodies	Unknown	Xu and Mendgen, (1997)
Hydrolytic enzymes	*Bgh*-barley *F. graminearum*- wheat	Tissue fixation, preparation of whole-mount coleoptiles with appropriate dye	Unknown	Takahashi et al., (1985)
Hydroxyproline-rich glycoproteins	*F. graminearum*- wheat	Immunogold labelling with specific monoclonal antibodies	Unknown	(Kang & Buchenauer, 2003)
Guanidine and arginine-rich proteins	*Bgh*-barley *Bgt*- wheat	Sakaguchi staining test	Anti-microbial activity	Wei et al., (1994)
Thionin proteins	*Bgh*-barley *F. graminearum*- wheat	Immunogold labelling with thionin antiserum	Anti-microbial activity	Ebrahim-Nesbat and Bohl, (1993)
Inorganic elements (Ca, Mn, Mg, Si)	*Bgh*-barley	X-ray microanalysis & Scanning electron microscopy	Unknown	Zeyen et al., (1993)
ROS	*Bgh*-barley	Staining with diaminobenzidine (DAB)-HCL pH 3.8	Involved in defence signaling, SAR, cell wall reinforcement, program cell death, phytoalexin production and have direct anti-microbial effects	ThordalChristensen et al., (1997)

Trafficking of defence components to papillae in the powdery mildew-cereal system

Upon infection, actin filaments are rearranged and focused on the attempted penetration sites. This targets vesicles and other membrane materials towards the developing papillae (Figure 2). Plants possibly use multiple secretory pathways for trafficking different wall components in order to assemble a papilla, making them a robust means of defence against biotrophs. Golgi-derived vesicular traffic is supposed to be the main secretion pathway through which non-cellulosic polysaccharides, phytoalexins, defensins and fungal enzyme inhibitor proteins could be incorporated into papillae (Albersheim *et al.*, 2011).

Forward genetics approaches have revealed that, for successful infection, powdery mildew pathogens require a wild type variant of *Mildew Locus O* (*MLO*). In both dicot and monocot species, recessive or mutant alleles of *mlo* mediate broad spectrum resistance against the pathogens (Jørgensen, 1992; Consonni *et al.*, 2006; Fondevilla *et al.*, 2006). In *mlo*-based powdery mildew resistance lines, trafficking of defence components to the infection site via Golgi-derived vesicles relies on some members of the *N*-ethylmaleimide-sensitive factor adaptor protein receptor (SNARE)-domain containing protein family (Collins *et al.*, 2003; Consonni *et al.*, 2006; Gu & Innes, 2011). Qa-SNARE proteins, PEN1 in *Arabidopsis* and its ortholog ROR2 in barley, are plasma membrane localized syntaxins (Collins *et al.*, 2003). PEN1/ROR2 forms a complex with another membrane-anchored adaptor Qb+ Qc SNARE (SNAP25/ SNAP34) and with Golgi vesicle-bound R-SNARE (VAMP721/722) for the exocytosis of cell wall precursors and antimicrobial compounds into the papillae (Lipka *et al.*, 2008; Underwood & Somerville, 2008).

Defence related materials may also be transported into the papillae through a peroxisome-localized thioglucosidase, PEN2, and a membrane located ABC transporter, PEN3 (Lipka & Panstruga, 2005; Stein *et al.*, 2006). PEN2 removes the glucose residue from a toxic metabolite, glucosinolate, in the cytoplasm and releases a product that is incorporated into the papillae

through PEN3 in the membrane of the papillae matrix (Bednarek, 2009; Frey & Robatzek, 2009).

How the phenolics and monolignols are exported into the papillae has not been determined yet. However, such components along with defence related plasma membrane proteins, membrane and lipid materials, and wall remodelling materials are thought to be delivered into the papillae by endosome mediated secretion (Albersheim *et al.*, 2011). The endocytic trafficking system becomes active upon PAMP perception of the fungal pathogens, while endosomes are formed by invaginations of the plasma membrane. Multivesicular bodies (MVBs) later fuse with the PM and release the small membrane vesicles as exosomes (Bednarek, 2009; Albersheim *et al.*, 2011).

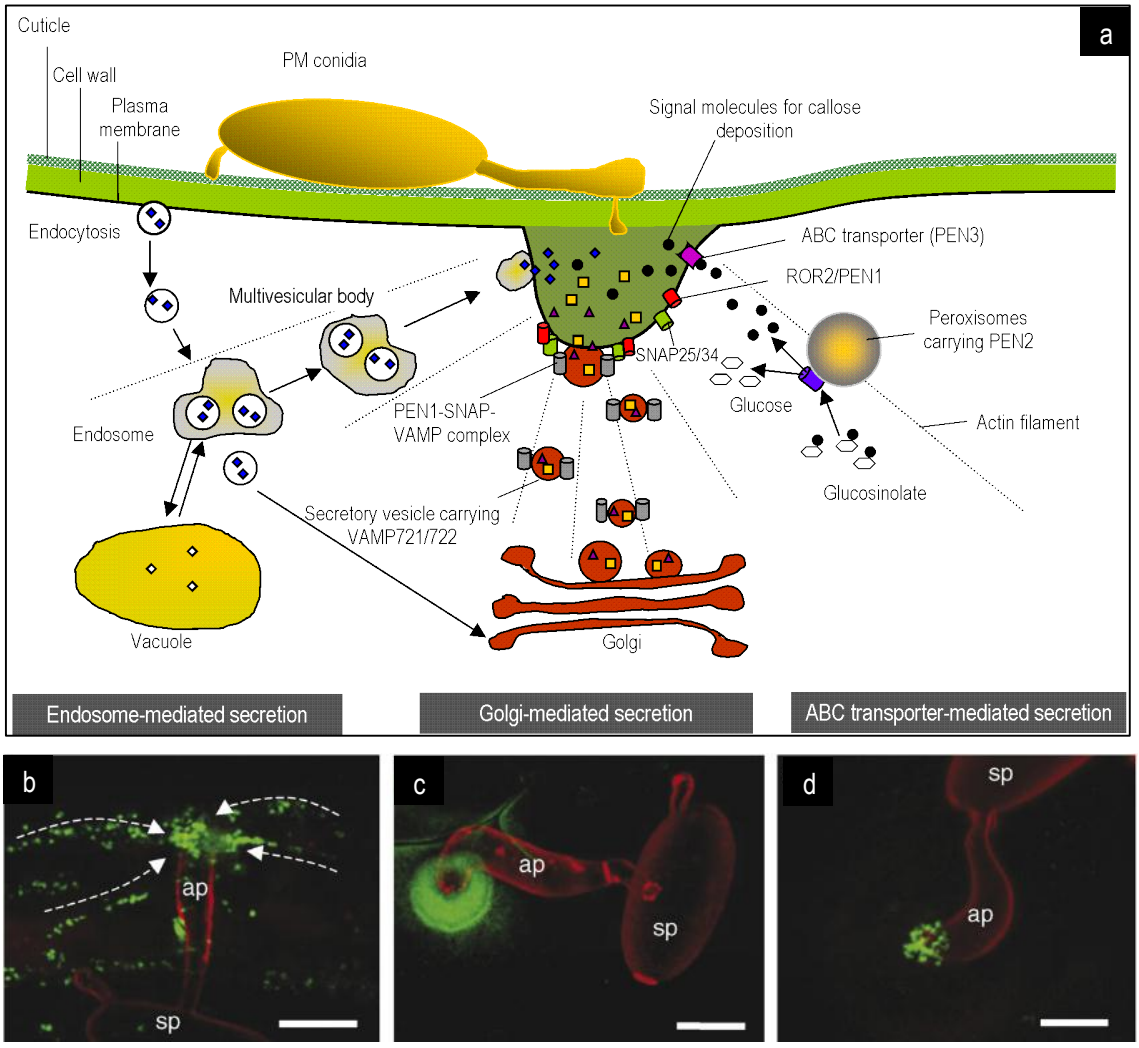

Figure 2. Pre-invasion papilla development against powdery mildew fungus. (a) The schematic view illustrates three possible polarized secretory events to ensure deposition of all the components needed to build up effective papillae (Modified from Albersheim *et al*. (2011), (b) secretory vesicles tagged with *Arabidopsis* GFP:VAMP22 moving towards the papillae (indicated by dashed arrows), (c) Focal accumulation of GFP:PEN3 fusion at the plasma membrane microdomain under the infection hypha, (d) Peroxisome carrying GFP:PEN2 fusions accumulated near the contact site of plant-fungus interaction. Photo (b, c, d) taken from Lipka *et al*. (2008)

Altered disease resistance triggered by alteration of cell wall polysaccharide composition

Outcomes of a number of studies suggest that alteration of cell wall composition might potentially lead to altered disease resistance of host plants. Both the gain-of-function and loss-of-function approaches have been utilized to examine the effects of altered wall composition on plant disease resistance. Some of these studies have been summarised below (Table 3). However, the majority have been dedicated to the improvement of digestibility of forage crops to render lignocellulose less recalcitrant for bioprocessing, but this has led to the concern that altered cell wall properties might make the plant more susceptible to pests and diseases. Transgenic lines with altered transcript levels of candidate genes involved in cellulose, non-cellulosic polysaccharides and lignin biosynthetic pathways have been studied with potential pathogen infection in mind.

Typically, reduction of cellulose biosynthesis by means of genetics or chemical treatments leads to increased lignification and such plant lines show enhanced disease resistance; this suggests that plants have cell wall integrity maintenance mechanisms (Hamann, 2012). The *Gsl5*/*PMR4* gene is associated with callose biosynthesis in papillae of *Arabidopsis* that form during pathogen infection. Counterintuitively, the *gls5* mutant shows increased resistance against the powdery mildew pathogen, possibly through hyperactivation of SA genes (Jacobs *et al.,* 2003; Nishimura *et al.,* 2003). Over-expression of *Gsl5* in *Arabidopsis* results in higher papillary callose accumulation, leading to complete penetration resistance against pathogens (Ellinger *et al.,* 2013).

Table 3: Host disease resistance phenotype with altered cell wall composition

Gene name	Mutant/ over-expression	Host species	Observed wall phenotype	Pathogen species	Phenotype	Reference
CesA3	Mutation	*Arabidopsis thaliana*	Reduced cellulose in primary wall, lignification, enhanced defence signalling	*Erysiphe cichoracearum* *Erysiphe orontii* *Oidium lycopersicum*	R	(Ellis & Turner, 2001; Caño-Delgado *et al.*, 2003)
CesA4(irx5) *CesA7(irx3)* *CesA8(irx1)*	Mutation	*A. thaliana*	Defective secondary cell wall, enhanced defence signalling	*Ralstonia solanacearum* *Plectosphaerella cucumerina* *Pseudomonas syringae* *Botrytis cinerea*	R	(Hernández-Blanco *et al.*, 2007)
WAT1	Mutation	*A. thaliana*	Defective secondary cell wall, enhanced defence signalling	*Xanthomonas campestris* *Verticillium dahlia* *V. alboatrum* *R. solanacearum* *P. cucumerina*	R	(Denancé *et al.*, 2013)
Gsl5 (PMR4)	Mutation	*A. thaliana*	Reduced callose accumulation in papillae, hyperactivation of SA responsive genes	*Sphaerotheca fusca* *Golovinomyces orontii* *E. cichoracearum* *Peronospora parasitica*	R	(Jacobs *et al.*, 2003; Nishimura *et al.*, 2003)
Gsl5 (PMR4)	Over-expression	*A. thaliana*	Increased callose accumulation in papillae	*Golovinomyces cichoracearum*	R	(Ellinger *et al.*, 2013)
CslF6	Mutation	Oryza sativa	Reduced mixed-linkage glucan in primary wall, activation of marker PR genes & SA responsive genes	*X. oryzae* pv. *oryzae*	R	(Vega-Sánchez *et al.*, 2012)
G-proteins	Mutation	*A. thaliana*	Reduced xylose content in the wall	*P. cucumerina*	R	(Delgado-Cerezo *et al.*, 2012)
PMR6	Mutation	*A. thaliana*	Enhanced pectin accumulation	*E. cichoracearum*	R	(Vogel *et al.*, 2002)
PMR5	Mutation	*A. thaliana*	Enhanced pectin accumulation	*E. cichoracearum* *E. orontii*	R	(Vogel *et al.*, 2004)
RWA2	Mutation	*A. thaliana*	Decreased levels of acetylated cell wall polymers	*B.cinerea*	R	(Manabe *et al.*, 2011)
PMEI-1	Over-expression	*A. thaliana*	Increased pectin methyl-esterification activity	*B.cinerea*	R	(Vega-Sánchez *et al.*, 2012)
PAL, CCoAOMT, COMT, CAD	RNAi	*Triticum monococcum*	Putatively reduced lignification in papillae and epidermal cell wall	*Blumeria graminis* f. sp. *tritici*	*S*	(Bhuiyan, Selvaraj et al. 2009)

References

Abdel-Ghani AH, Al-Ameiri NS, Karajeh MR. 2008. Resistance of barley landraces and wild barley populations to powdery mildew in Jordan. *Phytopathologia Mediterranea* **47**(2): 92-97.

Aist JR, Bushnell WR. 1991. *Invasion of plants by powdery mildew fungi, and cellular mechanisms of resistance*. London, New York: Plenum press.

Aist JR, Gold RE, Bayles CJ, Morrison GH, Chandra S, Israel HW. 1988. Evidence that molecular-components of papillae may be involved in ml-o resistance to barley powdery mildew. *Physiological and Molecular Plant Pathology* **33**(1): 17-32.

Aist JR, Israel HW. 1977. Papilla formation - timing and significance during penetration of barley coleoptiles by *Erysiphe graminis* hordei. *Phytopathology* **67**(4): 455-461.

Albersheim P, Darvill A, Roberts K, Sederoff R, Staehelin A. 2011. *Cell Walls and Plant-Microbe Interactions*: Garland Science, Taylor & Francis, 270 Madison Avenue, New York, Ny 10016 USA.

Bechinger C, Giebel K-F, Schnell M, Leiderer P, Deising HB, Bastmeyer M. 1999. Optical Measurements of Invasive Forces Exerted by Appressoria of a Plant Pathogenic Fungus. *Science* **285**(5435): 1896-1899.

Bednarek P. 2009. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* **323**: 101-106.

Bhuiyan NH, Selvaraj G, Wei Y, King J. 2009. Gene expression profiling and silencing reveal that monolignol biosynthesis plays a critical role in penetration defence in wheat against powdery mildew invasion. *Journal of Experimental Botany* **60**(2): 509-521.

Boller T, Felix G 2009. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annual Review of Plant Biology*, 379-406.

Bolton MD. 2009. Primary Metabolism and Plant Defense—Fuel for the Fire. *Molecular Plant-Microbe Interactions* **22**(5): 487-497.

Bracker CE. 1964. Ultrastructure of the haustorial apparatus of Erysiphe graminis and the relationship of the haustorium to the epidermal cell of Hordeum vulgare. *Phytopathology* **54**(8): 889-889.

Brisson LF, Tenhaken R, Lamb C. 1994. Function of oxidative cross-linking of cell-wall structural proteins in plant-disease resistance. *Plant Cell* **6**(12): 1703-1712.

Caño-Delgado A, Penfield S, Smith C, Catley M, Bevan M. 2003. Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis* thaliana. *The Plant Journal* **34**(3): 351-362.

Celio G, Mims C, Richardson E. 2004. Ultrastructure and immunocytochemistry of the host pathogen interface in poinsettia leaves infected with powdery mildew. *Canadian Journal of Botany* **82**(4): 421-429.

Chan KC, Boyd WJR, Khan TN. 1990. Distribution, severity and economic importance of powdery mildew of barley in western-australia. *Australian Journal of Experimental Agriculture* **30**(3): 379-385.

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, Huckelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P. 2003. SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* **425**(6961): 973-977.

Consonni C, Humphry ME, Hartmann HA, Livaja M, Durner J, Westphal L, Vogel J, Lipka V, Kemmerling B, Schulze-Lefert P, Somerville SC, Panstruga R. 2006. Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. *Nature Genetics* **38**(6): 716-720.

Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD. 2012. The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* **13**(7): 804-804.

Delgado-Cerezo M, Sánchez-Rodríguez C, Escudero V, Miedes E, Fernández PV, Jordá L, Hernández-Blanco C, Sánchez-Vallet A, Bednarek P, Schulze-Lefert P. 2012. *Arabidopsis* heterotrimeric G-protein regulates cell wall defense and resistance to necrotrophic fungi. *Molecular Plant* **5**(1): 98-114.

Denancé N, Ranocha P, Oria N, Barlet X, Rivière MP, Yadeta KA, Hoffmann L, Perreau F, Clément G, Maia-Grondard A. 2013. *Arabidopsis* wat1 (walls are thin1)-mediated resistance to the bacterial vascular pathogen, Ralstonia solanacearum, is accompanied by cross-regulation of salicylic acid and tryptophan metabolism. *The Plant Journal* **73**(2): 225-239.

Ebrahim-Nesbat F, Bohl S. 1993. Thionin in cell walls and papillae of barley in compatible and incompatible interactions with Erysiphe graminis f. sp. hordei. *Physiological and Molecular Plant Pathology* **43**(5): 343-352.

Ebrahim-Nesbat F, Heitefuss R, Rohringer R. 1986. Ultrastructural and histochemical-studies on mildew of barley (*Erysiphe-graminis* dc f-sp *hordei* Marchal) .4.

Characterization of papillae in fifth leaves exhibiting adult-plant resistance. *Journal of Phytopathology-Phytopathologische Zeitschrift* **117**(4): 289-300.

Ellinger D, Naumann M, Falter C, Zwikowics C, Jamrow T, Manisseri C, Somerville SC, Voigt CA. 2013. Elevated early callose deposition results in complete penetration resistance to powdery mildew in *Arabidopsis*. *Plant Physiology* **161**(3): 1433-1444.

Ellis C, Turner JG. 2001. The *Arabidopsis* mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *The Plant Cell* **13**(5): 1025-1033.

Enrique R, Siciliano F, Alejandra Favaro M, Gerhardt N, Roeschlin R, Rigano L, Sendin L, Castagnaro A, Vojnov A, Rosa Marano M. 2011. Novel demonstration of RNAi in citrus reveals importance of citrus callose synthase in defence against Xanthomonas citri subsp. citri. *Plant Biotechnology Journal* **9**(3): 394-407.

Fondevilla S, Carver TLW, Moreno MT, Rubiales D. 2006. Macroscopic and Histological Characterisation of Genes er1 and er2 for Powdery Mildew Resistance in Pea. *European Journal of Plant Pathology* **115**(3): 309-321.

Frey NFd, Robatzek S. 2009. Trafficking vesicles: pro or contra pathogens? *Current Opinion in Plant Biology* **12**(4): 437-443.

Gan PHP, Dodds PN, Hardham AR 2012. Plant Infection by Biotrophic Fungal and Oomycete Pathogens. In: Perotto S ed. *Signaling and Communication in Plant Symbiosis*, 183-212.

Garcion C, Lamotte O, Métraux J-P 2007. Mechanisms of Defence to Pathogens: Biochemistry and Physiology. *Induced Resistance for Plant Defence*: Blackwell Publishing, 109-132.

Glawe DA 2008. The powdery mildews: A review of the world's most familiar (yet poorly known) plant pathogens. *Annual Review of Phytopathology*, 27-51.

Glazebrook J 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, 205-227.

Gold RE, Aist JR, Hazen BE, Stolzenburg MC, Marshall MR, Israel HW. 1986. Effects of calcium nitrate and chlortetracycline on papilla formation, ml-o resistance and susceptibility of barley to powdery mildew. *Physiological and Molecular Plant Pathology* **29**(1): 115-129.

Green JR, Carver TLW, Gurr SJ. 2002. The formation and function of infection and feeding structures. *Powdery Mildews: A Comprehensive Treatise*: 66-82.

Gu Y, Innes RW. 2011. The KEEP ON GOING Protein of *Arabidopsis* Recruits the ENHANCED DISEASE RESISTANCE1 Protein to Trans-Golgi Network/Early Endosome Vesicles. *Plant Physiology* **155**(4): 1827-1838.

Hamann T. 2012. Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms. *Frontiers in plant science* **3**.

Hernández-Blanco C, Feng DX, Hu J, Sánchez-Vallet A, Deslandes L, Llorente F, Berrocal-Lobo M, Keller H, Barlet X, Sánchez-Rodríguez C. 2007. Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. *The Plant Cell* **19**(3): 890-903.

Huckelhoven R. 2005. Powdery mildew susceptibility and biotrophic infection strategies. *Fems Microbiology Letters* **245**(1): 9-17.

Huckelhoven R, Fodor J, Preis C, Kogel KH. 1999. Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiology* **119**(4): 1251-1260.

Hueckelhoven R. 2007. Cell wall - Associated mechanisms of disease resistance and susceptibility. *Annual Review of Phytopathology* **45**: 101-127.

Inoue S, Aist JR, Macko V. 1994. Earlier papilla formation and resistance to barley powdery mildew induced by a papilla-regulating extract. *Physiological and Molecular Plant Pathology* **44**(6): 433-440.

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB. 2003. An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* **15**(11): 2503-2513.

Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* **444**(7117): 323-329.

Jørgensen IH. 1992. Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. *Euphytica* **63**(1-2): 141-152.

Kang Z, Buchenauer H. 2003. Immunocytochemical Localization of Cell Wall-Bound Thionins and Hydroxyproline-Rich Glycoproteins in Fusarium culmorum-Infected Wheat Spikes. *Journal of Phytopathology* **151**(3): 120-129.

Katagiri F, Tsuda K. 2010. Understanding the Plant Immune System. *Molecular Plant-Microbe Interactions* **23**(12): 1531-1536.

Lamb C, Dixon RA. 1997. The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* **48**: 251-275.

Lipka U, Fuchs R, Lipka V. 2008. *Arabidopsis* non-host resistance to powdery mildews. *Current Opinion in Plant Biology* **11**(4): 404-411.

Lipka V, Panstruga R. 2005. Dynamic cellular responses in plant-microbe interactions. *Current Opinion in Plant Biology* **8**(6): 625-631.

Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, DeVera ME, Liang X, Toer M, Billiar T. 2007. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunological Reviews* **220**: 60-81.

Manabe Y, Nafisi M, Verhertbruggen Y, Orfila C, Gille S, Rautengarten C, Cherk C, Marcus SE, Somerville S, Pauly M. 2011. Loss-of-function mutation of REDUCED WALL ACETYLATION2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to Botrytis cinerea. *Plant Physiology* **155**(3): 1068-1078.

Mellersh DG, Foulds IV, Higgins VJ, Heath MC. 2002. H2O2 plays different roles in determining penetration failure in three diverse plant-fungal interactions. *Plant Journal* **29**(3): 257-268.

Micali CO, Neumann U, Grunewald D, Panstruga R, O'Connell R. 2011. Biogenesis of a specialized plant-fungal interface during host cell internalization of Golovinomyces orontii haustoria. *Cellular Microbiology* **13**(2): 210-226.

Neuenschwander U, Vernooij B, Friedrich L, Uknes S, Kessmann H, Ryals J. 1995. Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? *The Plant Journal* **8**(2): 227-233.

Nicholson RL, Hammerschmidt R. 1992. Phenolic-compounds and their role in disease resistance. *Annual Review of Phytopathology* **30**: 369-389.

Nicholson RL, Kunoh H. 1995. Early interactions, adhesion, and establishment of the infection court by *Erysiphe graminis*. *Canadian Journal of Botany-Revue Canadienne De Botanique* **73**: S609-S615.

Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC. 2003. Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* **301**(5635): 969-972.

Pryce-Jones E, Carver T, Gurr SJ. 1999. The roles of cellulase enzymes and mechanical force in host penetration by Erysiphe graminis f.sp hordei. *Physiological and Molecular Plant Pathology* **55**(3): 175-182.

Ride JP, Barber MS. 1987. The effects of various treatments on induced lignification and the resistance of wheat to fungi. *Physiological and Molecular Plant Pathology* **31**(3): 349-360.

Rodriguezgalvez E, Mendgen K. 1995. Cell-wall synthesis in cotton roots after infection with *Fusarium-oxysporum*. *Planta* **197**(3): 535-545.

Schweizer P, Kmecl A, Carpita N, Dudler R. 2000. A soluble carbohydrate elicitor from Blumeria graminis f. sp. tritici is recognized by a broad range of cereals. *Physiological and Molecular Plant Pathology* **56**(4): 157-167.

Smart MG, Aist JR, Israel HW. 1986. Structure and function of wall appositions .1. General histochemistry of papillae in barley coleoptiles attacked by *Erysiphe-graminis* f-sp *hordei*. *Canadian Journal of Botany-Revue Canadienne De Botanique* **64**(4): 793-801.

Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S. 2006. *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. *Plant Cell* **18**(3): 731-746.

Stergiopoulos I, de Wit PJGM 2009. Fungal Effector Proteins. *Annual Review of Phytopathology*, 233-263.

Takahashi K, Aist JR, Israel HW. 1985. Distribution of hydrolytic enzymes at barley powdery mildew encounter sites - implications for resistance associated with papilla formation in a compatible system. *Physiological Plant Pathology* **27**(2): 167-184.

ThordalChristensen H, Zhang ZG, Wei YD, Collinge DB. 1997. Subcellular localization of H2O2 in plants. H2O2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant Journal* **11**(6): 1187-1194.

Torres MA, Jones JDG, Dangl JL. 2006. Reactive oxygen species signaling in response to pathogens. *Plant Physiology* **141**(2): 373-378.

Tsuda K, Sato M, Stoddard T, Glazebrook J, Katagiri F. 2009. Network Properties of Robust Immunity in Plants. *PLoS Genet* **5**(12): e1000772.

Underwood W. 2012. The plant cell wall: a dynamic barrier against pathogen invasion. *Frontiers in plant science* **3**: 85-85.

Underwood W, Somerville SC. 2008. Focal accumulation of defences at sites of fungal pathogen attack. *Journal of Experimental Botany* **59**(13): 3501-3508.

Vega-Sánchez ME, Verhertbruggen Y, Christensen U, Chen X, Sharma V, Varanasi P, Jobling SA, Talbot M, White RG, Joo M. 2012. Loss of Cellulose synthase-like F6 function affects mixed-linkage glucan deposition, cell wall mechanical properties, and defense responses in vegetative tissues of rice. *Plant Physiology* **159**(1): 56-69.

Vogel JP, Raab TK, Schiff C, Somerville SC. 2002. PMR6, a pectate lyase–like gene required for powdery mildew susceptibility in *Arabidopsis*. *The Plant Cell* **14**(9): 2095-2106.

Vogel JP, Raab TK, Somerville CR, Somerville SC. 2004. Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. *The Plant Journal* **40**(6): 968-978.

von Ropenack E, Parr A, Schulze-Lefert P. 1998. Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *Journal of Biological Chemistry* **273**(15): 9013-9022.

Wang W, Wen Y, Berkey R, Xiao S. 2009. Specific targeting of the *Arabidopsis* resistance protein RPW8.2 to the interfacial membrane encasing the fungal Haustorium renders broad-spectrum resistance to powdery mildew. *Plant Cell* **21**: 2898-2913.

Wei YD, Deneergaard E, Thordalchristensen H, Collinge DB, Smedegaardpetersen V. 1994. Accumulation of a putative guanidine compound in relation to other early defense reactions in epidermal-cells of barley and wheat exhibiting resistance to *Erysiphe-graminis* f sp *hordei*. *Physiological and Molecular Plant Pathology* **45**(6): 469-484.

Zeyen RJ, Ahlstrand GG, Carver TLW. 1993. X-ray microanalysis of frozen-hydrated, freeze-dried, and critical point dried leaf specimens: determination of soluble and insoluble chemical elements at Erysiphe graminis epidermal cell papilla sites in barley isolines containing Ml-o and ml-o alleles. *Canadian Journal of Botany* **71**(2): 284-296.

Zeyen RJ, Bushnell WR. 1979. Papilla response of barley epidermal-cells caused by erysiphe graminis - rate and method of deposition determined by microcinematography and transmission electron-microscopy. *Canadian Journal of Botany-Revue Canadienne De Botanique* **57**(8): 898-913.

Zeyen RJ, Carver TLW, Lyngkjaer MF. 2002. Epidermal cell papillae. *Powdery Mildews: A Comprehensive Treatise*: 107-125.

CHAPTER 3

Differential accumulation of callose, arabinoxylan and cellulose in non-penetrated versus penetrated papillae on leaves of barley infected by *Blumeria graminis* f. sp. *hordei.*

Title of Paper	Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated versus penetrated papillae on leaves of barley infected with *Blumeria graminis* f. sp. *hordei*
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	New Phytologist 204(3): 650-660. doi: 10.1111/nph.12974

Principal Author

Name of Principal Author (Candidate)	Jamil Chowdhury		
Contribution to the Paper	Performed all sample analysis and microscopy, interpreted data, wrote manuscript and acted as principal author.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30 March 2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and acted as corresponding author		
Signature		Date	29 March 2016

Link to chapter 3

The review of the literature presented in Chapter 2 captures the research conducted on various aspects of cell wall reinforcement during pathogen infection. It is clear that cell wall reinforcement in the form of localised papillae deposition is an important factor determining the outcomes of plant pathogen interactions. However, the actual role of papillae might be complex and require further research to better understand the papillae-based penetration resistance mechanisms of host plants. In monocots, callose is the only polysaccharide that has been reported in papillae during plant-pathogen interactions. However, other polysaccharides contribute mechanical support to the cell so it is important to explore whether they are also present in papillae during pathogen infection. As stated in Chapter 1, one of the broad objective of this study is to investigate the composition, structure and dynamics of the polysaccharides in the papillae which confer altered penetration resistance in the barley-powdery mildew host-pathogen system. The research described in this chapter was undertaken using new immunocytochemical techniques, coupled with specific stains and monoclonal antibodies, to investigate the polysaccharide composition of effective and ineffective papillae that form during the barley-powdery mildew interaction. The outcome of the experiments is published as follows: Chowdhury J, Henderson M, Schweizer P, Burton RA, Fincher GB, Little A. 2014. Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated versus penetrated papillae on leaves of barley infected with *Blumeria graminis* f. sp. *hordei*. New Phytologist 204 (3): 650-660. The published version of the manuscript has been presented in Chapter 3.

Differential accumulation of callose, arabinoxylan and cellulose in non-penetrated versus penetrated papillae on leaves of barley infected by *Blumeria graminis* f. sp. *hordei*.

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Summary

- In plants, cell walls are one of the first lines of defence for protecting cells from successful invasion by fungal pathogens and are a major factor in basal host resistance. For the plant cell to block penetration attempts, it must adapt its cell wall to withstand the physical and chemical forces applied by the fungus.
- The polysaccharide composition of papillae that have been effective in preventing penetration by pathogens are traditionally believed to contain callose as the main polysaccharide component. Here, we have re-examined the composition of papillae of barley attacked by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) using a range of antibodies and carbohydrate-binding modules that are targeted to cell wall polysaccharides.
- The data show that barley papillae induced during infection with *Bgh* contain in addition to callose, significant levels of cellulose and arabinoxylan. Higher concentrations of callose, arabinoxylan and cellulose are found in effective papillae, compared with ineffective papillae. The papillae have a layered structure with the inner core consisting of callose and arabinoxylan and the outer layer containing arabinoxylan and cellulose.
- The association of arabinoxylan and cellulose with penetration resistance opens new targets for the improvement of papillae composition and enhanced disease resistance.

Key words: callose / arabinoxylan / cellulose / papillae / *Blumeria graminis* / powdery mildew / penetration

Introduction

Barley powdery mildew, a foliar disease caused by *Blumeria graminis* f. sp. *hordei* (*Bgh*), results in severe yield reduction almost every year (Oerke and Dehne, 2004). Like most of the powdery mildew pathogens, *Bgh* has adapted mechanisms to penetrate the cuticle and cell wall to directly draw nutrients from epidermal cells. As a defence response, plants reinforce the cell wall near the site of penetration by producing a dome-shaped apposition between the epidermal wall and the plasma membrane. This is commonly known as a papilla (Zeyen *et al*., 2002). Papillae are a front line defence against adapted and non-adapted *formae speciales* of powdery mildew fungi in a number of different plant species (Aghnoum *et al*., 2010) and are believed to provide mechanical and chemical barriers to halt penetration of pathogens or to delay the infection process while the plant's other defences become active (Huckelhoven, 2005; Huckelhoven, 2007; Albersheim *et al*., 2011). However, the formation of a papilla does not guarantee that penetration will be prevented and, based on the outcome of the penetration attempt, papillae can be classified as either effective or ineffective (Figure 1). The classification of papillae as effective is open for debate as there is still no direct evidence that the papilla itself is capable of acting as a defensive barrier preventing fungal penetration. Papillae formed in resistant host and non-host species appear more effective at blocking penetration than those formed in a susceptible host, but the observed resistance may be due to an alternate defence mechanism (Zeyen *et al*., 2002). In general, most studies have focused on correlating papillae effectiveness with the timing of papillae formation, their architecture, size and, even more intensively, on papillae composition. Faster papillae formation has been reported in naturally resistant lines and in chemically-induced resistant lines compared with wild type susceptible cultivars. Chemically-induced large papillae were found to be more effective in halting penetration compared to normal size papillae formed in wild type lines (Israel *et al*., 1980; Aist & Israel, 1977).

Barley papillae have been shown to contain callose, phenolics (lignin and phenolic conjugates), arabinogalactan proteins, antimicrobial components, inorganic elements and reactive oxygen species (ROS) (Aist, 1976; Zeyen *et al*., 2002; Underwood, 2012). The role of papillary callose has received the most research attention, because callose in papillae was believed to act as a mechanical barrier to penetration (Aist, 1976). However, this concept has been re-examined for several plant-pathogen interactions. The inhibition of *Arabidopsis* papillary callose deposition through disruption of the callose synthase gene GSL5/PMR4 showed a marginally decreased penetration rate in the *Arabidopsis*-*Golovinomyces cichoracearum* interaction (Jacobs *et al*., 2003) and in the Arabidopsis-*Erysiphe cichoracearum* interaction (Nishimura *et al*., 2003). Conversely, when the *PMR4* gene is overexpressed, higher and early accumulation of papillary callose occurs and host plants show complete penetration resistance against the well-adapted pathogens *G. cichoracearum* (Ellinger *et al*., 2013) and Barley-*Bgh* interactions (Blumke *et al*., 2013). Chemical inhibition of papillary callose synthesis also increases penetration efficiency of fungal pathogens on non-host grass species including rice, wheat and oat (Zeyen *et al*., 2002). Apart from callose, barley papillae formed in incompatible interactions have significantly higher accumulation of thionin proteins (Ebrahim-Nesbat *et al*., 1993), the phenolic conjugate *p*-coumaroyl-hydroxyagmatine (*p*-CHA) (von Ropenack, *et al*., 1998) and hydrogen peroxide (Huckelhoven *et al*., 1999) as compared with papillae formed in compatible interactions. All these components have been suggested to possess anti-fungal activities and in the case of hydrogen peroxide may also be involved in oxidative cross-linking of cell wall polysaccharides via phenolic compounds (Iiyama *et al*., 1994; Schopfer, 1996).

Despite these findings, the cell wall components responsible for making papillae an effective barrier to fungal penetration are not fully understood. The overall susceptibility or resistance of a barley genotype is determined by the frequency of papillae that halt penetration effectively, compared with papillae that are ultimately penetrated by the fungus. To gain more

information in understanding the structural and compositional basis of papillae effectiveness, we compared the composition of papillae in susceptible cells with that of papillae in resistant cells, within the same barley line. Callose is a (1,3)-β-glucan that is easy to detect with the aniline blue fluorochrome (Stone *et al*., 1984), and we now have access to a number of polysaccharide-specific antibodies that can be used to examine papillae for other polysaccharides that have been previously overlooked (Knox, 2008; Pattathil *et al*., 2010). Thus, in this study, we present a comprehensive immunohistochemical screen of the polysaccharide composition of barley papillae, using polysaccharide-specific probes.

Materials and Methods

Plants and pathogen growth conditions and inoculation

A field isolate of *Bgh* was kindly donated by Professor Richard Oliver (The Australian Centre for Necrotrophic Fungal Pathogens, Curtin University, W.A.). The isolate was maintained on a susceptible barley cultivar, 'Sloop SA', in a growth chamber at 20°C with a photoperiod of 16 h. Barley plants were grown in plastic pots containing a 'cocopeat' soil mixture. To obtain a uniform distribution of conidia, inoculation was performed using a settling tower at a height of 50 cm. Inoculation was carried out on the adaxial leaf surface of ten day old detached leaves pinned on agarose gel in petri dishes. Conidia were inoculated at an average density of 100 per mm^2.

Whole-mount tissue preparation for light microscopy

Inoculated leaves were sampled and cut into 2 cm pieces and decolorized in boiling 96% v/v ethanol for 10 min. The tissues were clarified for 16 h in saturated chloral hydrate solution. Clarified tissues were rinsed with water and washed with phosphate buffer solution (PBS, pH7.5). Fungal structures were stained with wheat germ agglutinin (WGA) conjugated with Alexa Fluor 488 (Life Technologies, Carlsbad, U.S.A.), which selectively binds to N−acetylglucosaminosyl residues in the fungal wall (Wright, 1984). Cellulose was labelled with a 0.01% solution Pontamine Fast Scarlet 4B (Aldrich Rare Chemicals Library, USA, catalogue no. S479896) in PBS (Anderson *et al*., 2010) for 2 h. Callose and arabinoxylan antibodies were labelled in the whole-mount tissue using the immunolocalization technique of Hervé et al. (2011), with the following modification: Tissues were permeabilized by immersion in 0.5% v/v Triton X-100 in PBS (PBST) for 20 min to allow access to the antigens in the epidermal papillae and halo areas. Nonspecific binding sites were blocked using PBS containing 2% bovine serum albumin (BSA) and 2% normal goat serum (PBSBG) for 30 min at room temperature. The callose-specific antibody, LAMP2H12H7 (Biosupplies Australia Pty Ltd.),

was conjugated to Alexa Fluor 555 using the Zenon® Mouse IgG1 labelling kit (Life Technologies, Carlsbad, U.S.A.) following the manufacturer's instructions. The LM11 antibody (specific for unsubstituted xylan and arabinoxylan) and LAMP2H12H7-Alexa Fluor 555 were diluted in PBST (1:100). The tissues were incubated with diluted antibodies in a humid chamber for 2 h protected from light. The tissues labelled with LM11 were washed with PBSBG for 30 min before incubating with secondary antibody conjugated with Alexa Fluor 555 for 4 h. The tissues labelled with callose-Alexa Fluor 555 were washed twice with PBST for 30 min. Callose was also detected using the aniline blue fluorochrome following the protocol previously described in Soukup (2014).

Sectioned tissue preparation for light and electron microscopy

For light and electron microscopy tissues were fixed, embedded and sectioned following the protocol described in Burton *et al.*, (2011). Immunofluorescence labelling for light microscopy and immunogold labelling for electron microscopy were performed as described in Burton *et al.*, (2011). Immunogold labelling for light microscopy was performed following the same procedure as used for electron microscopy with the following modifications: after incubating the samples with gold-conjugated secondary antibodies, samples were washed in PBS-gelatin buffer (pH 7.6) and repeatedly washed with distilled water. Gold particles were enhanced with the 'Nanoprobes gold enhancement kit' (Nanoprobes, Inc., USA, catalogue number 2112) following the manufacturer's instructions. To stop enhancement reactions and reduce background labelling, samples were repeatedly washed with deionized water and incubated in freshly prepared sodium thiosulphate (1-2% w/v) for 30 sec.

Microscopic image acquisition and analysis

Two dimensional (2D) images of the whole mount and cross sectioned tissues were examined using a Carl Zeiss Fluorescence microscope (Axio Imager M2, Zeiss, Germany) pre-aligned with an Hg-mercury system. Signals from Pontamine Fast Scarlet 4B and the

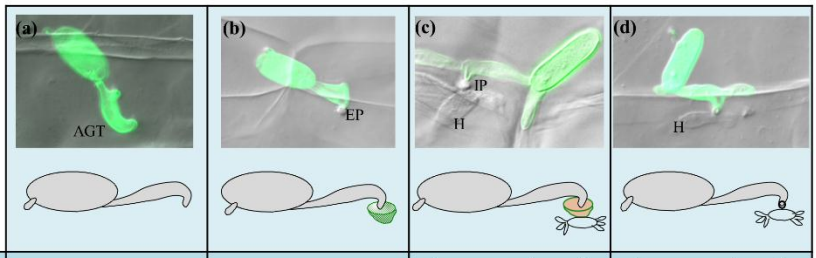
Alexa Fluor 555-conjugated secondary antibody were observed under the dsRED filter set at 545/25 nm excitation and 605/70 nm emission wavelengths. Signals from the Alexa Fluor 488 conjugated secondary antibody were observed under the GFP filter set at 470/40 nm excitation and 525/50 nm emission wavelengths. Phenolic acid-associated autofluorescence was observed under the DAPI filter set at 365 nm excitation and 445/50 nm emission wavelengths. After setting the exposure time using the 'measure' function, it was adjusted to 100-150 ms ± 20 ms for each specimen. Images were captured using an AxioCam HRc camera and Leica Zen software. For quantitative assessment of fluorescence location and distribution, the fluorescence intensity profiles were measured by drawing a line across the papillae and halo area using the 'curve spline' function of the Zen software. The intensity values were normalized for each cell by subtracting the background staining of the wall from the staining intensity of the papilla. The region for calculating the background staining was selected where the intensity profile radiating from the centre of the papilla had plateaued.

For 3D imaging a series of images across the Z plane were captured using the Leica SP5 spectral scanning confocal microscope with a 100X oil-immersion objective. Tissues were stained with the same fluorophores as used for fluorescence microscopy, as described above, and mounted between a microscope slide and coverslip in 70% v/v glycerol. Pontamine fast scarlet 4B and Alexa Fluor 555 were observed using a 561 nm excitation wavelength from a diode-pumped solid-state (DPSS) diode laser and detector slits were configured for emission from 572 nm to 700 nm. Alexa Fluor 488 was observed using a 488 nm excitation wavelength from an argon laser and detector slits were configured for emission from 499–551 nm. Aniline blue fluorochrome was observed using a 405 nm excitation wavelength from a diode laser and detector slits were configured for emission from 472–490-nm. For 3-D image reconstruction and *in silico* cross-sectioning, ImageJ software was used (http://imagej.nih.gov/ij/).

Results

Importance of effective papillae in pre-invasion resistance in the Barley/Bgh interaction

After the successful formation of the appressorial germtube (AGT), a number of possible outcomes were observed in the barley/*Bgh* interaction (Fig. 1). These included unsuccessful penetration without any visible host responses (Fig. 1a), unsuccessful penetration due to the formation of an effective host papilla (Fig. 1b), successful penetration through an ineffective host papilla (Fig. 1c) and successful penetration in the absence of a detectable papilla (Fig. 1d). Conidia that did not form AGT were not counted in the susceptibility calculations. The formation of effective papillae was the most common outcome in both the susceptible barley line (Golden Promise) and resistant line (Galaxy, *mlo 11*) with penetration blocked in 75% and 90% of attempts respectively (Fig. 1). The formation of ineffective papillae was the next highest outcome, with penetration of the papillae observed at 12% and 2% of attempts respectively. Once an effective papilla was formed it successfully halted subsequent penetration attempts during the remainder of the infection period observed.



Bgh structure		Appressorium	Appressorium	Appressorium & haustorium	Appressorium & haustorium
Papilla		None	Effective	Ineffective	None
Susceptible line % (Golden Promise)	24 h	12.5	76.5	4.9	6.1
	48 h	6.9	79.4	6.2	7.5
	72 h	4.4	74.5	12.3	8.8

Fig. 1 Possible infection outcomes from barley/*Blumeria graminis* f. sp. *hordei* interactions after the formation of an appressorium germ tube (AGT). Overlay images show *Blumeria graminis* f. sp. *hordei* conidia labeled with chitin-specific WGA-AF 555 (green) and underlying papillae and haustoria visible using the reflective index of differential interference contrast (DIC) microscopy. Percent frequencies were estimated from 200 conidial observations at each time point using the susceptible barley cultivar, Golden Promise. AGT, appressorium germ tube; EP, effective papilla; IP, ineffective papilla; H, haustorium.

Presence of wall polysaccharides in effective barley papillae

A screen was conducted in transverse sections of barley papillae using a range of polysaccharide-specific probes, including monoclonal antibodies and carbohydrate-binding modules (CBMs). The results were confirmed with two light microscopy techniques: 'Immunofluorescence labelling' allowed us to measure fluorescence intensity in both the papilla core and halo area. 'Enhanced immunogold labelling' allowed us to ensure the observation of target epitopes in the papillae was not attributable to background autofluorescence associated with phenolic acid wall constituents. Both techniques detected significant labelling in the papillae and the surrounding 'halo' areas when callose-, arabinoxylan- and crystalline-cellulose-specific probes were used (Table 1 and Fig. 2). Very weak labelling was also observed using unsubstituted xylan-, mannan- and homogalacturonan-specific antibodies. Labelling with (1,3;1,4)-β-glucan-specific antibodies was completely absent in papillae, but these polysaccharides were strongly detected in normal epidermal walls. No significant signals were detected with rhamnogalacturonan-specific probes. To confirm the presence of callose and crystalline cellulose within the papillae, the polysaccharides were chemically stained using aniline blue fluorochrome and Pontamine fast scarlet 4B, respectively, and the same patterns of fluorescence were observed (Fig. 3).

Table 1 Relative signal intensity of cell wall specific probes in the barley papillae and halo regions formed against *Blumeria graminis* f. sp. *hordei.*

Probe	Target antigen	Papillae		Halo region		Uninfected epidermal wall	
		IF	IGE-LM	IF	IGE-LM	IF	IGE-LM
BG1	(1,3;1,4)-β-glucan	NS	NS	NS	NS	+++	+++
BGM C6	(1,4)-β-Mannan; Galactomannan	NS	+	NS	NS	+	+
CBM3a	Crystalline cellulose	+++	+++	+	+	+++	+++
LAMP2H12H7	(1,3)-β-glucan	++	++	+	+	NS	NS
LM5	(1,4)-β-galactan	NS	NS	NS	NS	NS	NS
LM6	(1,5)-α-arabinan	NS	NS	NS	NS	NS	NS
LM7	Methylesterfied homogalacturonan	+	NS	+	NS	+	NS
LM8	Xylogalacturonan	NS	NS	NS	NS	NS	NS
LM9	Feruloylated galactan	NS	NS	NS	NS	NS	NS
LM10	(1,4)-β-Xylan	NS	NS	NS	NS	NS	NS
LM11	(1,4)-β-Xylan, Arabinoxylan	+++	+++	++	++	+	+
LM15	Xyloglucan	+	NS	NS	NS	++	+
LM19	De-esterified homogalacturonan	+	+	NS	NS	+	+

It is important to note that the avidity of each probe is different and therefore only qualitative comparisons can be made between tissues that have been treated with the same probe. All intensities were observed in 1 µm thick transverse sections using the susceptible barley cultivar, Golden Promise. NS, No signal detected; +, limited labelling; ++, moderate labelling; +++, strong labelling; IF, immunofluorescence; IGE-LM, immunogold enhancement in light microscopy.

Location of polysaccharides in effective barley papillae

High resolution transmission electron microscopy (TEM) immunogold labelling was used to confirm the presence of callose, arabinoxylan and crystalline cellulose in effective papillae and to determine if they were evenly distributed within the papillae structure. Consistent with the light microscopy results, all three target antibodies were detected using TEM in barley papillae that were believed to be effective in preventing penetration (Fig. 2). However, it was not possible to conclusively determine if the papillae were non-penetrated due to the chance that the cutting plane of the section may not have hit the penetration pore or haustorium. The structure of the papillae appeared layered, with callose concentrated in a central core, arabinoxylan distributed throughout the papillae and crystalline cellulose restricted to the outer layer of the papillae.

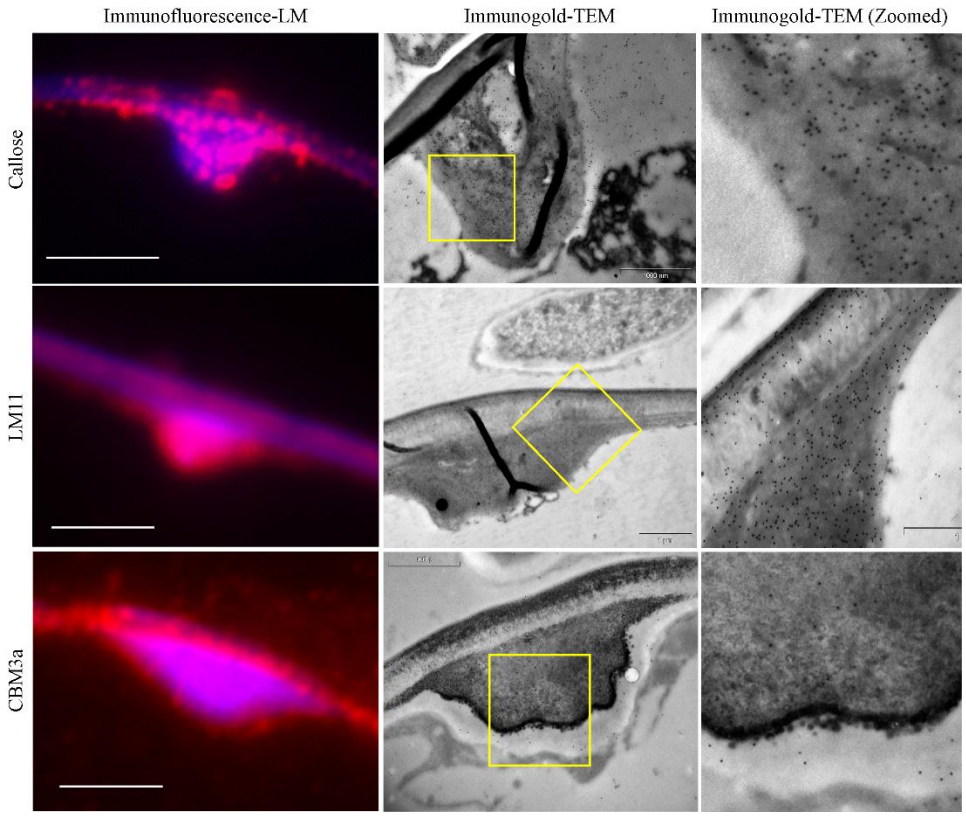


Fig. 2 Transverse sections of barley papillae formed against *Blumeria graminis* f. sp. *hordei* at 24 hours after inoculation (hai) labelled with callose-, arabinoxylan- and crystalline cellulose- specific probes. All intensities were observed in 1 µm thick transverse sections using the susceptible barley cultivar, Golden Promise. First column; red fluorescence, AF555-secondary antibodies attached to respective primary antibodies; blue fluorescence, phenolic acid-associated autofluorescence; scale bar = 5 µm. Second column, immunogold labelled transmission electron micrograph, scale bar = 1 µm. Third column, magnified view of boxed area from second column. LM, light microscopy; TEM, transmission electron microscopy.

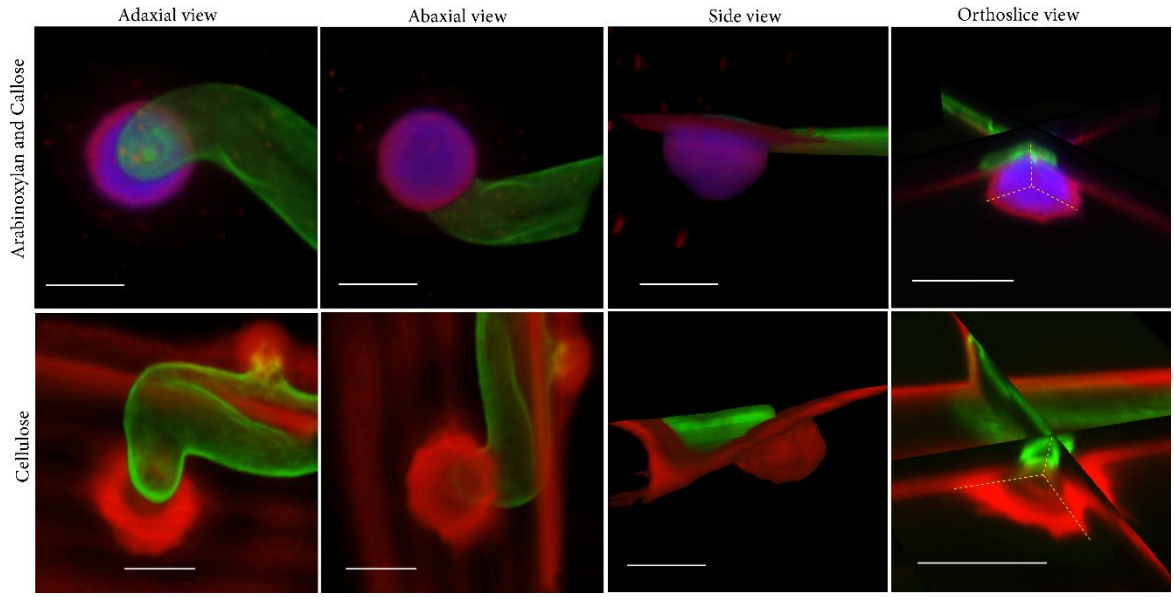


Fig. 3 Confocal microscopy of polysaccharide deposition patterns in effective barley papillae formed against *Blumeria graminis* f. sp. *hordei* at 24 hai. All intensities were observed using the susceptible barley cultivar, Golden Promise. Fungal appressoria labelled with WGA-AF488 (green). First row, different views of a papilla labelled with an arabinoxylan probe (LM11, red) and a callose probe (aniline blue, blue). Second row, different views of a papilla labelled with a cellulose probe (Pontamine fast scarlet 4B, red). Scale bar = 5 µm.

Polysaccharide deposition pattern in effective barley papillae

The overall polysaccharide deposition pattern was observed in fully grown effective barley papillae, by co-staining papillae with multiple fluorophores using an adapted whole mount tissue labelling technique (Fig. 3). The arabinoxylan probe (LM11) and the callose probe (aniline blue fluorochrome) showed that both of the polysaccharides were present in the inner core of the papillae. However, in most cases, the diameter of the arabinoxylan fluorescence area is greater than that of the callose (Fig. 3). Labelling with Pontamine fast scarlet 4B showed the presence of cellulose in the outer layer of papillae, with some labelling within the papillae core (Fig. 3). These observations were consistent with the TEM observations that CBM3a epitopes, which were used as probes for crystalline cellulose, were mainly concentrated in the outer layer of papillae cross sections. When the papillae were co-stained with Pontamine fast scarlet and aniline blue fluorochrome, the presence of callose was mainly detected in the inner layer and cellulose in the outer layer and minimal overlap was observed (Fig. 4). Papillae co-stained with LM11 and Pontamine fast scarlet revealed the presence of arabinoxylan throughout the papillae with cellulose in the outer layer and thus a significant overlap was observed (Fig. 4). The effective papillae are therefore composed of two layers; the innermost layer containing callose and arabinoxylan, and the second outer layer containing cellulose and arabinoxylan.

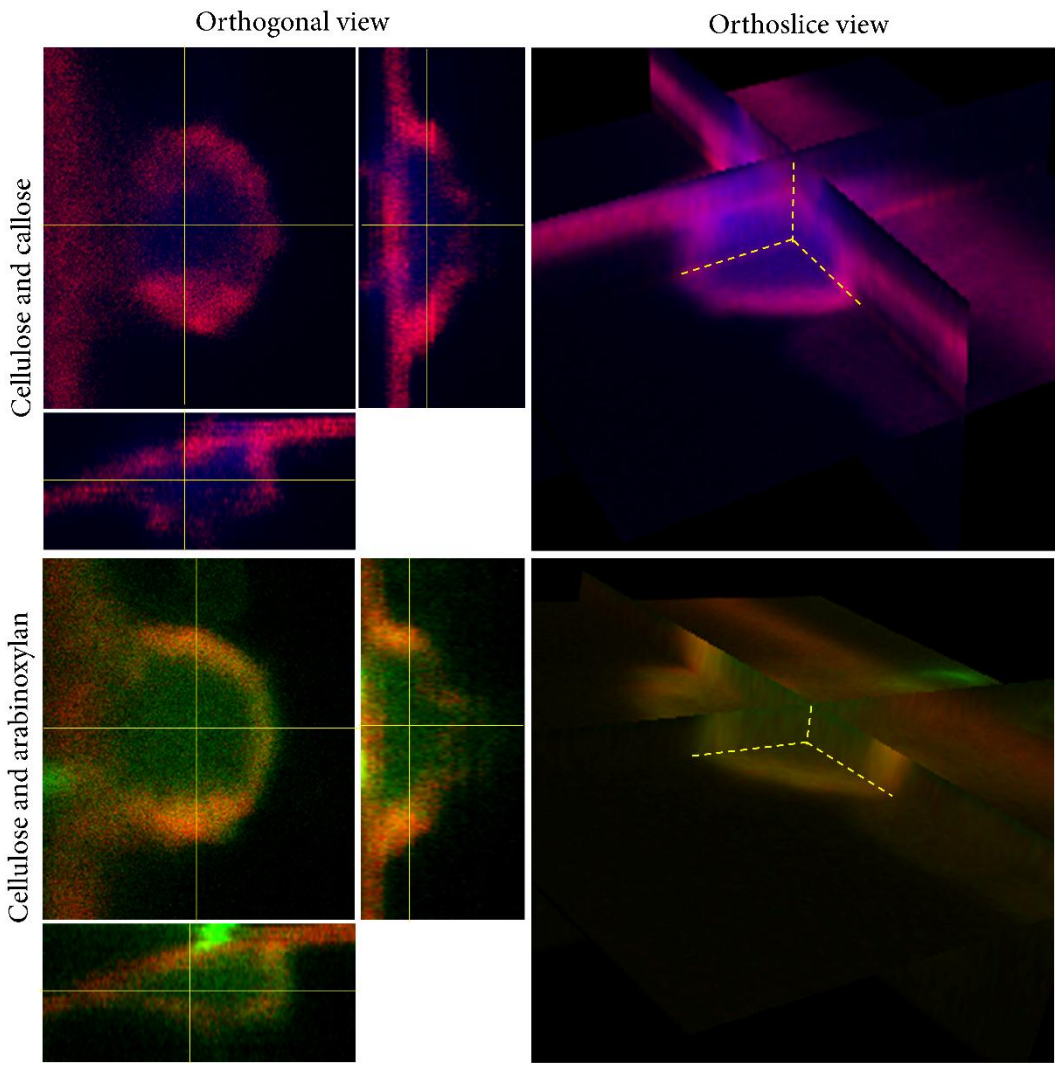


Fig. 4 Confocal microscopy of polysaccharide deposition patterns in effective barley papillae formed against *Blumeria graminis* f. sp. *hordei* at 24 hai. All intensities were observed using the susceptible barley cultivar, Golden Promise. Orthogonal views of virtual sections showing papilla labelling from top and side angles. Papilla labelled with the same probes as Fig. 3, but with different colours; callose (blue), cellulose (red) and arabinoxylan (green).

Association of polysaccharide-specific probes with effective and ineffective barley papillae

The labelling intensity for callose, arabinoxylan and cellulose was observed in all papillae, either those which were successful in preventing penetration, or in ineffective papillae, which were not successful in blocking fungal growth (Fig. 5). Ineffective papillae were identified through the presence of haustorial structures underneath them, inside the cell. The relative staining of each fluorophore on different papillae was compared by quantifying the fluorescence intensity of 50 effective and 50 ineffective papillae (Fig. 6). The average normalized fluorescence intensity was significantly higher in effective papillae for callose, arabinoxylan and cellulose, as compared with the intensity in the ineffective papillae. No significant difference was found between the effective and ineffective papillae when callose was stained with the aniline blue fluorochrome, probably due to non-specific binding of the stain. Strong fluorescence is observed with (1,3)-ß-glucans although a weaker fluorescence is observed with some other glucans (Evans *et al*., 1984). Phenolic acid-associated autofluorescence was observed to be distributed throughout both types of papillae at a similar intensity, although ineffective papillae seemed to have slightly reduced intensity in the core region.

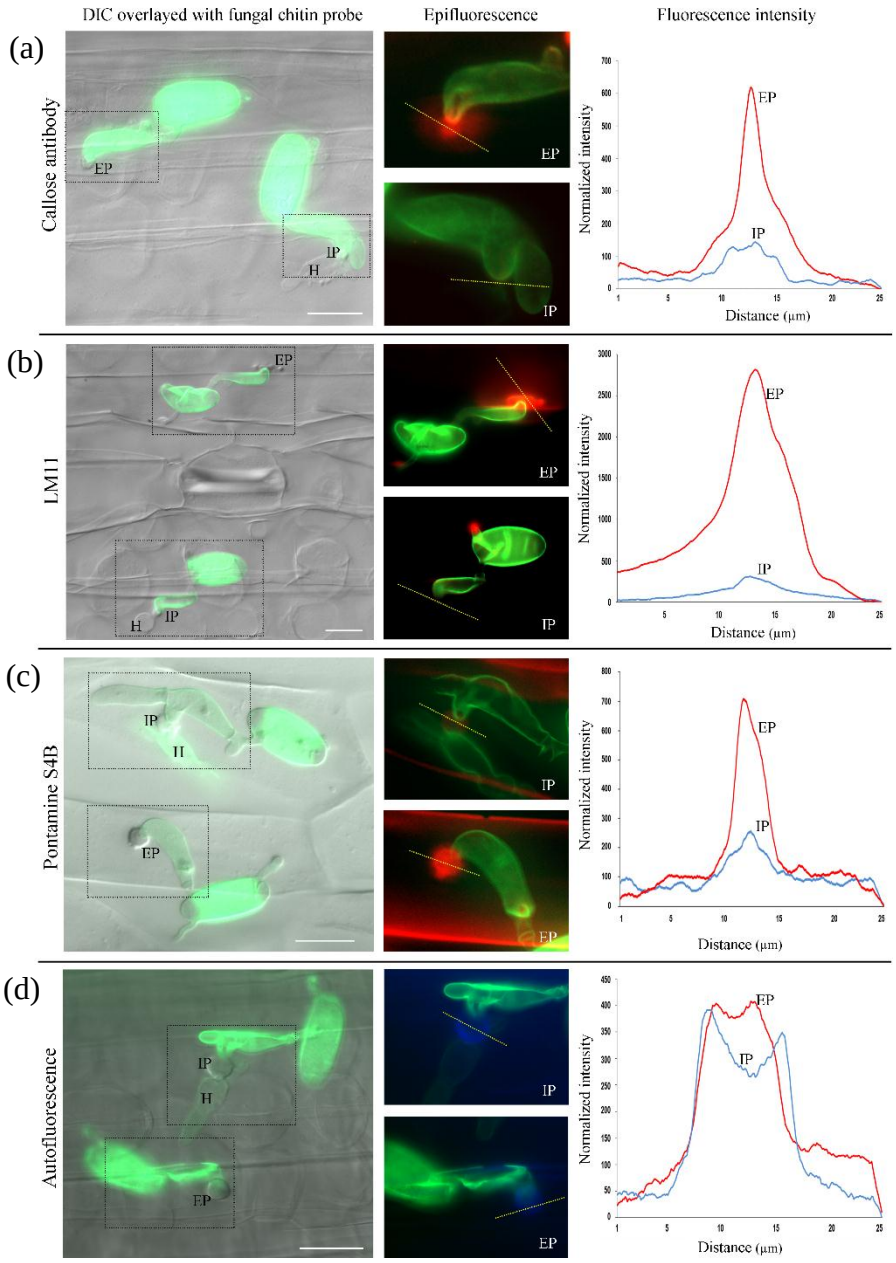


Fig. 5 Labelling of callose-, arabinoxylan-, cellulose specific- probes and phenolic acid-associated autofluorescence in effective and ineffective barley papillae formed against *Blumeria graminis* f. sp. *hordei* at 24 hai. All intensities were observed using the susceptible barley cultivar, Golden Promise. (a-d) Fungal appressoria labelled with WGA-AF488 (green). (a-c) Polysaccharide-specific probes (red) and (d) autofluorescence (blue). Fluorescence intensity profiles correspond to yellow dashed lines showing the distribution of observed labelling in effective and ineffective papillae. EP, effective papilla; IP, ineffective papilla; H, haustorium. Scale bar = 20 µm.

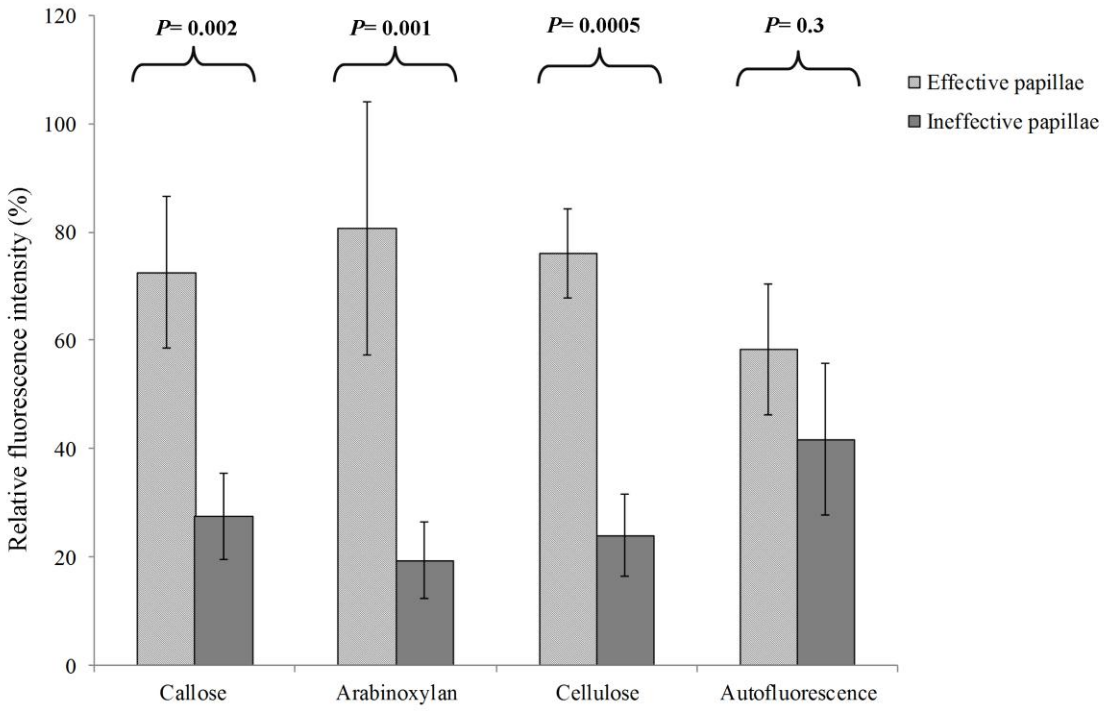


Fig. 6 Relative fluorescence intensity in effective and ineffective barley epidermal papillae at 24 hai. All intensities were observed using the susceptible barley cultivar, Golden Promise. For each probe the average maximum intensity was calculated using the maximum intensity found in the fluorescence intensity profile of fifty papillae and normalized against background tissue staining. In order to directly compare the different probes in one graph the relative fluorescence intensity was calculated as a percentage of the highest maximum found for each probe. The error bars show the standard deviations of the mean. *P* values of student's t-test are indicated above each set.

Discussion

Callose is a well-known component of papillae in a number of plant species, where intensive research has been conducted into the defence response mounted during powdery mildew infection. Such studies are exemplified by those carried out in barley and Arabidopsis (Pirselova & Matusikova, 2013). The proposed role of callose in penetration resistance originally resulted from observations correlating effective papillae to callose staining in a wide variety of plant-pathogen interactions (Stone & Clarke, 1992). Early deposition and an increased size of the host callose deposit were thought to create a more effective papilla. This assumption is supported by the results presented here where a higher callose staining intensity in effective papillae is observed when compared to ineffective papillae. However, the importance of callose has been questioned due to the number of callose-rich ineffective papillae found (Aist, 1976) and more recently by the behaviour of callose-deficient mutants in Arabidopsis that show enhanced pathogen resistance (Jacobs *et al*., 2003; Nishimura *et al*., 2003). The *glucan synthase like 5* (*Gsl5*) gene, responsible for the production of callose in Arabidopsis papillae, was silenced in an insertion mutant (Jacobs *et al*., 2003). This led to a significant reduction in callose deposition levels, but an unexpected increase in fungal resistance. One possible explanation for this involved a potential role for callose in the encasement of the fungal haustoria, where the callose may be required for structural integrity or transport of essential nutrients (Jacobs *et al*., 2003). The role of callose as a key component in penetration resistance was confirmed following the overexpression of *Gsl5* in Arabidopsis (Ellinger *et al*., 2013) resulting in early callose deposition and complete penetration resistance. The significantly higher levels of callose in the effective papillae compared to the ineffective papillae observed here in barley, suggest a similar role for callose in penetration resistance against *Bgh*. Some members of the *Gsl* gene family in barley have been identified (Schober *et al*., 2009), but the isoform responsible for pathogen-induced callose deposition is not known.

The question remains as to whether silencing the particular *Gsl* gene responsible for callose synthesis in the barley papillae will lead to an increase or decrease in susceptibility.

A significantly enhanced amount of arabinoxylan labelling was detected in effective compared with ineffective papillae and in the epidermal cell wall (Fig. 2). The LM11 antibody has been reported to bind to unsubstituted xylan and arabinoxylans with a low degree of arabinose substitution (McCartney *et al*., 2005). It can also accommodate more extensive substitution of a xylan backbone, binding strongly to wheat arabinoxylan and weakly to maize glucuronoarabinoxylan. In order to narrow down the potential epitopes being labelled in the papillae, the LM10 antibody was used. LM10 can recognise unsubstituted and relatively low-substituted xylans in several species, but has no cross-reactivity with wheat arabinoxylan. The precise epitope that LM11 is binding to in the papillae is not known, but it is clear that there is some form of arabino-substituted xylan present. The arabinoxylan location pattern extends throughout the papillae and overlaps the regions containing callose, cellulose and phenolic acids. Arabinoxylan has a linear (1,4)-β-xylan backbone substituted at either the C(O)2 and/or C(O)3 position with single arabinofuranosyl residues. Non-covalent bonds between the arabinoxylan chains and cellulose fibres are likely to play a part in maintaining the barley cell wall (McNeil *et al*., 1975). A single arabinoxylan molecule may contain unsubstituted regions that bind to other arabinoxylan molecules and regions that bind to cellulose. This potentially leads to a highly cross linked cell wall. Such a tight wall structure would not easily lend itself to mechanical penetration. In addition to this, the presence of phenolic components, such as ferulic and *p*-coumaric acids, in the papillae gives significant potential for further covalent crosslinking. Ester linkages between the phenolic acids and the arabinose substitutions of arabinoxylan, followed by oxidation-induced dihydrodimer formation of the phenolic acids, has been demonstrated to account for substantial crosslinking and strength in cereal grain tissues (Harris & Trethewey, 2010; Philippe *et al*., 2007).

Cellulose is detected in effective barley papillae using two specific probes; CBM3a (Blake *et al*., 2006) and Pontamine fast scarlet 4B (Anderson *et al*., 2010). Two different cellulose probes were used since CBM3a has been shown to bind non-specifically to xyloglucan and callose (Vincent Bulone, pers. comm.). The observed labelling with the xyloglucan-specific antibody was low removing the issue of cross detection. Whilst Pontamine fast scarlet 4B shows some non-specific binding to xyloglucan, binding to callose is minimal (Anderson et al., 2010). Therefore, by using the two probes in combination and observing the same patterning we are confident that we detected cellulose in these papillae. Some previous studies of barley papillae reported the absence of cellulose (Smart *et al*., 1986; Russo & Bushnell, 1989), but in these studies basic staining methods that rely on the cellulose swelling in order for staining to be successful were used. Thus, false negatives could occur if other components in the papillae prevented the cellulose from swelling, such as the arabinoxylan crosslinking with phenolic acids. The presence of cellulose around the outside of papilla has been reported previously in the interaction between *Zea mays* roots and *Phytophthora cinnamomi*, however, the results were inconclusive due to the non-specific nature of the stains that were used (Hinch & Clarke, 1982). In addition, the presence of 1,3:1,4-ß-glucan within the *Zea mays* papilla was also suggested, but our screens with 1,3:1,4-ß-glucan specific antibodies provided evidence for the absence of 1,3:1,4-ß-glucan in the barley papillae (Table 1). The observation that cellulose is present in the outer layer of papillae suggests that it accumulates at the last stage of papillae development (Fig. 7). The encapsulation of a papilla core with cellulose and arabinoxylan could provide the structural strength required for the formation of an effective papilla. Given that the encapsulation occurs late in the formation of the papilla it could explain why the penetration efficiency decreases significantly if the papilla has been able to fully form prior to the penetration attempt (Zeyer *et al*., 1979).

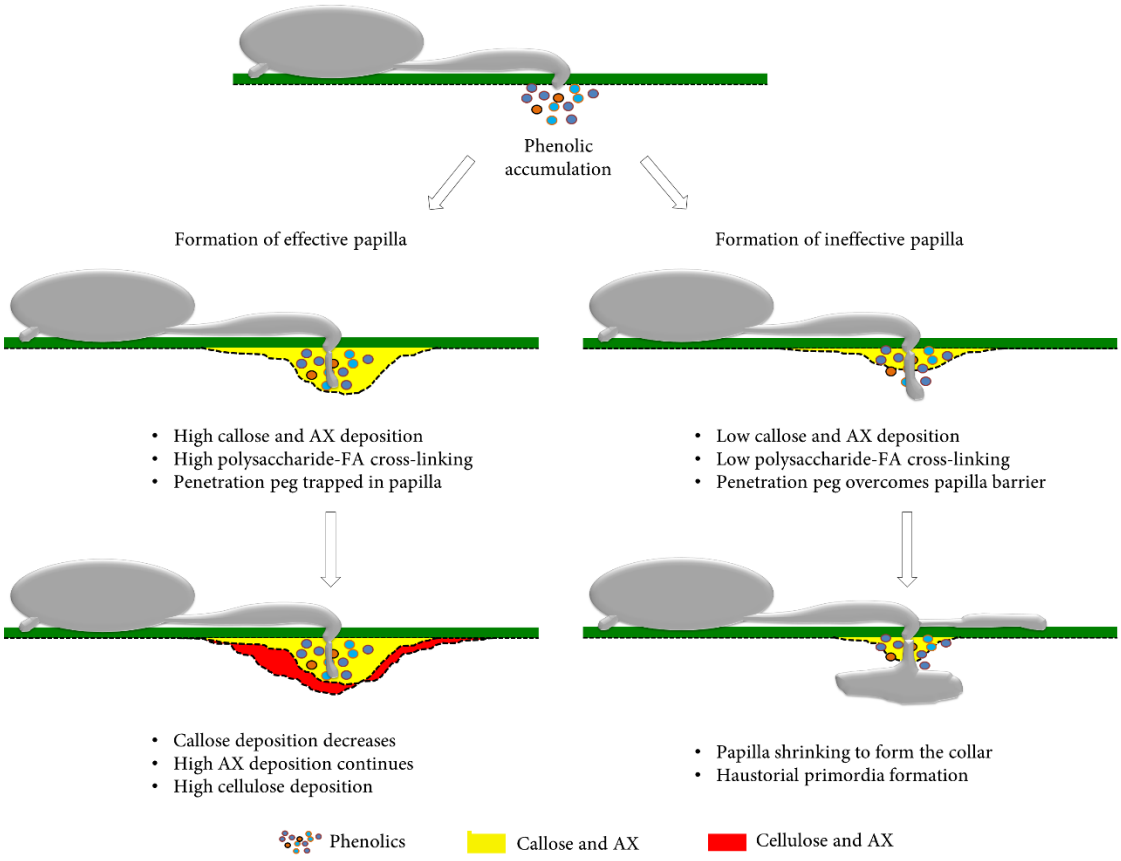


Fig. 7 Putative model depicting deposition of polysaccharides and phenolics in effective and ineffective papillae during the barley/*Blumeria graminis* f. sp. *hordei* interaction. AX=arabinoxylan, FA=Ferulic acid.

The intensity of phenolic acid-associated autofluorescence in effective and ineffective papillae does not show any significant differences (Fig. 6). In contrast to this, previous studies found a positive correlation between autofluorescence and penetration resistance (Clark *et al*., 1994) and showed enhanced susceptibility of barley and wheat against powdery mildew attack by silencing enzymes of the shikimate and phenylpropanoid lignin-precursor pathways (Bhuiyan *et al*., 2009; Hu *et al*., 2009). However, effective papillae were compared between different barley lines where there may be extra factors to consider such as additional underlying resistance alleles that may have obscured underlying changes. In addition, the shikimate pathway is essential for the biosynthesis of other compounds including

phytohormones. In conclusion, when comparing the effective and ineffective papillae in a single barley line, as presented here, the main difference appears to be in polysaccharide composition rather than in phenolic acid levels.

The primary cell walls of the Poaceae are mainly composed of cellulose, arabinoxylans and (1,3;1,4)-β-glucans whereas other species, including dicotyledonous plants, contain mainly cellulose, xyloglucan and pectin (Carpita & Gibeaut, 1993; Somerville *et al*., 2004; Burton *et al*., 2010). It is unclear whether differences between the non-cellulosic polysaccharide components of the primary cell wall of Poaceae and dicots may also be reflected in the composition of papillae, with xyloglucan and pectin replacing the arabinoxylan observed in barley. However, xyloglucan has been detected in ineffective papillae of the poinsettia (*Euphorbia pulcherrima)*-powdery mildew (*Pseudoidium poinsettiae*) interaction (Celio *et al*., 2004) where it could play a similar role as arabinoxylan in the barley papilla by binding to and crosslinking cellulose microfibrils. The methods reported here that have been developed to interrogate papillae structure and composition can now be adopted for use in many other pathogen/host interactions encompassing a broad range of plant types.

In this study, we have shown that the major polysaccharides found in barley papillae are callose, arabinoxylan and cellulose. The association of arabinoxylan and cellulose with penetration resistance provides new targets for the improvement of papillae composition and for the generation of novel crop lines with greater disease resistance.

Acknowledgements

We thank Professor Richard Oliver from The Australian Centre for Necrotrophic Fungal Pathogens for providing the *Blumeria graminis* f. sp. *hordei* isolate. We would like to acknowledge Dr Agatha Labrinidis from Adelaide Microscopy for her assistance with the confocal microscopy. This work was supported by grants from the Australian Research Council.

References

Aghnoum R, Niks RE. 2010. Specificity and levels of nonhost resistance to nonadapted *Blumeria graminis* forms in barley. *New Phytologist* **185**(1): 275-284.

Aist JR, Israel HW. 1977. Papilla formation - timing and significance during penetration of barley coleoptiles by *Erysiphe graminis* hordei. *Phytopathology* **67**(4): 455-461.

Aist JR. 1976. Papillae and related wound plugs of plant-cells. *Annual Review of Phytopathology* **14**: 145-163.

Albersheim P, Darvill A, Roberts K, Sederoff R, Staehelin A. 2011. *Cell Walls and Plant-Microbe Interactions*: Garland Science, Taylor & Francis, 270 Madison Avenue, New York, Ny 10016 USA.

Anderson CT, Carroll A, Akhmetova L, Somerville C. 2010. Real-time imaging of cellulose reorientation during cell wall expansion in Arabidopsis roots. *Plant Physiology* **152**(2): 787-796.

Blake AW, McCartney L, Flint JE, Bolam DN, Boraston AB, Gilbert HJ, Knox JP. 2006. Understanding the biological rationale for the diversity of cellulose-directed carbohydrate-binding modules in prokaryotic enzymes. *Journal of Biological Chemistry* **281**(39): 29321-29329.

Blümke A, Somerville SC, Voigt CA. 2013. Transient expression of the Arabidopsis thaliana callose synthase PMR4 increases penetration resistance to powdery mildew in barley. *Advances in Bioscience & Biotechnology* **4**(8): 810-813.

Burton RA, Gidley MJ, Fincher GB. 2010. Heterogeneity in the chemistry, structure and function of plant cell walls. *Nature Chemical Biology* **6**(10): 724-732.

Burton RA, Collins HM, Kibble NAJ, Smith JA, Shirley NJ, Jobling SA, Henderson M, Singh RR, Pettolino F, Wilson SM, Bird AR, Topping DL, Bacic A, Fincher GB. 2011. Over-expression of specific *HvCslF* cellulose synthase-like genes in transgenic barley increases the levels of cell wall (1,3;1,4)-beta-glucans and alters their fine structure. *Plant Biotechnology Journal* **9**(2): 117-135.

Bhuiyan NH, Selvaraj G, Wei YD, King J. 2009. Gene expression profiling and silencing reveal that monolignol biosynthesis plays a critical role in penetration defence in wheat against powdery mildew invasion. *Journal of Experimental Botany* **60**: 509-521.

Carpita NC, Gibeaut DM. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **3**(1): 1-30.

Celio G, Mims C, Richardson E. 2004. Ultrastructure and immunocytochemistry of the host pathogen interface in poinsettia leaves infected with powdery mildew. *Canadian Journal of Botany* **82**(4): 421-429.

Clark TA, Zeyen RJ, Smith AG, Carver TLW, Vance CP. 1994. Phenylalanine ammonia lyase mRNA accumulation, enzyme activity and cytoplasmic responses in barley isolines, differing at Ml-a and Ml-o loci, attacked by *Erysiphe graminis* f.sp. *hordei*. *Physiological and Molecular Plant Pathology* **44**(3): 171-185.

Ebrahim-Nesbat F, Bohl S, Heitefuss R, Apel K. 1993. Thionin in cell walls and papillae of barley in compatible and incompatible interactions with *Erysiphe graminis* f. sp. *hordei*. *Physiological and Molecular Plant Pathology* **43**(5): 343-352.

Ellinger D, Naumann M, Falter C, Zwikowics C, Jamrow T, Manisseri C, Somerville SC, Voigt CA. 2013. Elevated early callose deposition results in complete penetration resistance to powdery mildew in Arabidopsis. *Plant Physiology* **161**(3): 1433-1444.

Evans NA, Hoyne PA, Stone BA. 1984. Characteristics and specificity of the interaction of a fluorochrome from aniline blue for the histochemical detection of callose. *Carbohydrate Polymers* **4**: 215-230.

Harris P, Trethewey JK. 2010. The distribution of ester-linked ferulic acid in the cell walls of angiosperms. *Phytochemistry Reviews* **9**(1): 19-33.

Hervé C, Marcus S, Knox JP. 2011. Monoclonal antibodies, carbohydrate-binding modules, and the detection of polysaccharides in plant cell walls. In: Popper ZA ed. *The Plant Cell Wall*. Berlin, Germany: Humana Press, 103-113.

Hinch JM, Clarke AE. 1982. Callose formation in *Zea mays* as a response to infection with *Phytophthora cinnamomi*. *Physiological Plant Pathology* **21**(1): 121-124.

Hu PS, Meng Y, Wise RP. 2009. Functional contribution of chorismate synthase, anthranilate synthase, and chorismate mutase to penetration resistance in barley-powdery mildew interactions. *Molecular Plant-Microbe Interactions* **22**: 311-320.

Huckelhoven R, Fodor J, Preis C, Kogel KH. 1999. Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiology* **119**(4): 1251-1260.

Huckelhoven R. 2005. Powdery mildew susceptibility and biotrophic infection strategies. *Fems Microbiology Letters* **245**(1): 9-17.

Huckelhoven R. 2007. Cell wall - Associated mechanisms of disease resistance and susceptibility. *Annual Review of Phytopathology* **45:** 101-127.

Iiyama K, Lam TB-T, Stone BA. 1994. Covalent cross-links in the cell wall. *Plant Physiology* **104**(2): 315-320.

Israel H, Wilson R, Kunoh H. 1980. Cell wall appositions and plant disease resistance: Acoustic microscopy of papillae that block fungal ingress. *Proceedings of the National Academy of Sciences* **77**(4): 2046-2049.

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB. 2003. An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* **15**(11): 2503-2513.

Knox JP. 2008. Revealing the structural and functional diversity of plant cell walls. *Current Opinion in Plant Biology* **11**: 306-313.

McCartney L, Marcus SE, Knox JP. 2005. Monoclonal antibodies to plant cell wall xylans and arabinoxylans. *Journal of Histochemistry & Cytochemistry* **53**(4): 543-546.

McNeil M, Albersheim P, Taiz L, Jones RL. 1975. The structure of plant cell walls VII. Barley aleurone cells. *Plant Physiology* **55**(1): 64-68.

Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC. 2003. Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* **301**(5635): 969-972.

Oerke EC, Dehne HW. 2004. Safeguarding production - losses in major crops and the role of crop protection. *Crop protection* **23**: 275-285.

Pattathil S, Avci U, Baldwin D, Swennes AG, McGill JA, Popper Z, Bootten T, Albert A, Davis RH, Chennareddy C, Dong R, O'Shea B, Rossi R, Leoff C, Freshour G, Narra R, O'Neil M, York WS and Hahn MG. 2010. A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiology* **153**: 514-525.

Pirselova B, Matusikova I. 2013. Callose: the plant cell wall polysaccharide with multiple biological functions. *Acta Physiologiae Plantarum* **35**: 635-644.

Philippe S, Tranquet O, Utille J-P, Saulnier L, Guillon F. 2007. Investigation of ferulate deposition in endosperm cell walls of mature and developing wheat grains by using a polyclonal antibody. *Planta* **225**(5): 1287-1299.

Russo VM, Bushnell WR. 1989. Responses of barley cells to puncture by microneedles and to attempted penetration by Erysiphe-graminis f sp hordei. *Canadian Journal of Botany* **67**(10): 2912-2921.

Schober MS, Burton RA, Shirley NJ, Jacobs AK, Fincher GB. 2009. Analysis of the (1, 3)-β-D-glucan synthase gene family of barley. *Phytochemistry* **70**(6): 713-720.

Schopfer P. 1996. Hydrogen peroxide-mediated cell-wall stiffening *in vitro* in maize coleoptiles. *Planta* **199**(1): 43-49.

Smart MG, Aist JR, Israel HW. 1986. Structure and function of wall appositions .1. General histochemistry of papillae in barley coleoptiles attacked by *Erysiphe-graminis* f-sp *hordei*. *Canadian Journal of Botany* **64**(4): 793-801.

Somerville C, Bauer S, Brininstool G, Facette M, Hamann T, Milne J, Osborne E, Paredez A, Persson S, Raab T, Vorwerk S, Youngs H. 2004. Toward a systems approach to understanding plant cell walls. *Science* **306**(5705): 2206-2211.

Soukup A. 2014. Selected simple methods of plant cell wall histochemistry and staining for light microscopy. In: Zarsky V, Cvrckova F eds. *Plant Cell Morphogenesis: Methods and Protocols*. Berlin, Germany: Human Press, 25-40.

Stone BA, Evans NA, Bonig I, Clarke AE. 1984. The application of Sirofluor, a chemically defined fluorochrome from aniline blue for the histochemical detection of callose. *Protoplasma* **122**(3): 191-195.

Stone BA, Clarke AE. 1992. *Chemistry and Biology of (1→3)-β-Glucans*. Victoria, Australia: La Trobe University Press.

Underwood W. 2012. The plant cell wall: a dynamic barrier against pathogen invasion. *Frontiers in Plant Science* **3**: 85-85.

von Ropenack E, Parr A, Schulze-Lefert P. 1998. Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *Journal of Biological Chemistry* **273**(15): 9013-9022.

Wright CS. 1984. Structural comparison of the two distinct sugar binding sites in wheat germ agglutinin isolectin II. *Journal of Molecular Biology* **178**(1): 91-104.

Zeyen RJ, Carver TLW, Lyngkjaer MF. 2002. Epidermal cell papillae. In: Bélanger RR, Bushnell WR, Dik AJ, and Carver TLW, eds. *Powdery Mildews: A Comprehensive Treatise*. St Paul, MN: APS Press, 107-125.

CHAPTER 4

Down-regulation of the *glucan synthase-like 6* gene (*HvGsl6*) in barley leads to decreased callose accumulation and increased susceptibility to *Blumeria graminis* f. sp. *hordei.*

Title of Paper	Down-regulation of the *Glucan synthase-like 6* gene (*HvGsl6*) in barley leads to decreased callose accumulation and increased susceptibility to *Blumeria graminis* f. sp. *hordei*.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript submitted to New Phytologist journal. Summittion ID: NPH-MS-2016-21945.

Principal Author

Name of Principal Author (Candidate)	Jamil Chowdhury		
Contribution to the Paper	Bioinformatics analysis, perfromed transgenic sample analysis and microscopy, interpreted data, wrote manuscript and acted as principal author.		
Overall percentage (%)	60		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30 March 2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate in include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature	Unable to get signature due to his recent passing away	Date	

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Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation		
Signature		Date	30 March 2016

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Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and act as corresponding author		
Signature		Date	29 March 2016

Link to chapter 4

In Chapter 3, it was reported that callose, arabinoxylan and cellulose are the three major polysaccharides found in barley papillae that form against the powdery mildew pathogen and that they are potentially important factors of host penetration resistance mechanisms. To further understand the role of these polysaccharides in the host defence mechanism, identification of the genes associated with their biosynthesis is essential. Thus, Chapter 4 reports the outcomes of the experiments conducted to identify and characterise genes associated with callose biosynthesis in barley papillae during powdery mildew infection. The identification of the barley functional homolog of the *Arabidopsis Glucan synthase-like 5* gene, which was previously shown to be involved in callose biosynthesis of *Arabidopsis* papillae, was a particular focus. This chapter has been written in manuscript format and has been submitted to the peer reviewed journal 'New Phytologist'.

Down-regulation of the *glucan synthase-like 6* gene (*HvGsl6*) in barley leads to decreased callose accumulation and increased susceptibility to *Blumeria graminis* f. sp. *hordei.*

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Summary

- The recent characterisation of the polysaccharide composition of papillae deposited at the barley cell wall during infection by the powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei (Bgh),* has provided new targets for the generation of enhanced disease resistance. The role of callose in papilla-based penetration resistance of crop species is largely unknown because the genes involved in the observed callose accumulation have not been identified unequivocally.
- We have employed both comparative and functional genomics approaches to identify the functional ortholog of *AtGsl5* in the barley genome. *HvGsl6,* which has the highest sequence identity to *AtGsl5,* is the only *Bgh* induced gene among the *HvGsls* examined in this study. Through dsRNAi-mediated silencing of *HvGsl6*, we have shown that down regulation of *HvGsl6* is associated with lower accumulation of papillary and wound callose and higher susceptibility to penetration of the papillae by *Bgh*, compared with control lines.
- The results indicate that the *HvGsl6* gene is a functional ortholog of *AtGsl5* and is involved in papillary callose accumulation in barley. The increased susceptibility of *HvGsl6* dsRNAi transgenic lines to infection indicates that callose positively contributes to the barley fungal penetration resistance mechanism.

Key words: glucan synthase like / *Gsl* / callose / papillae / *Blumeria graminis* / powdery mildew / penetration

Introduction

In the plant and pathogen co-evolutionary battleground, host plants have evolved a wide range of defence strategies against attacking pathogens. One of the earliest observed defence responses, which has been analysed at a cellular level for more than 150 years, is the formation of papillae at the site of infection. The papillae are cellular reinforcements formed by depositing polysaccharides between the wall and the plasma membrane (Zeyen *et al.*, 2002). Barley papillae, which are the main focus of this study, have been reported to contain callose, phenolic compounds, arabinogalactan-proteins, inorganic elements, antimicrobial components and reactive oxygen species (Aist, 1976; Zeyen *et al.*, 2002). Recently, two more polysaccharides, arabinoxylan and cellulose, have been identified in barley papillae formed against the powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei (Bgh)* (Chowdhury *et al.*, 2014).

Since the presence of callose in papillae was first detected by Mangin (1895), the polymer comprised of (1,3)-β-linked glucosyl residues has been identified in papillae from a wide range of plant species during infection. Given the fact that callose is found at high levels in papillae and that papillae are believed to act as mechanical barriers to prevent pathogen ingress (Aist, 1976; Stone & Clarke, 1992), it was widely held that callose contributes to plant innate immunity. The hypothesis was examined in *Arabidopsis thaliana glucan synthase-like 5* (*AtGsl5*) mutant lines that produce significantly reduced levels of callose in papillae and wounded tissue. The mutants paradoxically showed increased resistance against the pathogen *Golovinomyces cichoracearum* (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003), possibly through an indirect activation of the salicylic acid-dependent defence pathway. On the other hand, over-expression of *AtGsl5* lead to a higher and early accumulation of papillary callose, which results in complete penetration resistance against *G. cichoracearum* (Ellinger *et al*., 2013). These results suggest that manipulating the callose level induced through pathogen infection would be a potential target in achieving plant immunity. It is important to utilize the

information gained from working with model plant species such as *Arabidopsis* and translate the findings to economically important crop plant species such as barley.

In barley, callose has been detected in both penetrated and non-penetrated papillae, using fluorescent stains and specific antibodies (Aist, 1976; Chowdhury *et al.*, 2014). However, the observation that higher callose staining intensity is associated with non-penetrated papillae compared to penetrated papillae suggests the accumulation of callose might contribute to the barley fungal penetration resistance mechanism (Chowdhury *et al.*, 2014). Additionally, it is unknown whether any alteration in the amount of callose in the papillae of a monocotyledonous species would lead to the same effects observed in *Arabidopsis.* In order to answer this question using barley, it was important to first identify the gene associated with papillary callose accumulation.

Increasing evidence suggests that many members of the *glucan synthase-like* (*Gsl*) family, also known as callose synthases, *CalS*, are involved in callose biosynthesis in plants (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003; Enns *et al.*, 2005; Nishikawa *et al.*, 2005; Chen *et al.*, 2009; Xie *et al.*, 2011). The *Gsls* are the functional homologs of the yeast glucan synthase, FKS (Hong *et al.*, 2001) and belong to the glycosyltransferase 48 family (GT48) (Stone *et al.*, 2010). The (1,3)-β-glucan polymer plays an important role in many plant growth and developmental processes (Stone & Clarke, 1992). Transcript profiling of the seven published *HvGsl* gene family members demonstrated differential tissue-specific expression of individual *HvGsl* genes in vegetative and reproductive tissues (Schober *et al.*, 2009). Perhaps the best characterised to date, *HvGsl1* has been linked with callose biosynthesis in the barley developing grain, florets, coleoptile and root tissues (Li *et al.*, 2003). In searching the putative ortholog(s) of *AtGsl5* in barley we have utilized a comparative genomics approach that includes phylogenetic analysis, transcript profiling of the *HvGsls* during pathogen infection,

and functional analysis using loss-of-function strategies in transient and stable transgenic plants.

Materials and methods

Plant-Bgh growth conditions and spore inoculation procedure

A field isolate of *Bgh* provided by Professor Richard Oliver (Centre for Crop Disease Management, Curtin University, WA, Australia) was used in this study. For generation of spores the isolate was maintained on a highly susceptible Australian barley variety 'Baudin' in a growth chamber at 21°C with 80% relative humidity and 16 h photoperiod. Transgenic lines were grown in a separate PC2 growth room at 25°C with a 16 h photoperiod. All the plants were grown in plastic pots containing 'cocopeat' soil mixture. For microscopic observations, *Bgh* spore inoculation was carried out on 10 days old detached leaves as described in Chowdhury *et al.* (2014).

Phylogenetic analysis

The putative *Gsls* of *Arabidopsis,* barley, sorghum, *Brachypodium* and rice were extracted from the EnsemblGenomes database (http://plants.ensembl.org/) for each respective genome using the glucan_synthase Pfam (PF02364) as the query sequence. Additionally, two putative *Gsl* genes from barley and one gene from rice were found when searching annotated genes for high homology to full length *HvGsl* sequences. In total, 12 *Arabidopsis,* 11 sorghum, 11 rice, 11 *Brachypodium* and 19 barley genes containing the glucan synthase Pfam domains were obtained. Where possible, partial gene annotations were combined into one predicted open reading frame through alignments with barley genome sequences extracted from the Institute of Plant Genetics and Crop Plant Research (IPK) BAC library database (Deng *et al.*, 2007) (Supplemental Table S1). A phylogenetic tree was constructed using 15 barley sequences along with *Arabidopsis,* sorghum, rice and *Brachypodium Gsl* sequences. The nomenclature of seven barley *Gsl*s was previously assigned (Li *et al.*, 2003; Schober *et al.*,

2009). The phylogenetic tree was constructed using the longest protein sequence of every *Arabidopsis*, barley, sorghum and rice gene available in EnsemblGenomes. The sequences were aligned using the 'Muscle' program using default parameters. BMGE was used to strip the alignment of uninformative sites (Criscuolo & Gribaldo, 2010). Phylogenies were constructed using the Bayesian MCMC package BEAST v 2.3.1 (Drummond *et al.*, 2012) with the GTR + G substitution model. In order to test the reliability of the Bayesian Tree, a Maximum Likelihood Tree was constructed using the RAxML program with the protein GTR substitution model with gamma rate heterogeneity (Stamatakis, 2014). The potential orthologs are predicted from their relationship within the Bayesian Tree.

Reverse-transcription quantitative PCR (RT-qPCR) of HvGsl *transcripts*

10 day old barley primary leaves were used for inoculation. Total RNA was isolated from infected and uninfected leaf epidermal tissue using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St Louis, U.S.A.) following the manufacturer's protocol. cDNA was synthesized from total RNA using SuperScript® III Reverse Transcriptase (RT) enzyme (Life Technologies, Carlsbad, U.S.A.) following the recommended protocol. *HvGsl* transcript profiling was carried using RT-qPCR as described previously in Burton *et al.* (2010). The *HvGsl* gene-specific primers and RT-qPCR product sequences are presented in Supplemental Table S2. The data were normalized against the geometric mean of the four housekeeping genes, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cyclophilin, tubulin and HSP70 (Vandesompele *et al.*, 2002).

Transient-induced gene silencing

Barley seedlings were grown in a glass house under a 25°C day and 17°C night temperature regime, with a minimum of 13 h light per day. The micro-projectile bombardment procedure for transient transformation has been outlined in (Schweizer *et al.*, 1999b), with the following modifications. The youngest emerged leaf blades were excised from 8 day old

seedlings and placed with the abaxial side upwards onto 1.5% agar containing 10% sucrose in petri dishes. The plates were sealed with parafilm and placed in the glasshouse for 4 h. The leaves were transferred to 1.5% agar plates containing 85 μM benzimidazole, a senescence inhibitor, immediately prior to bombardment. A total of 1 mg gold particle micro-carriers were added to 3.5 μg of the *HvGsl6* dsRNAi plasmid DNA, the *HvGsl7* dsRNAi plasmid DNA or the empty pHannibal vector DNA, along with 3.5 μg pUbi-GUS-NOS DNA, 50 μl 2.5 M $CaCl_2$ and 20 μl 0.1 M spermidine. Using a He Biolistic® Particle Delivery System (Model PDS-1000, BIORAD, Hercules, U.S.A.), the gold micro-carriers were fired at a pressure of 900 psi, with the stopping screen 10 cm from the excised leaves. Each plate of excised leaves was bombarded twice. The plates were sealed with parafilm and returned to the glass-house for 72 h. The bombarded, excised leaf material was inoculated with *Bgh* spores at a density of around 150 mm^{-2} before they were incubated in the growth room for 24 h. The transformed cells were identified by GUS staining (Schweizer *et al.*, 1999a). Presence of callose in the transformed cells was detected by staining with 0.02% aniline blue fluorochrome (Biosupplies Australia Pty Ltd.).

Construction of dsRNAi vector and stable plant transformation

An *HvGsl6* gene-specific region was identified through alignment of the *HvGsl* family members in the poorly conserved 3' untranslated region. Primer sequences used for cloning are shown in Table S3. The amplified region was cloned into the pHannibal vector (Wesley *et al.*, 2001), confirmed through sequencing and sub-cloned into a binary vector pPG1 (Paul Gooding, unpublished) based on pPZP200 (Hajdukiewicz *et al.*, 1994). Restriction digestion, DNA ligation and plasmid DNA preparation were performed using standard protocols. The pPG1 vector was introduced into *Agrobacterium* strain AGL1 using a standard heat shock protocol (Hofgen & Willmitzer, 1988). Agrobacterium was used to transform into immature embryos collected from developing spikes of the Golden Promise cultivar as described

previously (Tingay *et al.*, 1997; Matthews *et al.*, 2001). Transgenic status of the plants was confirmed by standard Southern blotting and PCR procedures to detect the presence of the hygromycin phosphotransferase (HPT) sequence (data not shown). Seedlings from the T3 generation were used in this study and the sequences of the primers used to screen transcript abundance of the *HvGsl6* gene in the transgenic lines are given in Supplemental Table S2.

Wounding treatment

Leaves of 10 day-old barley plants were wounded using a razor blade while the leaves remained attached to the plants (Jacobs *et al.*, 2003). After 24 h, the wounded leaves were detached from the plants, cleared and processed for immunolabelling following the protocol described in Chowdhury *et al.* (2014).

Aniline blue staining and measurement of callose intensity

Callose was detected in papillae using colourless aniline blue (0.02% w/w) dissolved in 0.1 M K_2HPO_4 buffer, pH 11. After 2 h staining with aniline blue, the tissues were rinsed briefly with 0.1 M K_2HPO_4 buffer, pH 11, before they were observed under a Carl Zeiss fluorescence microscope (Axio Imager M2; Carl Zeiss, Oberkochen, Germany). Fluorescence emitted from aniline blue was observed using the DAPI filter set at 365 nm excitation and 445/50 nm emission wavelengths. The quantitative measurements of fluorescence intensities were performed following the method described in Chowdhury *et al.* (2014).

Results

Phylogenetic analysis demonstrates homology between the HvGsl6 *and* HvGsl7 *genes and the Arabidopsis papillary callose synthase* AtGsl5

To identify the putative orthologs of the *AtGsl5* gene in monocot species, the phylogenetic relationships of *Gsl* genes in the *Arabidopsis*, barley, *Brachypodium*, sorghum and rice genome sequences were investigated. After an initial phylogenetic tree was constructed, we found that there were six clades that consisted of two or more barley sequences where the other monocot species only had one representative sequence. Similar chromosomal locations and transcript expression profiles (JHI RNAseq expression database, Supplemental Table S1 and Supplemental Figure S1) were also observed for genes across different tissues. The barley *Gsl* sequences within each clade could be aligned against closely related full length *Gsl* sequences from other species without sequence overlap (data not shown) suggesting that most of the barley sequences found in individual clades may represent a single gene. Where possible the partial *Gsl* gene annotations were confirmed to refer to one single gene through alignments with barley genomic sequences extracted from the Webblast database of the Institute of Plant Genetics and Crop Plant Research (IPK). The initial number of 19 *HvGsl* members of the barley family was thereby reduced to 15, however, with further genome sequence coverage we predict that the number could decrease to 11 or 12 genes, which would be consistent with the gene family numbers observed in dicot and other monocot plants.

A phylogenetic tree of full-length protein sequences was constructed using the 15 predicted barley *Gsl* members (Figure 1). The clade of interest, containing the *AtGsl5* gene, consists of two genes from *Arabidopsis*, sorghum and rice and three from barley. Within the clade two Arabidopsis genes, *AtGsl1* and *AtGsl5*, are separated and form a sub-clade from the monocot *Gsl* members. Within the clade, all four monocot species sequences show the highest homology with the *AtGsl5* sequence compared with other members of the genome, with

sequence identities ranging from 53 to 75%. Among the barley genes, *HvGsl6* (MLOC_75948) showed maximum homology (65%) with *AtGsl5*, followed by *HvGsl7* (MLOC_11267) and MLOC_21519 (63%). Based on gene structure modelling, most *AtGsl* genes have 40–50 exons, except for *AtGsl1* and *AtGsl5*, which have one and two exons, respectively (Enns *et al.*, 2005). The gene structure of the barley *Gsl* genes follows a similar pattern with most having a large number of exons, except for *HvGsl6* (MLOC_75948) and *HvGsl7* (MLOC_11267) which have one and two exons respectively (data not shown). The gene annotation MLOC_21519 was removed from further investigation because we were unable to find a full length protein sequence or any significant level of transcript in different tissues in the JHI RNAseq database, suggesting that it may be a pseudogene. Therefore, only *HvGsl6* and *HvGsl7* were considered for further analysis.

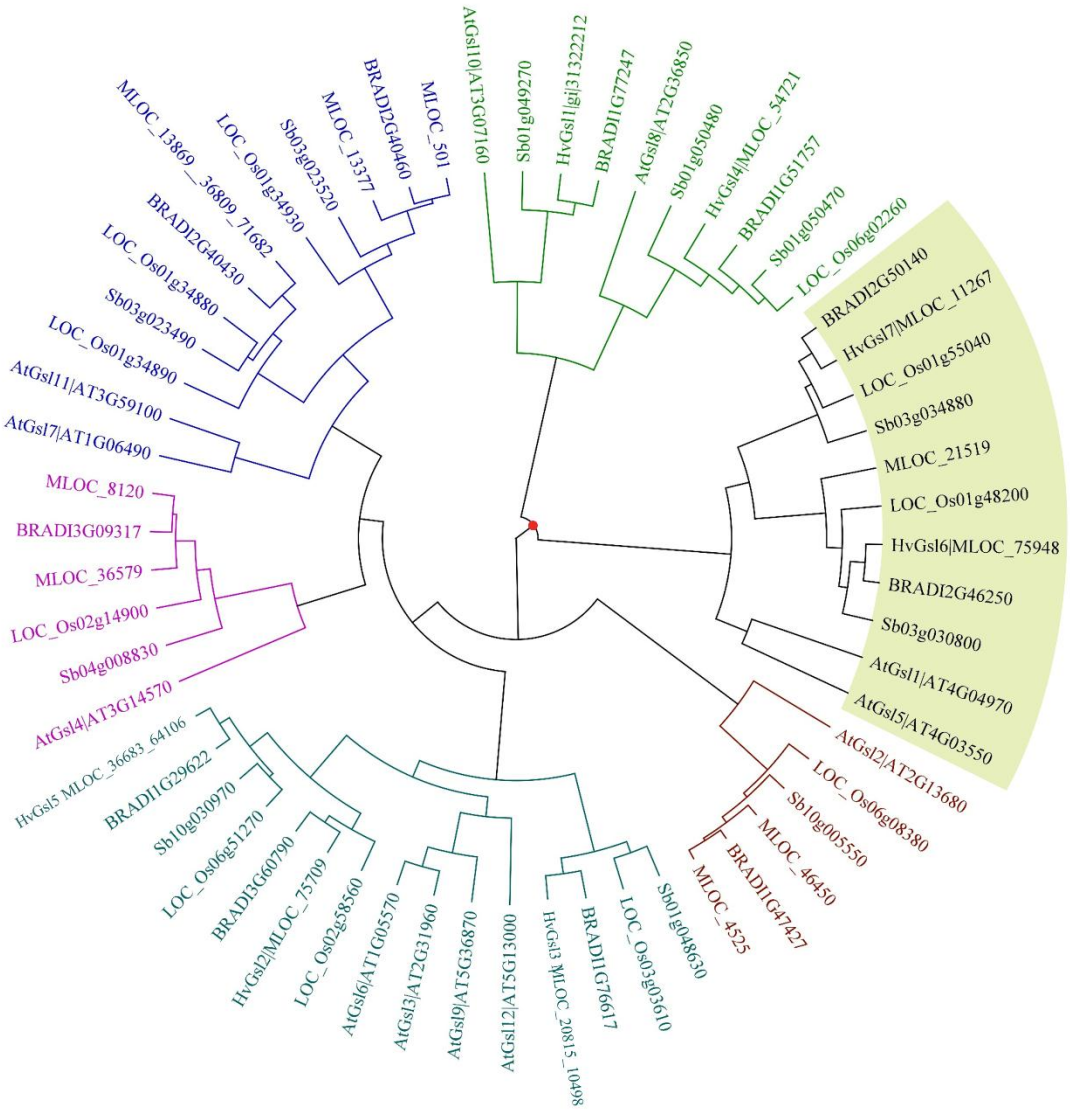


Figure 1. A phylogenetic tree depicting the relationships among the GLUCAN SYNTHASE-LIKE proteins from *Arabidopsis*, barley, rice, sorghum and *Brachypodium*. All deep nodes are supported with posterior probability support values > 0.85 for the Bayesian maximum clade credibility tree along with maximum likelihood bootstrap support values > 85%, except for single highlighted node (red) which has a lower posterior probability support value of 0.48 and a maximum likelihood bootstrap support value of 100%. The clade containing the *AtGsl5* pathogen responsive gene is highlighted in the box.

HvGsl6 expression increases in the epidermal layer during papillae formation in response to Bgh infection

To examine the involvement of the *HvGsl6* and *HvGsl7* genes in callose biosynthesis in barley papillae, their transcript profiles during pathogen infection were examined using RT-qPCR (Figure 2). Expression levels were observed in a time course 'infection series' of barley epidermal peels at 4 h intervals across the first 24 h after inoculation. As observed in the profile, the transcript levels of the *HvGsl6* gene in the *Bgh* infected barley epidermal tissue were reached consistently higher levels compared with the uninfected control. The transcript level of *HvGsl6* transcription peaked at 12 h approximately four fold higher than the uninfected control. This time point is consistent with previous observations that callose deposition in the papillae occurs mainly between 8 and 12 h after inoculation (Chowdhury *et al*., 2014). The transcript levels of *HvGsl6* remained higher than the uninfected control for the next 8 h until dropping back close to the uninfected expression level at 24 h. The transcript abundance of *HvGsl7* varied largely over time in the uninfected control and was lower in *Bgh* infected barley epidermal tissue when compared with the control. In addition to *HvGsl7*, two other *Gsl* family members, *HvGsl1* and *4*, were also observed to be down-regulated during the defence response against *Bgh* infection (data not shown).

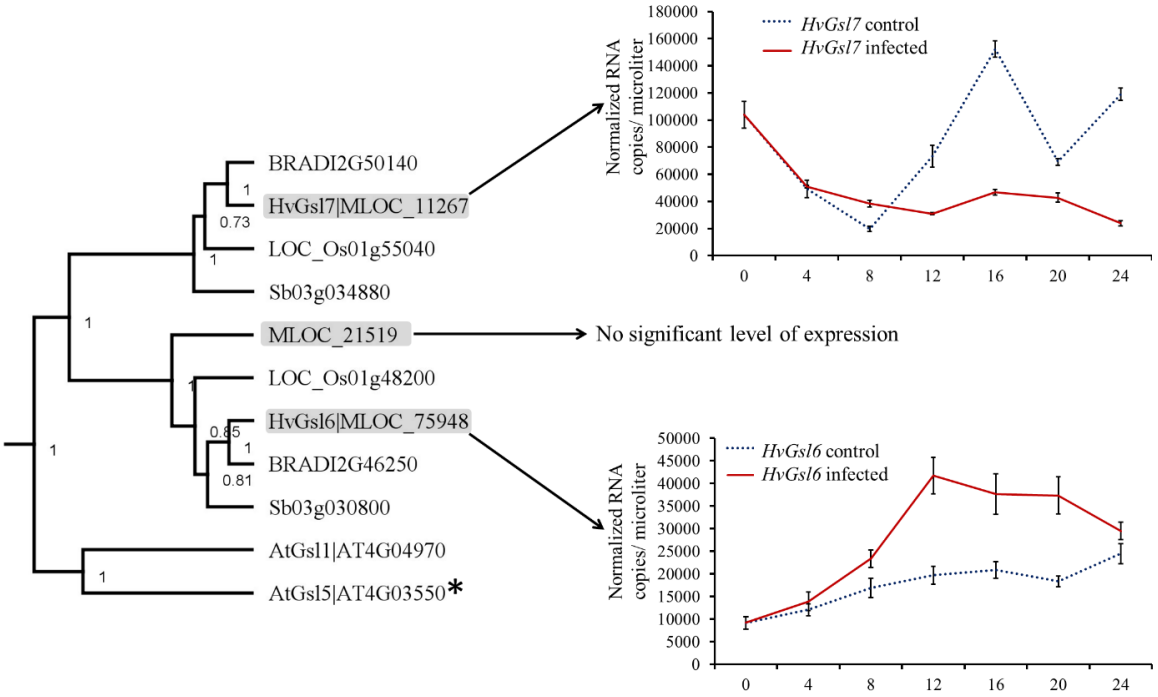


Figure 2. Transcript profiling of barley *glucan synthase-like* genes within the phylogenetic clade of interest. The *Arabidopsis AtGsl5* within the clade of interest is shown with an asterisk. Transcript patterns of *HvGsl6* and *HvGsl7* analysed by RT-qPCR in barley epidermal tissue in response to *Blumeria graminis* f. sp. *hordei* infection across a 24 h time course. Transcript of MLOC_21519 was not detected during infection. Transcript values are arbitrary units of comparison that have been normalized as described by Vandesompele *et al*. (2002).

Transient dsRNAi-mediated silencing of HvGsl6 resulted in an increased number of papillae lacking callose

In order to determine if expression of the *HvGsl6* gene is required for the deposition of callose in the papilla during infection, *HvGsl6* and *HvGsl7* dsRNAi constructs were transiently expressed in barley epidermal cells using biolistic bombardment. Transformed cells were detected through the expression of a *β-glucuronidase* reporter gene (GUS). The presence or absence of callose in papillae formed during *Bgh* infection were examined by staining with aniline blue. The frequency of papillae lacking callose formed in GUS stained cells was significantly higher for the *HvGsl6* dsRNAi construct compared with the empty vector control (Figure 3). No significant change in callose levels were observed with the cells transformed with the *HvGsl7* dsRNAi construct.

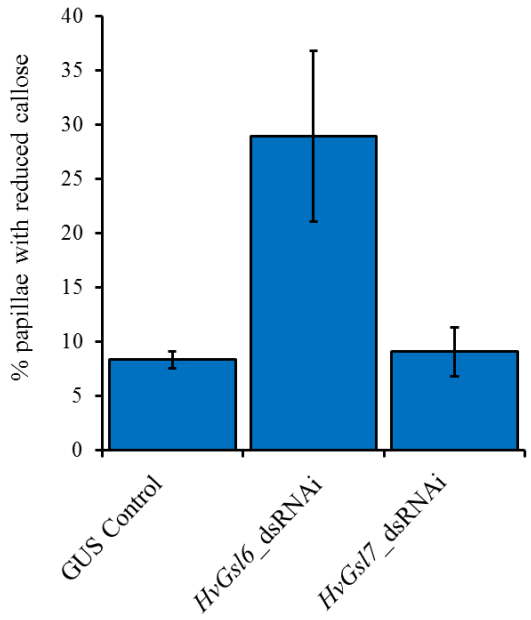


Figure 3. Frequency of callose-lacking papillae in transiently transformed barley epidermal cells in response to *Blumeria graminis* f. sp. *hordei* (*Bgh*) infection. The observations were made from >100 *GUS* transformed cells infected with *Bgh*. Error bars represent standard deviation. A significant increase was observed in *HvGsl6* dsRNAi transformed cells ($P < 0.05$, student T-test) compared to the GUS control and *HvGsl7* dsRNAi transformed cells.

Down regulation of HvGsl6 expression leads to significantly less callose accumulation in papillae and wounded tissue

Based on the Q-PCR transcript profile and observations from the transient-induced gene silencing experiments, stable transgenic lines were generated using a dsRNAi hairpin construct of *HvGsl6*. The T3 seedlings from three individual transformation events were screened for transgene copy number (Table S4) and examined for *HvGsl6* transcript abundance in non-inoculated seedlings using RT-qPCR analysis (Figure 4). For all the seedlings the *HvGsl6* transcript abundance was between four and twenty fold less than the wild type. The empty vector control lines showed no significant difference with wild type for *HvGsl6* transcript abundance. No visible morphological variation in growth and development was observed in the *HvGsl6* dsRNAi lines or in the empty vector control lines, compared with wild type Golden Promise. The transcript abundance of other members of the *HvGsl* family was checked in order to address possible off-target silencing effects (Figure S4). No significant off-target silencing was observed, however, in one event there was a large increase in transcript for *HvGsl5*.

The deposition of callose in wounded tissue was induced by cutting the leaf with a razor blade while they remained attached to the plants. After 24 h, the wounded leaves were detached from the plants and callose was observed by fluorescence microscopy using a callose specific antibody. Callose deposition was consistent between the wild type and empty vector lines (Figure 5A). Labelling was significantly reduced in the *HvGsl6* dsRNAi lines, but not completely eliminated from the wounded tissue, with levels slightly higher than the levels of autofluorescence observed in the negative control.

Callose deposition in the papillae induced by *Bgh* infection was screened using aniline blue fluorochrome (Figure 5B) and the maximum signal intensity within the papillae was measured (Figure 5C). Due to the heterogeneous nature of papillae, we observed a wide range

of fluorescence intensity levels in both wild type and transgenic lines. The median value of aniline blue fluorescence intensity from 150 papillae showed that the control plants contain significantly higher callose staining intensities compared with the *HvGsl6* dsRNAi lines (Figure 5D). This result indicates that the accumulation of callose has decreased in the papillae of *HvGsl6 dsRNAi* transgenic plants compared with that in the empty vector and wild type lines. Despite the reduction of callose accumulation in the *HvGsl6* dsRNAi transgenic plants to near background autofluorescence levels (Figure 5B), the papillae structure could still be observed clearly under the microscope. This may be due to the many other remaining components present in the papillae (Chowdhury *et al.*, 2014). In order to observe if there were any significant changes in the other components of the papillae, the infected lines were co-stained with the cellulose probe Pontamine fast scarlet 4B (PFS). The average intensity level of PFS did not change significantly in the *HvGsl6* dsRNAi lines, indicating that the cellulose level was not changed by the down regulation of *HvGsl6* transcripts (Figure S2).

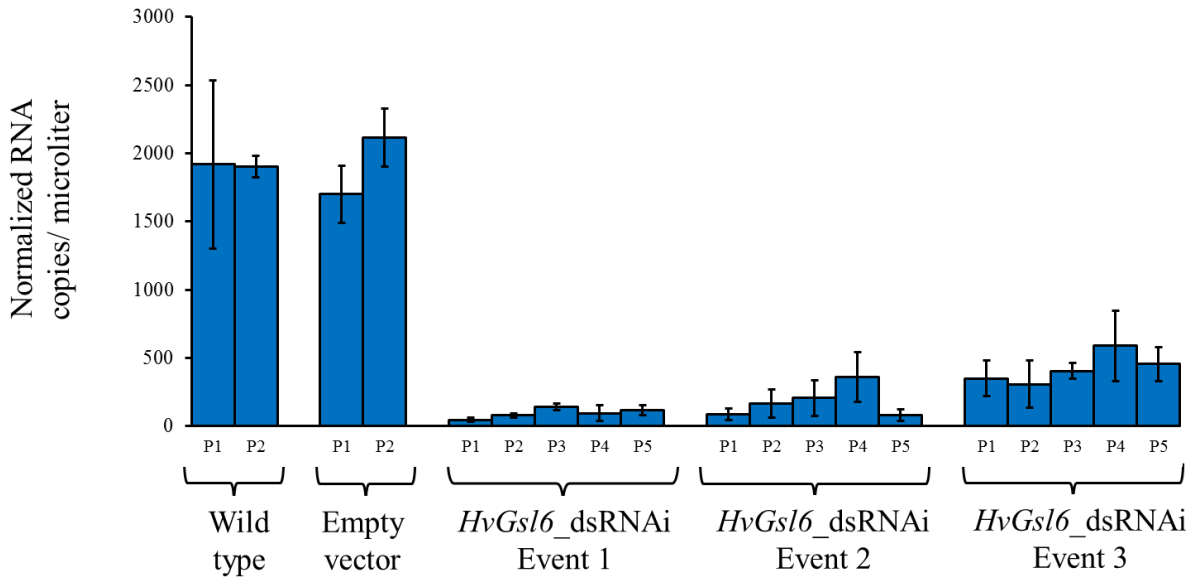


Figure 4. Transcript profiling of the barley *glucan synthase-like 6* (*HvGsl6*) gene in the second leaf of five plants (P1-P5) from three dsRNAi transgenic events compared to wild type and empty vector. Significant differences in transcript levels were observed comparing the *HvGsl6* dsRNAi events to wild type and empty vector lines ($P < 0.01$, student T-test). Transcript values are arbitrary units of comparison that have been normalized as described by Vandesompele *et al*. (2002).

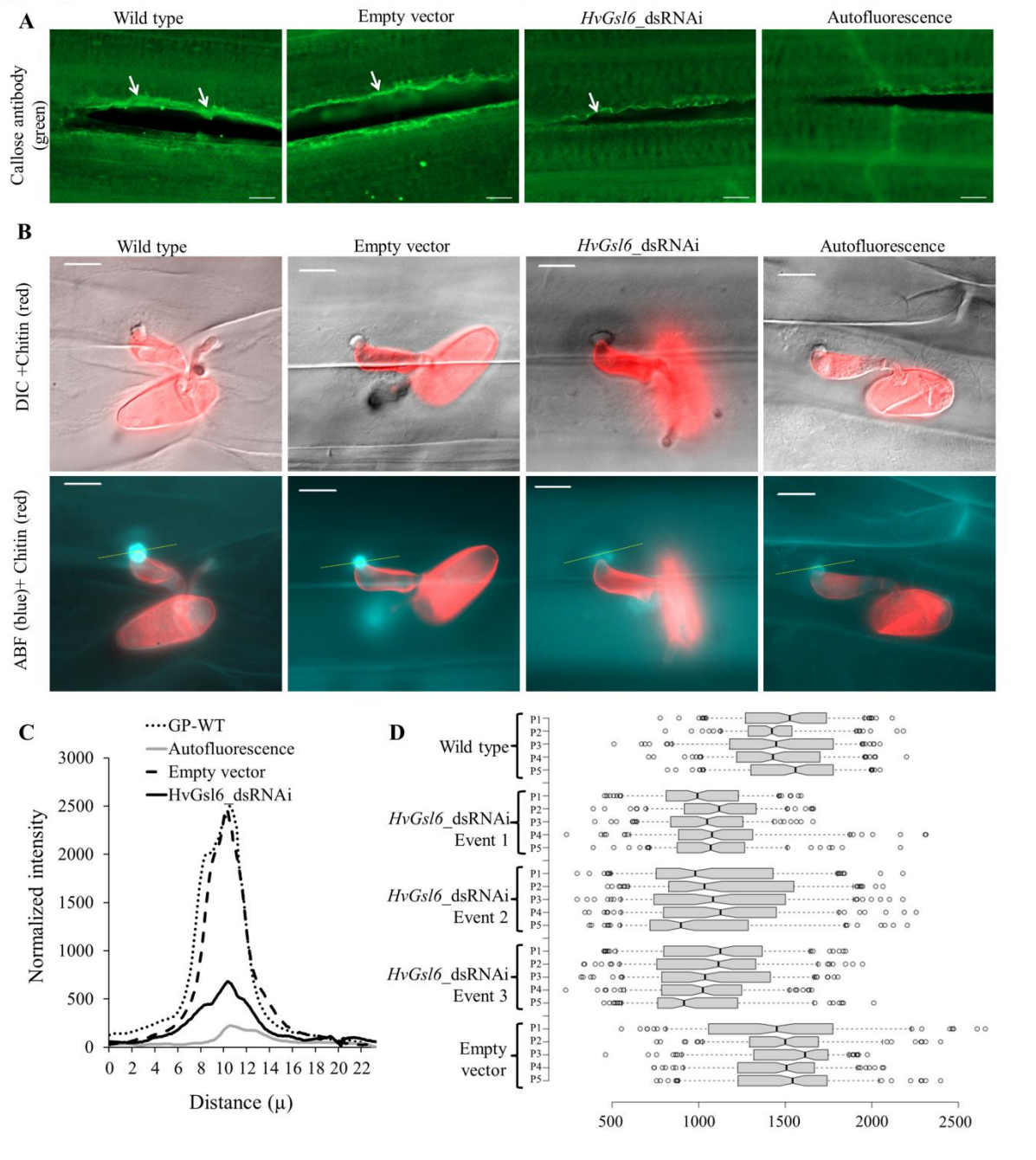


Figure 5. Down-regulation of barley *glucan synthase-like 6* in stable transgenic plants leads to lower accumulation of callose in the wound area and papillae formed in response to *Blumeria graminis* f. sp. *hordei* infection. A) Callose accumulation at an artificially wounded site 24 h after cutting with a razor. Detection of callose accumulation was performed using the callose specific antibody conjugated with AlexaFluor® 488. Bars= 100 µm. B) Callose accumulation in the papillae formed near the barley-*Bgh* interaction sites at 24 hours after inoculation. Callose was detected using the aniline blue fluorochrome (ABF) and fungal structure was stained with an AlexaFluor® 555 conjugate of Wheat germ agglutinin (WGA). C) Fluorescence intensity profiles correspond to yellow dashed lines in Figure 5B. Bars = 10

µm. D) Normalized fluorescence intensity of aniline blue stained callose in barley papillae during *Bgh* infection. The intensity values were normalized for each cell by subtracting the background staining of the wall from the staining intensity of the papilla. The centre lines show the median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend to 5th and 95th percentiles; outliers are represented by dots. The fluorescence intensity level observed in the three *HvGsl6* transgenic events were significantly different compared to that of wild type and empty vector lines ($P < 0.01$, student T-test), n = 150 sample points.

Reduction of the callose in papillae is associated with higher levels of susceptibility to penetration by Bgh

To assess if the observed reduction in papillary callose in the *HvGsl6* dsRNAi transgenic lines altered the susceptibility to *Bgh* infection, the levels of fungal growth were observed on the leaf surface at 3 days after inoculation. The greater abundance of *Bgh* hyphal growth was a clear indicator that the *HvGsl6* dsRNAi transgenic lines were more susceptible to *Bgh* infection (Figure 6A). The increased hyphal growth observed on the leaf surface was not a result of increased hyphal growth following each penetration, but was a direct result from the increase in the number of successful penetrations of papillae leading to more sites of hyphal growth. The relative susceptibility index (RSI) was calculated by dividing the number of cells containing haustoria by the total number of cells that were in contact with appressoria and the data were normalised against the empty vector control. The RSI revealed that all of the lines from the three *HvGsl6* dsRNAi transgenic events had a higher level of susceptibility to penetration by *Bgh* compared with wild type plants ($P < 0.05$, Figure 6B). The RSI of the wild type plants were similar to that of the empty vector control lines, indicating that the transformation vector had no significant effect on plant disease resistance.

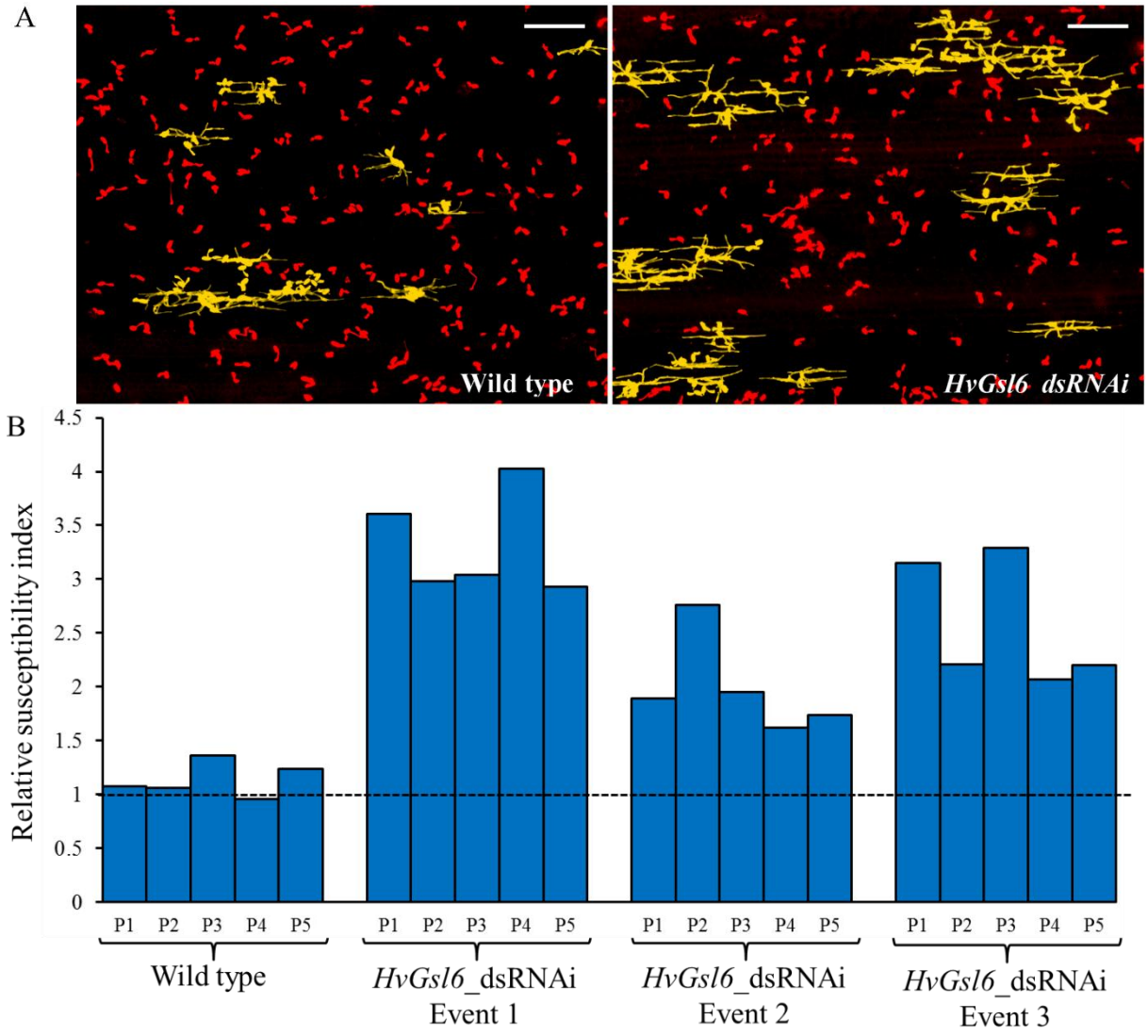


Figure 6. Level of susceptibility against *Blumeria graminis* f. sp. *hordei* (*Bgh*) infection of plants from three dsRNAi transgenic events. A) Micrographs show abundance of *Bgh* hyphal growth on barley leaves at 3 days after inoculation (dai). Fungal structures were stained with an AlexaFluor® 555 conjugate of Wheat germ agglutinin (WGA). Fungal staining from unsuccessful penetration events are artificially coloured in red and staining from hyphal growth resulting from successful penetration attempts are artificially coloured yellow. Bars = 200 µm. B) Relative susceptibility index of the dsRNAi transgenic lines against *Bgh* infection at 3 dai. The susceptibility index was calculated across the whole leaf area of five individual leaves from each event and normalized against the empty vector lines. Each transgenic event shows statistically significant differences compared to wild type ($P < 0.05$, student T-test). *Bgh* spore density was approximately 150 mm^{-2}.

Discussion

The *Glucan synthase-like* gene family members have been widely studied in a number of plant species for their role in callose deposition in specific tissues, at certain developmental stages and under different stress conditions. Previously, *AtGsl5* has been shown to be involved in the accumulation of papillary callose during pathogen infection and in wound callose accumulation (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). It is noteworthy that overexpression in barley of the stress-induced callose synthase gene *AtGsl5* from Arabidopsis induced penetration resistance to *Bgh* (Blümke *et al.*, 2013).

It was expected that there would be an *AtGsl5* ortholog present in monocot species like barley. We have re-examined the seven previously described *Gsl* genes (Schober *et al*., 2009) along with the *Gsl* genes annotated in the barley genome to identify any of these genes that might mediate callose accumulation during pathogen infection. To identify potential candidate genes involved in callose deposition in barley papillae, we have employed a comparative genomics approach between *Arabidopsis* and a series of representative monocot species including rice, barley, sorghum and *Brachypodium*.

A phylogenetic analysis revealed that *HvGsl6* and *HvGsl7* have the closest sequence similarity with *AtGsl5*, suggesting that both genes are potential orthologs of *AtGsl5* (Figure 1). Transcript profile analysis, conducted at different time points revealed that only the *HvGsl6* transcript is significantly up-regulated following infection by *Bgh*, with *HvGsl7* transcript levels significantly down-regulated (Figure 2). As *AtGsl5* was also the only *Gsl* induced during pathogen infection (Dong *et al.*, 2008) these results suggest that *HvGsl6* is the likely ortholog of *AtGsl5*.

The role of *HvGsl6* in the deposition of papillary callose induced during the *Bgh* infection was investigated using loss of function screens. Through transient-induced gene silencing, it was demonstrated that the frequency of papillae lacking normal levels of callose

in the *HvGsl6* dsRNAi transformed cells was significantly higher compared with that of the empty vector transformed cells (Figure 3). The *HvGsl7* dsRNAi transformed cells, however, were not significantly different from the control lines.

In order to confirm the preliminary evidence that *HvGsl6* is the ortholog of *AtGsl5*, stable *HvGsl6* dsRNAi transgenic lines were generated. Fluorescent labelling of the papillary callose deposited in the transgenic lines was significantly less compared with that of the control lines (Figure 5B-D). However, due to background autofluorescence we are unsure if the callose was completely eliminated from the papillae in the transgenic lines. The *HvGsl6* transcript levels have been quantified within the transgenic lines and are significantly reduced compared with wild type and empty vector controls, although not completely removed (Figure 4). It is possible that *HvGsl6* transcripts still expressed at low levels are responsible for some of the background fluorescence in the dsRNAi transgenic lines. A complete knock out line would be required to determine if any other *HvGsl* family member is also contributing to callose production. If so, a complete elimination of papillary callose might not be possible by knocking down only one gene. No off-target silencing is observed for the other members of the *Gsl* family, although in one of the transgenic lines (Event 1) there is a significant increase of the *HvGsl5* transcript (Figure S4). It is important to note that even though the transcript levels of *HvGsl5* have increased more than 20 fold, *HvGsl5* is unable to compensate for the silencing of *HvGsl6* because we see significant decreases in papillary callose and an increase in penetration by *Bgh*.

The observation that decreased accumulation of callose in the papillae leads to increased susceptibility of barley against the well adapted pathogen *Bgh* matches the outcome predicted from the early literature. For example, the result is supported by the demonstration that chemical inhibition of papillary callose synthesis through applying 2-deoxy-D-glucose (DDG) leads to significantly increased penetration of *Bgh* into a resistant *mlo* cultivar (Bayles *et al.*,

1990). However our result is not consistent with the observation in *Arabidopsis* that the loss of the papillary callose synthase (*AtGsl5*) gene led to increased resistance against well adapted powdery mildew pathogens (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). The increased resistance has been explained as an indirect phenotype where the loss of callose has somehow resulted in a hyperactivation of the salicylic acid-dependent defence pathways (Nishimura *et al.*, 2003). Although the reasons why differential outcomes are observed in *Arabidopsis* and barley have not been resolved, the debate over the role of callose in the prevention of penetration resistance has been supported following the recent overexpression of the *AtGsl5* in barley and *Arabidopsis* leading to the generation of complete penetration resistance to *Blumeria graminis* f.sp. *hordei* and *Golovinomyces cichoracearum* respectively (Blümke *et al.*, 2013; Ellinger *et al.*, 2013). Given that the silencing of the *HvGsl6* gene leads to a loss of function phenotype that is not clouded by an off-target increase in the salicylic acid-dependent defence pathways, this result complements the gain of function experiments in Arabidopsis and strengthens the suggestion that callose plays an important role in the prevention of penetration resistance.

In addition to the conclusion that *HvGsl6* is the functional ortholog of *AtGsl5*, we also propose that the *Gsl* members of rice (LOC_Os01g48200), *Brachypodium* (BRADI2G46250) and sorghum (Sb03g030800) present within the sub-clade containing *HvGsl6* are also potential candidates mediating papillary callose accumulation during different plant-pathogen interactions (Figure 2). This is supported by rice microarray data obtained from the Plant Expression Database (Plexdb), where transcripts of the *HvGsl6* ortholog, LOC_Os01g48200, were up-regulated during *Bgh* infection while the transcripts of the *HvGsl7* ortholog, LOC_Os01g55040, were slightly down-regulated (Supplemental Figure S3).

Based on the results presented here we propose that callose accumulation contributes to the penetration resistance mechanism of barley, which confirms previous observations that

there is more callose accumulation in effective papillae compared with ineffective ones in barley (Chowdhury *et al.*, 2014). Based on these findings, it can be predicted that higher accumulation of barley papillary callose would be possible in the near future through over-expression of the *HvGsl6* gene, especially if this could be linked with the defence response, and that this would lead to enhanced penetration resistance of barley against the powdery mildew fungus. If so, it would be interesting to see how those lines respond to different barley pathogens other than powdery mildew that also rely on penetration for successful infection. The combined expression of the *HvGsl6* gene with the barley genes required for the synthesis of the remaining papilla polysaccharide components, namely cellulose and arabinoxylan (Chowdhury *et al.*, 2014), could potentially create a papillae capable of withstanding the most aggressive fungal penetration attempts and lead to a high and durable level of resistance.

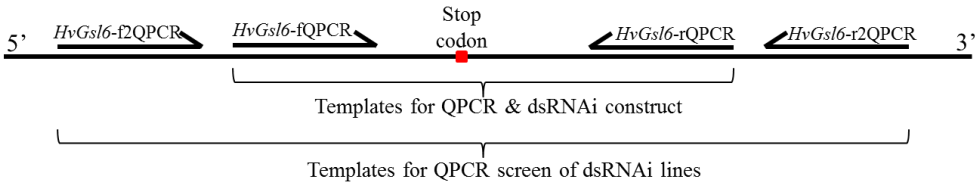
Supporting information

Supplemental Table S1. Combined gene annotation of multiple MLOC sequences into single *HvGsl* open reading frames.

Annotated gene name	MLOC number	Chromo number	Chromo position (cM)	JHI RNAseq expression profile in different tissue								BAC Genomic Sequence	Model protein
				Leaf	Root	Internode	Inflorescence 5mm	Inflorescence 15mm	Caryopsis 5DPA	Caryopsis 15DPA	Embryo		
	MLOC_4525	7	51	0	0	0	0.03	1.45	0.8	0.1	0	morex_contig_135227	BRADI1G47427
	MLOC_46450	7	51	0	0.13	0.08	0.12	1.33	0.67	0.04	0	morex_contig_290563	
	MLOC_13377	3	46	7.84	3.73	3.62	9.01	15.59	9.09	5.51	8.32	morex_contig_1565486	BRADI2G40460
	MLOC_501	3	46	9.4	6.95	7.2	12.63	22.03	12.24	9.14	13.03	morex_contig_103522	
HvGsl5	MLOC_36683	7	166	19.08	38.6	33.71	228.14	267.93	164.26	35.62	57.89	barke_contig_268978	BRADI1G29622
	MLOC_64106	7	144	11.31	31.21	31.32	167.9	192.74	133.89	29.92	50.23		
	MLOC_8120	6	52	1.19	5.63	4.3	6.5	7.27	1.35	0.73	3.08	morex_contig_141211	BRADI3G09317
	MLOC_36579	6	52	1.4	3.95	2.81	5.84	7.2	1.34	0.68	2.95	morex_contig_2547005	
HvGsl3	MLOC_20815	4	98	16.74	17	17.03	4.59	5.06	24.53	7.02	14.69	HVVMRXALLhA0600K20_c4	LOC_Os03g03610
	MLOC_10498	4	99	22.14	13.96	10.94	4.79	5.36	30.23	7.09	15.79	morex_contig_1559222	
	MLOC_13869	2	135	8.28	6.17	4.77	7.28	9.65	5.91	3.85	9.85	bowman_contig_125896	Sb03g023490
	MLOC_716682	2	135	2.71	3.06	2.5	5.08	7.47	4	3.41	3		
	MLOC_36809	3	44	17.01	11.61	10.16	17.49	19.4	12.85	16.34	12.13		

Supplemental Table S2. Primers used for Q-PCR transcript analysis and Q-PCR screening of *HvGsl6* dsRNAi transgenic lines.

Primer name	Sequence
HvGsl6-fQPCR	GGAGTGATTGTTATGACA
HvGsl6-rQPCR	GAATCAAGGTAAGGATGTCGC
HvGsl6-f2QPCR	GGCGCGCCTGTATGATATAC
HvGsl6-r2QPCR	TACGGAAATCCATGTGTACATCA
HvGsl7-fQPCR	AGAAATGCAGACAAGGGTA
HvGsl7-rQPCR	CCAAGACTCAATGCCTAAATCAC



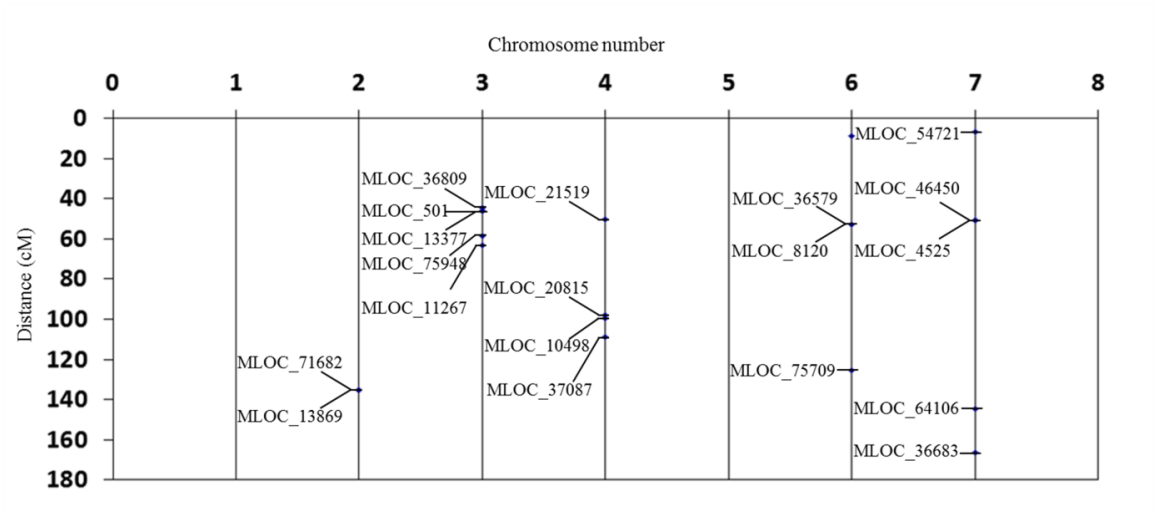
Supplemental Table S3. Primers used for *HvGsl6* dsRNAi vector construction.

*fHvGsl6-Eco*RI	CCGGAATTCGAATCAAGGTAAGGATGTCGC
*fHvGsl6-Xho*I	CCGCTCGAGGGAGTGATTGTTATGACACCTG
*rHvGsl6-Hin*dIIII	CCCAAGCTTGAATCAAGGTAAGGATGTCGC
*rHvGsl6-Xba*I	GCTCTAGAGGAGTGATTGTTATGACACCTG

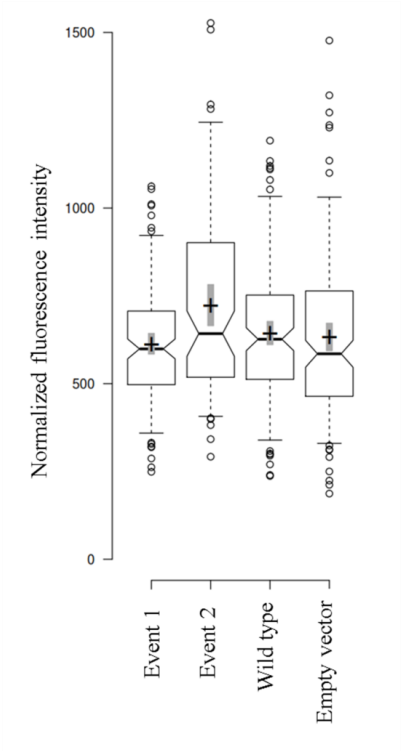
NB: restriction sites are underlined, gene specific regions are highlighted in yellow

Supplemental Table S4. Number of selectable marker gene insertion loci in the transgenic lines revealed by Southern blot analysis.

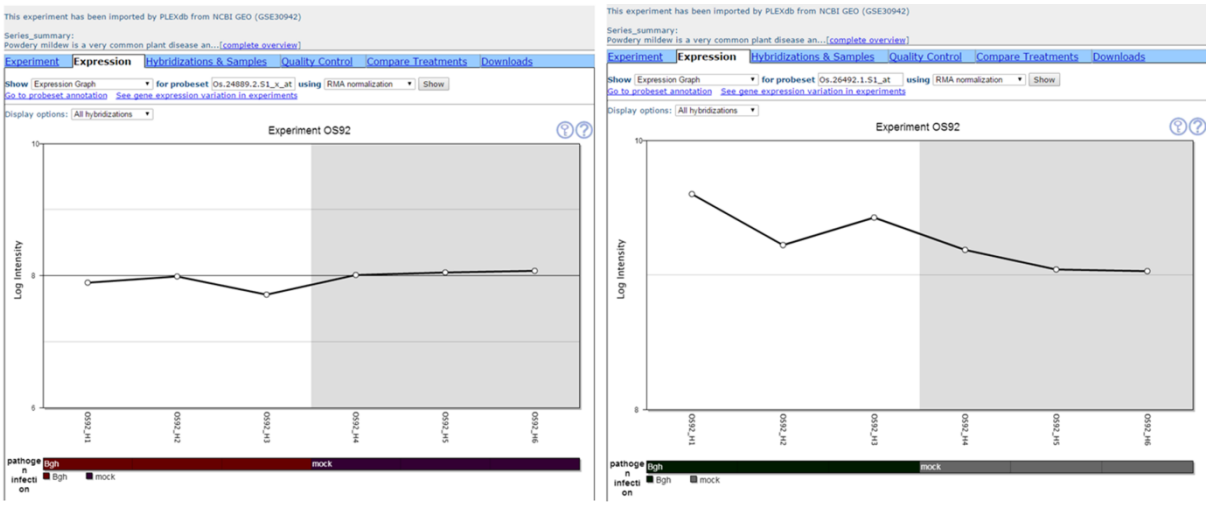
*HvGsl6*_dsRNAi Event number	Construct number	Number of selectable marker gene insertion loci
Event 1	G27-4-1	2
Event 2	G27-7-9	1
Event 3	G27-13-1	4



Supplemental Figure S1. Chromosomal location of barley sequences containing a glucan synthase domain.

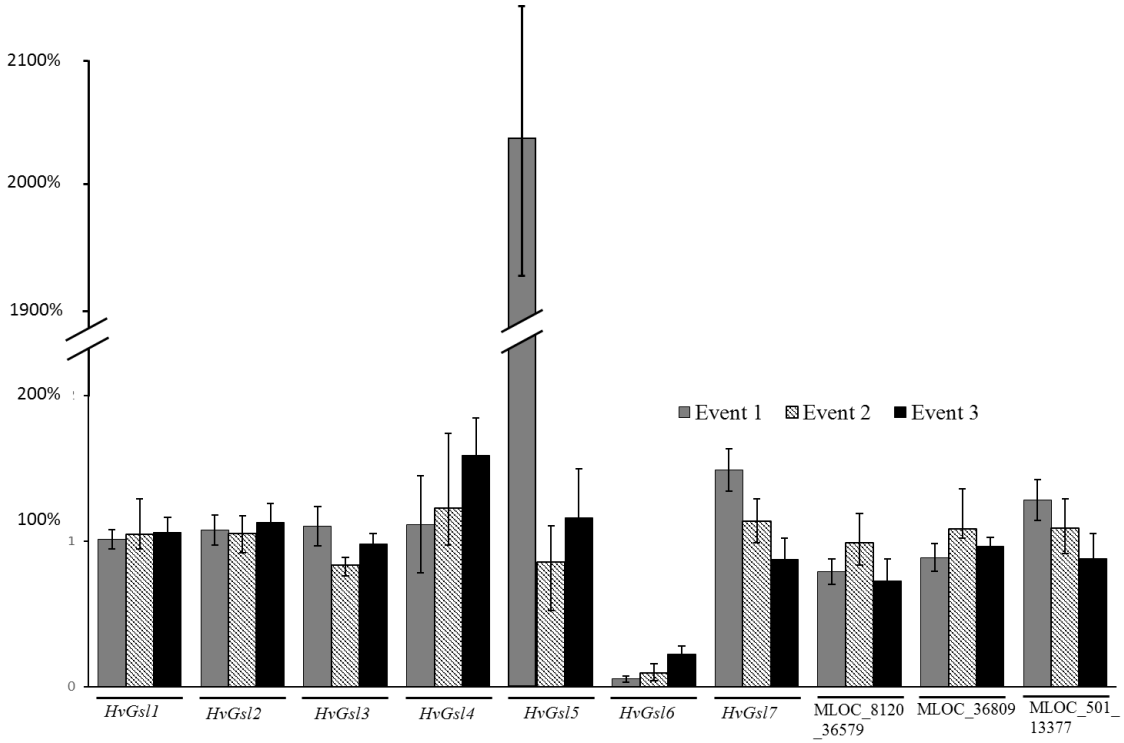


Supplemental Figure S2. Measurement of the cellulose staining intensity in papillae formed in response to *Blumeria graminis* f. sp. *hordei* infection using Pontamine fast scarlet 4B. Events 1 and 2 are transgenic lines overexpressing the HvGsl6 dsRNAi construct.



LOC_Os01g55040 LOC_Os01g48200

Supplemental Figure S3. Microarray transcript profiles of two rice *Gsls*, found in the same phylogenetic clade as *HvGsl6* and *HvGsl7*, in response to *Blumeria graminis* f. sp. *hordei* infection.



Supplemental Figure S4. Relative transcript levels of barley *Glucan synthase* (*Gsl*) members in the leaf tissue of three *HvGsl6*_dsRNAi transgenic events compared to control lines. The expression of MLOC_4525, MLOC_46450 and MLOC_21519 was not found in the leaf tissue and therefore, are not included in the graph.

Acknowledgements

We would like to thank Jason Eglington for providing us with the barley variety, Baudin, for the maintenance of our *Bgh* isolate. The work was supported by grants from the Australian Research Council. J.C. was supported by an International Scholarship Award from the University of Adelaide

References

Aist JR. 1976. Papillae and related wound plugs of plant cells. *Annual Review of Phytopathology* **14**(1): 145-163.

Bayles CJ, Ghemawat MS, Aist JR. 1990. Inhibition by 2-deoxy-D-glucose of callose formation, papilla deposition, and resistance to powdery mildew in an ml-o barley mutant. *Physiological and Molecular Plant Pathology* **36**(1): 63-72.

Blümke A, Somerville SC, Voigt CA. 2013. Transient expression of the Arabidopsis thaliana callose synthase PMR4 increases penetration resistance to powdery mildew in barley. *Advances in Bioscience & Biotechnology* **4**(8).

Burton RA, Collins HM, Kibble NAJ, Smith JA, Shirley NJ, Jobling SA, Henderson M, Singh RR, Pettolino F, Wilson SM, Bird AR, Topping DL, Bacic A, Fincher GB. 2010. Over-expression of specific HvCslF cellulose synthase-like genes in transgenic barley increases the levels of cell wall (1,3;1,4)-β-d-glucans and alters their fine structure. *Plant Biotechnology Journal* **9**(2): 117-135.

Chen X-Y, Liu L, Lee E, Han X, Rim Y, Chu H, Kim S-W, Sack F, Kim J-Y. 2009. The Arabidopsis callose synthase gene GSL8 is required for cytokinesis and cell patterning. *Plant Physiology* **150**(1): 105-113.

Chowdhury J, Henderson M, Schweizer P, Burton RA, Fincher GB, Little A. 2014. Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated versus penetrated papillae on leaves of barley infected with Blumeria graminis f. sp. hordei. *New Phytologist* **204**(3): 650-660.

Criscuolo A, Gribaldo S. 2010. BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. *Bmc Evolutionary Biology* **10**(1): 210.

Deng W, Nickle DC, Learn GH, Maust B, Mullins JI. 2007. ViroBLAST: a stand-alone BLAST web server for flexible queries of multiple databases and user's datasets. *Bioinformatics* **23**(17): 2334-2336.

Dong X, Hong Z, Chatterjee J, Kim S, Verma DPS. 2008. Expression of callose synthase genes and its connection with Npr1 signaling pathway during pathogen infection. *Planta* **229**(1): 87-98.

Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol* **29**(8): 1969-1973.

Ellinger D, Naumann M, Falter C, Zwikowics C, Jamrow T, Manisseri C, Somerville SC, Voigt CA. 2013. Elevated early callose deposition results in complete penetration resistance to powdery mildew in Arabidopsis. *Plant Physiology* **161**(3): 1433-1444.

Enns LC, Kanaoka MM, Torii KU, Comai L, Okada K, Cleland RE. 2005. Two callose synthases, GSL1 and GSL5, play an essential and redundant role in plant and pollen development and in fertility. *Plant Molecular Biology* **58**(3): 333-349.

Hajdukiewicz P, Svab Z, Maliga P. 1994. The small, versatilePZP family ofAgrobacterium binary vectors for plant transformation. *Plant Molecular Biology* **25**(6): 989-994.

Hofgen R, Willmitzer L. 1988. Storage of competent cells for Agrobacterium transformation. *Nucleic Acids Research* **16**(20): 9877.

Hong Z, Delauney AJ, Verma DPS. 2001. A Cell Plate-Specific Callose Synthase and Its Interaction with Phragmoplastin. *Plant Cell* **13**(4): 755-768.

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB. 2003b. An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* **15**(11): 2503-2513.

Li J, Burton R, Harvey A, Hrmova M, Wardak A, Stone B, Fincher G. 2003. Biochemical evidence linking a putative callose synthase gene with (1→3)-β-d-glucan biosynthesis in barley. *Plant Molecular Biology* **53**(1-2): 213-225.

Mangin LA. 1895. *Recherches anatomiques sur les Péronosporées*: Imp. Dejussieu pére et fils.

Matthews P, Wang M-B, Waterhouse P, Thornton S, Fieg S, Gubler F, Jacobsen J. 2001. Marker gene elimination from transgenic barley, using co-transformation with adjacent `twin T-DNAs' on a standard Agrobacterium transformation vector. *Molecular Breeding* **7**(3): 195-202.

Nishikawa S-i, Zinkl GM, Swanson RJ, Maruyama D, Preuss D. 2005. Callose (β-1, 3 glucan) is essential for Arabidopsis pollen wall patterning, but not tube growth. *BMC Plant Biology* **5**(1): 22.

Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC. 2003a. Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* **301**(5635): 969-972.

Schober MS, Burton RA, Shirley NJ, Jacobs AK, Fincher GB. 2009. Analysis of the (1,3)-[beta]-d-glucan synthase gene family of barley. *Phytochemistry* **70**(6): 713-720.

Schweizer P, Christoffel A, Dudler R. 1999a. Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance. *The Plant Journal* **20**(5): 541-552.

Schweizer P, Pokorny J, Abderhalden O, Dudler R. 1999b. A transient assay system for the functional assessment of defense-related genes in wheat. *Molecular Plant-Microbe Interactions* **12**(8): 647-654.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*: btu033.

Stone BA, Clarke AE. 1992. *Chemistry and Biology of (1→3)-β-D-Glucans*. Victoria, Australia: La Trobe University Press.

Stone BA, Jacobs AK, Hrmova M, Burton RA, Fincher GB. 2010. The biosynthesis of plant cell wall and related polysaccharides by enzymes of the GT2 and GT48 families. *Plant Polysaccharides Series: Annual Plant Reviews* **41**: 109-166.

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R. 1997. Agrobacterium tumefaciens-mediated barley transformation. *The Plant Journal* **11**(6): 1369-1376.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**(7): research0034.0031 - research0034.0012.

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. *The Plant Journal* **27**(6): 581-590.

Xie B, Wang X, Zhu M, Zhang Z, Hong Z. 2011. CalS7 encodes a callose synthase responsible for callose deposition in the phloem. *The Plant Journal* **65**(1): 1-14.

Zeyen R, Carver T, Lyngkjaer M, Bélanger R, Bushnell W, Dik A. 2002. Epidermal cell papillae. *The powdery mildews: a comprehensive treatise*: 107-125.

CHAPTER 5

Xylan biosynthesis in papillae for improved penetration resistance against *Blumeria graminis* f. sp. *hordei* in barley

Title of Paper	Xylan biosynthesis in papillae for improved penetration resistance against Blumeria graminis f. sp. hordei in barley
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	This work has not been submitted yet.

Principal Author

Name of Principal Author (Candidate)	Jamil Chowdhury		
Contribution to the Paper	Performed bioinformatics analysis, gene cloning, transient gene expression assays, interpreted data, and drafted manuscript.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30 March 2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

vii. the candidate's stated contribution to the publication is accurate (as detailed above);

viii. permission is granted for the candidate in include the publication in the thesis; and

ix. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dimitar Douchkov		
Contribution to the Paper	Supervised TIGS experiment and helped in data interpretation.		
Signature		Date	10.02.2016

Name of Co-Author	Stefanie Lück		
Contribution to the Paper	Prepared and cloned the dsRNAi constructs		
Signature		Date	10.02.2016

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Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation.		
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Signature		Date	29 March 2016

Link to chapter 5

As described in Chapter 3, arabinoxylan may play a significant role in the penetration resistance mechanisms of barley. Identifying genes associated with arabinoxylan biosynthesis in papillae is important to further characterise this potential new target for improving disease resistance in crop plants. In the cell wall during normal growth and development, several glycosyltransferase families have been implicated in arabinoxylan biosynthesis. In Chapter 5 the characterisation of candidate genes putatively involved in arabinoxylan biosynthesis in papillae is reported. By changing the transcript expression level of several candidate genes that have been implicated in arabinoxylan biosynthesis, using both loss-of-function and gain-of-function approaches, host penetration resistance levels can be significantly altered. This Chapter has been written in manuscript format in preparation for future submission to the New Phytologist journal.

Xylan biosynthesis in papillae for improved penetration resistance against *Blumeria graminis* f. sp. *hordei* in barley

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Summary

- Arabinoxylan has recently been identified as an important component of papillae and might be involved in penetration resistance during powdery mildew infection of barley leaves. Deposition of arabinoxylan near the sites of attempted fungal penetration in the epidermal cell wall is believed to enhance the physical resistance to the fungal penetration peg and hence to improve pre-invasion resistance.
- Several glycosyltransferase (GT) families are implicated in the assembly of arabinoxylan in the plant cell wall, and are likely to work together in a multi-enzyme complex. The microarray expression profile shows that members of key GT families associated with arabinoxylan biosynthesis are up-regulated in the epidermal layer during powdery mildew infection. Modulation of their expression leads to altered susceptibility levels, suggesting that these candidates are important for penetration resistance. The highest level of resistance was achieved when a GT43 gene was co-expressed with a GT47 candidate gene, both of which have been predicted to be involved in xylan backbone biosynthesis.
- Our data suggest that by altering the expression level of several GT43 and GT47 candidate genes, disease susceptibility can be altered significantly, probably through changes in the biosynthesis of arabinoxylan in barley papillae.

Introduction

The cell wall is a dynamic structural barrier that can determine the outcome of the interactions between plants and pathogens. At the sites of interaction, plants actively reinforce the cell wall though development of cell wall appositions, called papillae (Zeyen *et al.*, 2002). Papillae formation appears to be a consequence of PAMP/ DAMP -triggered immunity (PTI/DTI), that is observed during a number of plant-pathogen interactions (Boller & Felix, 2009; Malinovsky *et al.*, 2014). Although the role of papillae is not clearly understood, it is believed that they provide a physical and chemical barrier that can completely prevent pathogen entry or at least delay pathogen penetration into the cells so that other defence strategies can be activated (Stone & Clarke, 1992; Huckelhoven, 2005). The composition of papillae may vary among different species, but commonly found papillae components are cell wall polysaccharides, cell wall proteins, reactive oxygen species, phenolics and anti-microbial compounds (Zeyen *et al.*, 2002). Among the cell wall polysaccharides, callose has commonly been assumed to be the major polysaccharide in the papillae that form during plant/pathogen interactions, but it has recently been shown that arabinoxylan and cellulose are also major constituents of barley papillae that form during *Blumeria graminis* f. sp. *hordei* (*Bgh*) infection (Chowdhury *et al.*, 2014). The data presented in that study also indicated that earlier and heavier deposition of these polysaccharides aid in arresting the progress of the fungal penetration peg through effective papillae (Chowdhury *et al.*, 2014). Later we have shown that a glucan synthase gene (*HvGsl6*) is responsible for biosynthesis of callose in barley papillae and down regulation of this gene causes increased penetration susceptibility to powdery mildew pathogen (Chapter 4). Together these results suggest that polysaccharide deposition in papillae is essential for barley penetration mechanism against powdery mildew infection.

Arabinoxylan was detected in barley papillae using the LM11 monoclonal antibody (Chowdhury *et al.*, 2014). The arabinoxylan epitopes were distributed throughout the entire papillae, in which two zones could be readily recognized. An inner core consisted of

arabinoxylan, callose and phenolics, while an outer layer, or coat, contained arabinoxylan and cellulose. The LM11 antibody binds to unsubstituted xylans and arabinoxylans with a low degree of arabinose substitutions (McCartney *et al.*, 2005). Therefore, we can assume that some degree of substitution of the backbone xylan with arabinosyl residues is present in the arabinoxylan in barley papillae.

Cell wall reinforcement in response to biotrophic and necrotrophic pathogen attack can be mediated through oxidative cross-linking between polysaccharides, structural proteins and phenolic compounds (Passardi *et al.*, 2004; Deepak *et al.*, 2010). The presence of arabinoxylan in papillae provides an opportunity for increased cell wall polymer cross-linking through associated phenolic compounds, such as ferulic acid. More highly cross-linked wall polysaccharides would presumably toughen the wall and increase resistance against fungal penetration, whether that penetration is mediated through physical pressure or by enzymatic hydrolysis. In monocotyledonous plants, ferulic acid can be ester-linked to arabinofuranosyl substituents of the xylan backbone. The ferulic acid may undergo oxidative dimerization to covalently link adjacent feruloylated arabinoxylan chains or, alternatively, the arabinoxylan chains might be cross-linked with lignin (Burr & Fry, 2009; Marcia, 2009). There is evidence that arabinoxylans with low degrees of substitution can interact with cellulose microfibrils and other polymers via extensive intermolecular hydrogen bonding (Busse-Wicher *et al.*, 2014) and this is also likely to influence the strength and elasticity of walls, and contribute to the resistance of the walls to enzymatic degradation. It can be argued that arabinoxylan, as a major component of papillae, helps to provide mechanical strength against the fungal penetration process. Thus, genetic manipulation of arabinoxylan content or structure may impact upon the plant susceptibility to infection.

The complete process of arabinoxylan biosynthesis in the plant cell wall is not fully understood. However, there is evidence to suggest that the assembly of the xylan backbone

and its substituents involves at least six families of glycosyltransferases (GTs) found in the Carbohydrate-Active EnZymes (CAZy) database (Cantarel *et al.*, 2009), namely GT8, GT31, GT43, GT47, GT61 and GT75 (Rennie & Scheller, 2014). The predicted role for each of these GT families in assembling arabinoxylan in grasses is presented in Figure 1. Two members of the GT43 family, known as *Irregular Xylem* 9 (*IRX9*) and *IRX14*, and a member of the GT47 family, *IRX10*, have putative xylosyltransferase activities required for synthesising the xylan backbone (Brown *et al.*, 2007; Lee *et al.*, 2007; Wu *et al.*, 2009; Mortimer *et al.*, 2015a). Two members of the GT8 family, termed *Glucuronic Acid Substitution of Xylan* (*GUX*) *1* and *GUX2*, introduce glucuronyl (GlcA) substituents onto the xylan backbone (Mortimer *et al.*, 2010; Rennie *et al.*, 2012). Members of the GT75 family, which encode UDP-arabinose mutases (UAM) or Reversibly Glycosylated Protein (RGP), are responsible for converting UDP-arabinopyranose to UDP-arabinofuranose, which is the substrate for xylan arabinoxyltransferases (Rautengarten *et al.*, 2011; Hsieh *et al.*, 2015). Some members of the GT61 family also have xylan arabinosyltransferase activity that adds arabinosyl residues onto the xylan backbone (Anders *et al.*, 2012), while another member of the GT61 family is capable of adding feruloylated arabinoxyl residues onto the xylan backbone (Chiniquy *et al.*, 2012). The GT31 family was also included in our study due to their reported association with arabinoxylan synthesis, although there is no direct evidence that they are directly involved. Several GT31 genes encode galactosyltransferases that transfer galactosyl residues from UDP-galactose to arabinogalactan proteins (AGPs) (Nguema-Ona *et al.*, 2015). AGPs may be cross-linked with arabinoxylan (Tan *et al.*, 2013) and play a role in pathogen defence responses (Nguema-Ona *et al.*, 2013).

In the current study, we have investigated the potential roles in papillae formation of genes that have been implicated in arabinoxylan biosynthesis and more specifically, to determine if the arabinoxylans contribute to penetration resistance against the barley powdery mildew pathogen, *Bgh*. Up- or down-regulation of transcript levels of selected candidate genes

during infection indicated that the susceptibility against *Bgh* penetration is significantly altered in barley epidermal cells. The identification of these genes will provide new opportunities for the generation of novel crop lines with greater disease resistance.

Materials and methods

Plant and fungal materials

A field isolate of *Blumeria graminis* f. sp. *hordei* (*Bgh*) was provided by Professor Richard Oliver, Centre for Crop Disease Management, Curtin University, Western Australia. The isolate was maintained on seven day old seedlings of a susceptible barley cultivar, 'Baudin' as described previously (Chowdhury *et al.*, 2014).

Phylogenetic relationship analysis

Amino acid sequences of the selected GT families were retrieved from the Ensembl Plants database (http://plants.ensembl.org) using the respective Pfam domain IDs. Phylogenetic trees were constructed using FastTree from amino acid sequences aligned with the Muscle alignment program in the Geneious software version 8.1.3 (www.geneious.com). The putative functions of the gene clusters were annotated from 'The Arabidopsis Information Resource (TAIR)' and 'Rice genome annotation project' databases.

Microarray transcript profiling

RNA was isolated from the abaxial leaf epidermal peel of seven-day-old barley plants cv. Vada inoculated with *B. graminis* f. sp. *hordei* (*Bgh*) or *B. graminis* f. sp. *tritici* (*Bgt*) at 6 to 74 h after inoculation as described previously (Zellerhoff *et al.*, 2010). Total quality-controlled RNA was hybridized to a 44 K Agilent oligonucleotide array chip (Chen *et al.*, 2011). Details of the data normalization procedure have been described previously (Douchkov *et al.*, 2014).

Biolistic DNA delivery for transiently induced gene silencing and transient over-expression

To test the impacts of up- or down-regulation of the genes of interest on the penetration success of *Bgh* in barley leaf epidermal cells, DNA-coated gold particles carrying a reporter gene as well as a test gene construct were co-bombarded using a biolistic DNA delivery system. Although there is no direct evidence that the test gene transcript level changes to desired directions (Up- or down-regulated) in the reporter gene (GUS or GFP) transformed cells, there is an assumption that the gene of interest is co-expressed. Quantification of the pathogen susceptibility index in several previous studies in relation to a control gene transformation suggests the impact of the gene of interest on the outcome of the plant pathogen interaction (Nielsen *et al.*, 1999; Schweizer *et al.*, 1999; Panstruga, 2004; Douchkov *et al.*, 2014). For transiently induced gene silencing experiments, approximately 500 bp of the candidate gene sequences were PCR-amplified using the primers listed in Supplementary Table S1, and ligated into pIPKTA38 entry clones. The cloned fragments were recombined into the RNA interference (RNAi) destination vector pIPKTA30N as inverted repeats, using the Gateway LR clonase reaction (Invitrogen) according to the procedure described in Douchkov *et al.* (2005). Possible off-target effects from use of the transiently induced gene silencing constructs were predicted using si-Fi software (labtools.ipk-gatersleben.de). Several previously tested genes (*mlo*, *HvSNAP34* and *HvCslD2*) have been used as control genes for transient gene expression assay which have significant impact on pathogen susceptibility level upon their up- or down-regulation (Douchkov *et al.* 2005). A PDS-100/He microprojectile bombardment system was used to co-bombard seven-day-old detached barley leaves with the candidate gene dsRNAi constructs in a vector containing the reporter gene β-glucuronidase (GUS) (Douchkov *et al.*, 2005). Twenty four hours post bombardment the leaf segments were inoculated with powdery mildew conidia at a density of approximately 200 conidia/mm^2. Forty eight hours post inoculation, the relative susceptibility index (RSI, compared to the

empty-vector control that was set to 100%) was calculated, following the method described previously (Douchkov *et al.*, 2014).

In transient over-expression experiments, the longest coding sequence of the candidate genes was obtained from a publicly available database (plants.ensembl.org). Candidate genes of interest were PCR-amplified using the Phusion polymerase (New England Biolab® inc., Ipswich, USA) with primers flanking the predicted coding region (Supplementary Table S2). The amplified sequences were ligated into the pCR8 entry vector (Invitrogen) and recombined into a Gateway enabled destination vector, pEAQ-*HT*-DEST1 (Sainsbury *et al.*, 2009) where genes of interest are under the control of a CaMV 35S promoter. The same conditions were used for microprojectile bombardment in silencing and over-expression experiments, except that a Green Fluorescent Protein (GFP) expressing construct was used instead of the GUS reporter construct. Haustoria present in GFP-transformed cells were observed in live tissue under a GFP filter (excitation 485/20 nm, emission 530/25 nm) of a fluorescence microscope (Axio Imager M2; Carl Zeiss, Oberkochen, Germany) pre-aligned with a mercury system (Supplementary Figure 2).

Results

Transcript profiling of arabinoxylan biosynthetic gene family members during Bgh infection

The transcript expression profiles of candidate GT gene families induced in the barley epidermal layer during infection by the pathogenic *Blumeria graminis* f. sp. *hordei* (*Bgh*) and the non-pathogenic *Blumeria graminis* f. sp. *tritici* (*Bgt*) fungi were quantified using microarray analysis (Figure 2). A relatively small number of each GT family were significantly up-regulated, most members showed no significant changes while a few members were down-regulated. The development of papillae under the first and second appressorial lobes was completed within 24 hours after inoculation, and the gene candidates were therefore selected on the basis of their up-regulation patterns within these time points. With few exceptions, the transcript patterns of the up-regulated gene family members were comparable during *Bgh* and *Bgt* infection. Among the selected GT families, the GT43 family was the only family that did not show increases in expression during powdery mildew infection. Due to the predicted involvement of the GT43 family in xylan synthesis, one candidate gene from the GT43 family (MLOC_54026) was selected for further analysis. Given that the transcripts of this gene were highly abundant in both infected and uninfected epidermal tissue it could still play a role in the synthesis of the papillae.

To assign putative functions to the selected candidate genes, phylogenetic analyses of each GT families were performed, using protein sequences from the barley, rice and *Arabidopsis* genomes (Supplementary Figures 1 to 5). The annotated functions of the selected barley candidates are summarised in Table 1.

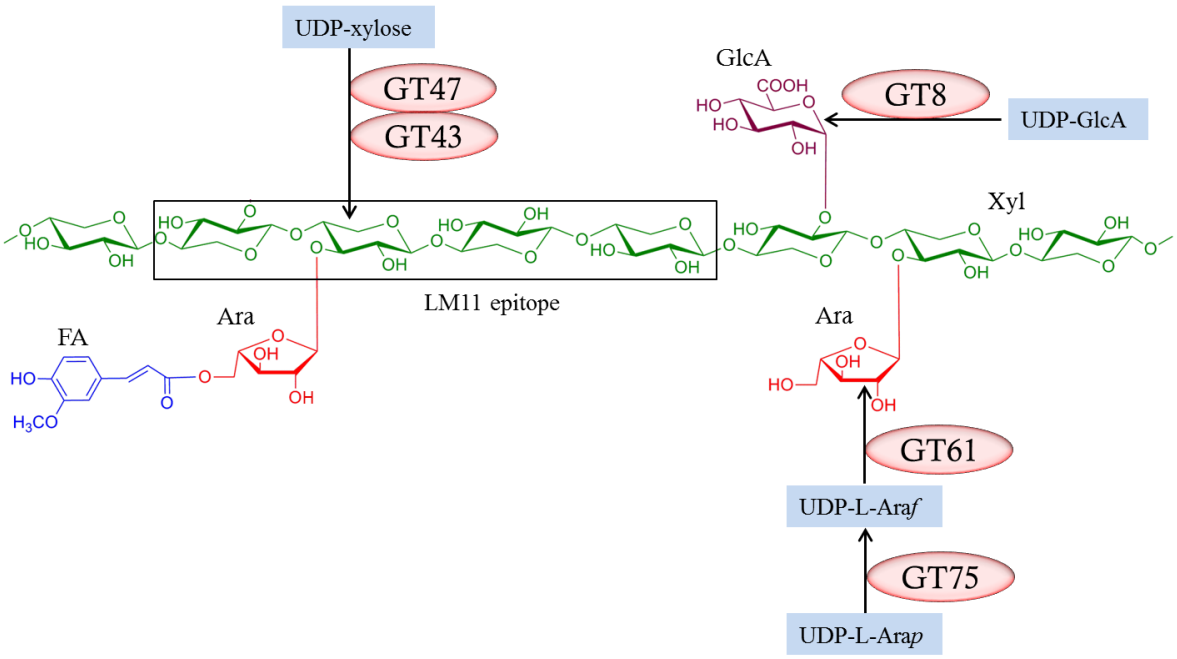

Figure 1. Predicted roles of glycosyltransferase (GT) gene families in arabinoxylan biosynthesis. The LM 11 antibody recognises an arabinoxylan backbone carrying a low degree of arabinose substitutions. Xyl = Xylose, Ara = Arabinose, GlcA= Glucuronic acid, FA=Ferulic acid. UDP-L-Ara*p* = UDP-L-arabinopyranose, UDP-L-Ara*f* = UDP-L-arabinofuranose.

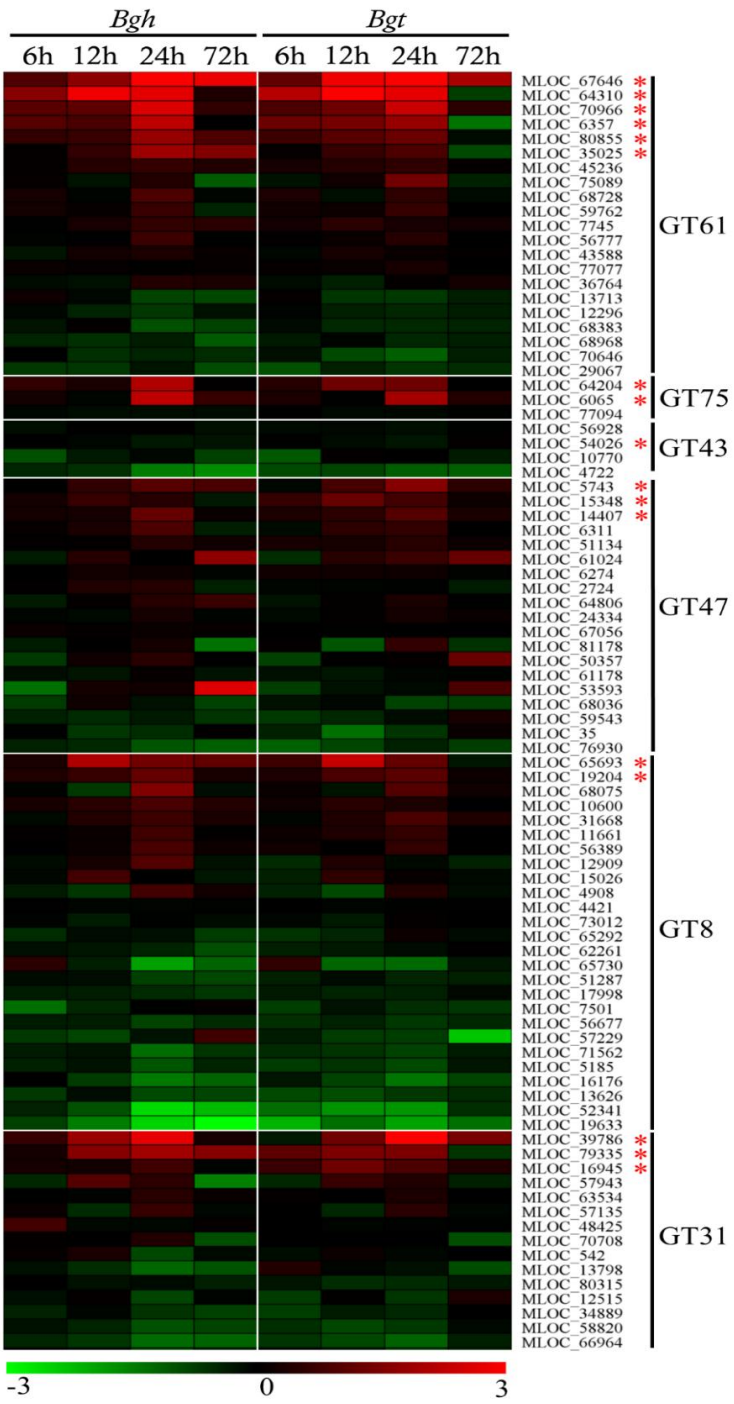

Figure 2. Microarray transcript profiling of the glycosyltransferase gene families in barley leaves infected with pathogenic *Blumeria graminis* f. sp. *hordei* (*Bgh*) and non-pathogenic *Blumeria graminis* f. sp. *tritici* (*Bgt*) fungi at different time points. The scale of this heatmap is given as standardised fold-changes (log2) of infected tissue relative to the uninfected control with a range from -3 (green) to 3 (red). * indicates the candidate genes selected for further analysis. The genes were chosen on the basis of their expression pattern up to 24 h after inoculation.

Table 1: Selected candidate genes and their proposed function in the synthesis of arabinoxylan.

MLOC ID	CAZy family	*Arabidopsis* homolog	Putative function	Chromosome number	Chromosome position (cM)	Host epi. transcript regulation (Log2 fold change 24hai)	Non-host epi. transcript regulation (Log2 fold change 24hai)	References
MLOC_54026	GT43	AT1G27600 (IRX9-L)	β-1,4 Xylosyl transferase	1H	116.501	NS (0.03)	NS (-0.17)	(Lee *et al.*, 2010; Mortimer *et al.*, 2015b)
MLOC_5743	GT47	AT3G45400	Exostosin family protein with unknown function	4H	51.404	UP (1.04)	UP (1.60)	(Andersson-Gunneråsa *et al.*, 2006)
MLOC_15348	GT47	AT2G20370 (MUR3)	Xyloglucan galactosyl transferase, actin organization	4H	91.713	UP (0.46)	UP (0.80)	(Chevalier *et al.*, 2010; Li *et al.*, 2013)
MLOC_14407	GT47	AT4G16745	Exostosin family protein with unknown function	3H	6.149	UP (1.20)	UP (1.01)	(Wang *et al*., 2008)
MLOC_65693	GT8	AT2G35710	UDP-glucuronyl transferase	1H	47.827	UP (1.37)	UP (1.19)	(Rennie *et al.*, 2012)
MLOC_19204	GT8	AT5G18480	UDP-glucuronyl transferase	2H	71.956	UP (1.19)	UP (1.08)	(Rennie *et al.*, 2012; Rennie *et al.*, 2014)
MLOC_67646	GT61	Not found	Predicted protein with unknown function	3H	141.918	UP (5.73)	UP (5.32)	-
MLOC_64310	GT61	AT3G18170 AT3G18180	Arabinofuranosyl transferase (homolog of TaXAT1)	6H	72.238	UP (2.70)	UP (2.73)	(Anders *et al.*, 2012)
MLOC_70966	GT61	Not found	Predicted protein with unknown function	7H	67.917	UP (2.62)	UP (2.41)	-
MLOC_6357	GT61	AT3G18170 AT3G18180	Arabinofuranosyl transferase	7H	69.263	UP (2.25)	UP (1.80)	(Anders *et al.*, 2012)
MLOC_80855	GT61	AT3G18170 AT3G18180	Arabinofuranosyl transferase	6H	49.787	UP (1.79)	UP (1.27)	(Anders *et al.*, 2012)
MLOC_35025	GT61	AT3G10320	Xylosyl transferase , decorates xylan with xylose side chains	1H	17.288	UP (1.86)	UP (0.91)	(Voiniciuc *et al.*, 2015)
MLOC_64204	GT75	Not found	UDP-Arabinose Mutase (*HvUAM3*)			UP (2.09)	UP (1.34)	(Hsieh *et al.*, 2015)
MLOC_6065	GT75	Not found	UDP-Arabinose Mutase (*HvUAM1*)	4H	51.274	UP (2.26)	UP (1.95)	(Hsieh *et al.*, 2015)
MLOC_39786	GT31	AT5G57500	Galactosyl transferase	5H	55.625	UP (2.75)	UP (3.42)	(Qu *et al.*, 2008)
MLOC_79335	GT31	AT1G27120	Hydroxyproline-O-galactosyl transferase	5H	143.403	UP (1.94)	UP (1.48)	(Qu *et al.*, 2008; Basu *et al.*, 2015)
MLOC_16945	GT31	AT1G27120	Hydroxyproline-O-galactosyl transferase	1H	18.272	UP (0.78)	UP (0.91)	(Qu *et al.*, 2008; Basu *et al.*, 2015)

* The physical location of the candidate genes derived from different mapping populations: Morex x Barke POPSEQ 2013, Oregon Wolfe POPSEQ 2013 extracted from morexGenes - Barley RNA-seq Database of The James Hutton Institute, hai= hours after inoculation

Altered pathogen susceptibility following transiently induced gene silencing of candidate genes

To examine the potential roles of candidate genes for arabinoxylan biosynthesis, we conducted transiently induced gene silencing (TIGS) experiments (Schweizer *et al.*, 1999; Douchkov *et al.*, 2014), which allow the examination of the susceptibility of papillae in barley epidermal cells to penetration by *Bgh* when the candidate genes are down-regulated. In this experiment dsRNAi silencing constructs targeting each candidate gene were expressed in the barley leaf following biolistic bombardment. The bombarded cells were subsequently tested for their ability to produce papillae that were effective in preventing fungal penetration. The relative susceptibility index (RSI) was used to measure the ratio of successful fungal penetrations compared with the empty vector control. Down-regulation of seven candidate genes led to significantly increased susceptibility (Figure 3). The maximum shift in the susceptibility level was achieved by silencing a GT43 gene (MLOC_54026), which resulted in a RSI of 235%, followed by a GT61 (MLOC_6357) at 215%, another GT61 gene (MLOC_70966) at 204% and a GT8 gene (MLOC_65693) at 199% (Figure 3). Slightly increased penetration resistance was observed by silencing a GT47 gene (MLOC_15348). No significant difference was found in the RSI following silencing of the remaining candidate genes. As expected, the down-regulation of the control genes *HvCslD2* and *HvSNAP34* resulted in an increase in susceptibility and down-regulation of *HvMlo* also resulted in an increase in resistance, which is consistent with previous work (Douchkov *et al.*, 2014).

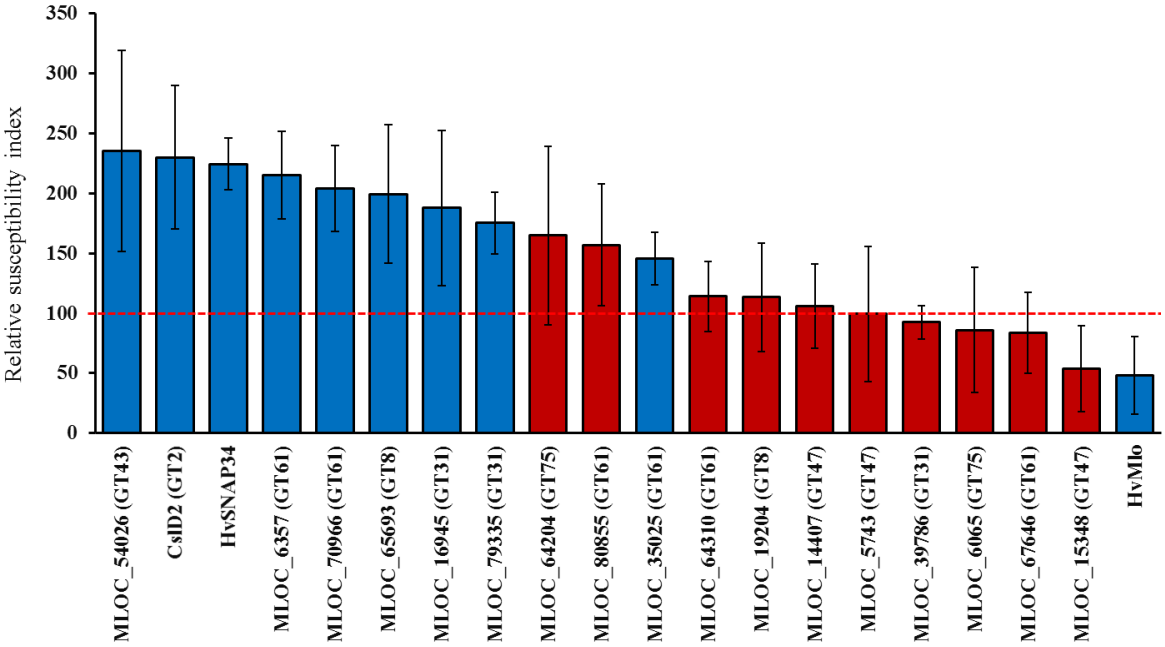

Figure 3. Transiently induced gene silencing of candidate genes and the changes in susceptibility to fungal penetration. The relative susceptibility index (RSI) was calculated from four biological replications and normalized against the empty vector control. Individual constructs with blue bars displayed a statistically significant ($p < 0.05$, Student's t-test) difference compared to the empty vector construct. Error bars indicate standard errors.

Altered pathogen susceptibility following transient over-expression of candidate genes

Transient over-expression of candidate genes was also performed to examine the effects of putatively-increased polysaccharide levels on penetration resistance. Transient over-expression of ten candidate genes led to significant decreases in the RSI compared with the empty vector (Figure 4). The maximum decrease in the RSI was observed by over-expressing a GT31 gene (MLOC_39786) followed by another GT31 (MLOC_70966) and a GT75 gene (MLOC_64204). The resulting RSI values were 46%, 52% and 66% of control levels, respectively. However, over-expression of three candidate genes, MLOC_67646 (GT61), MLOC_54026 (GT43) and MLOC_5743 (GT47), resulted in significant increases in RSI.

For a few candidate genes, down-regulation and up-regulation did not lead to reciprocal phenotypes, suggesting that the changes in RSI may be due to aberrant or off-target effects. However, these data might also suggest that these genes require ancillary factors that need to be co-expressed for normal activity. Silencing of the GT43 gene (MLOC_54026) resulted in the highest level of susceptibility (RSI of 235%), suggesting that this protein plays an integral role in papillae penetration resistance against the invading fungal pathogens. However, over-expression of this gene also resulted in the second highest observed increase in RSI. Given that any xylan synthase complex might contain both GT43 and GT47 family members, various combinations of GT43 and GT47 candidate genes were over-expressed to see if we could find any significant increase in penetration resistance. When the GT43 gene (MLOC_54026) was co-expressed with three individual members of the GT47 family, a significant increase in penetration resistance was observed in all cases. The most striking change was observed when co-expressing the two GT43 genes, MLOC_54026 and MLOC_5743, which individually led to increased susceptibility (RSI of 215% and 175% respectively), resulted in a significant increase in resistance (RSI of 55%) (Figure 5). The highest level of resistance (RSI of 41%) was observed when the GT43 (MLOC_54026) and GT47 (MLOC_14407) genes were co-expressed; this value is significantly lower than the RSI value of 72% observed for GT47

(MLOC_14407). These data suggest that interactions might be occurring between the two gene products and provide indirect evidence that the two genes (MLOC_54026; GT43 and MLOC_5743; GT47) might be partners in a xylan synthase complex and also that the GT47 gene (MLOC_14407) may interact with another GT43 expressed in epidermal cells.

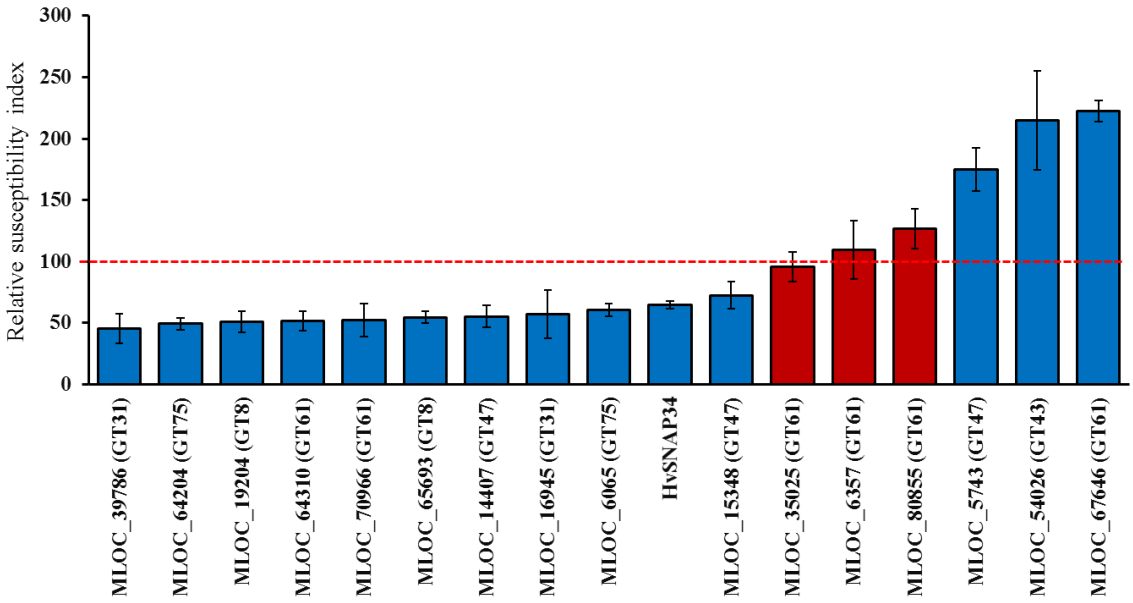

Figure 4. Transient over-expression of candidate genes and the changes in susceptibility to fungal penetration. The relative susceptibility index (RSI) was calculated from four biological replications and normalized against the empty vector control. Individual constructs with blue coloured bars displayed a statistically significant ($p < 0.05$, Student's t-test) difference in comparison to the empty vector control. Error bars show standard errors.

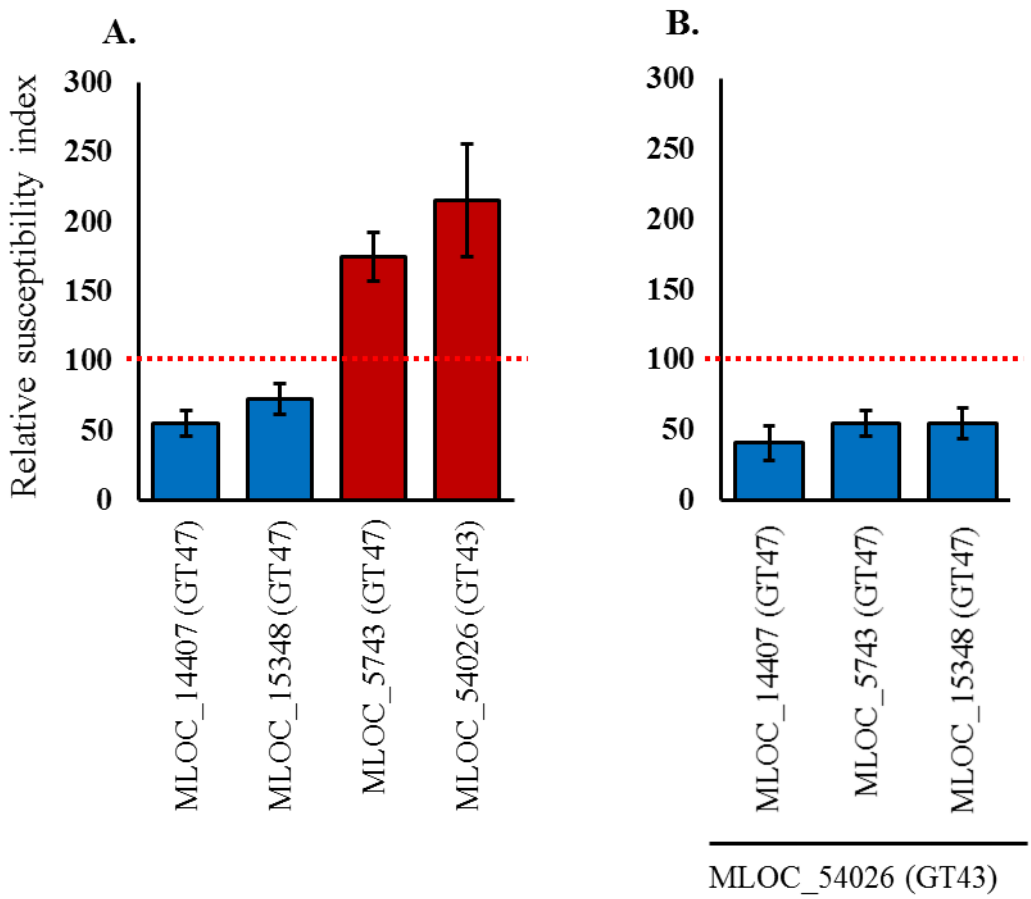

Figure 5. Effects of individual and combined expression of GT43 and GT47 members on host susceptibility level. The left hand panel shows the effects of the genes individually (A), and the right hand panel shows the effects of the three GT47 genes in the presence of the single GT43 gene (B). The relative susceptibility index (RSI) was calculated from four individual replicates and normalized against the empty vector control. Individual constructs with blue coloured bars displayed increased resistance and red coloured bars shows increased susceptibility at statistically significant ($p < 0.05$, Student's t-test) levels compared with empty vector constructs. Error bars indicate standard errors.

Discussion

The dynamics of arabinoxylan biosynthesis represents an area of untapped potential for studying cell wall associated plant defence mechanisms. Arabinoxylan is an important component of barley papillae and is associated with penetration resistance against powdery mildew pathogens (Chowdhury *et al.*, 2014). The precise structure of arabinoxylans in papillae is not known, because it is technically difficult to isolate individual papillae for chemical analysis. However, the arabinoxylans in cell walls of grasses and cereals has been shown to contain a (1,4)-β-xylan backbone and, depending upon the species and tissue type, the backbone is substituted to varying degrees with α-arabinofuranosyl (Ara*f*) residues, α-glucuronosyl residues (GlcA) and with feruloylated arabinofuranosyl residues (Carpita & Gibeaut, 1993; Ebringerová *et al.*, 2005; Burton & Fincher, 2012). Due to the inherent capability of feruloylated arabinoxylans to form covalent cross-links via their phenolic acid residues not only with other arabinoxylan molecules, but also with other wall polymers, one might expect that this cross-linking might lead to an increase in mechanical strength of the walls and that the presence of feruloylated arabinoxylans in barley papillae could be of great importance in plant resistance to fungal penetration.

To understand the role of arabinoxylan in papillae-based cell wall reinforcement, we examined candidate genes selected from various glycosyltransferase (GT) families that have been implicated in arabinoxylan biosynthesis (Rennie & Scheller, 2014). A number of candidate genes from arabinoxylan-associated GT families were significantly up-regulated in the barley epidermal layer during powdery mildew infection, at times corresponding to the period when arabinoxylan was being synthesised in the papillae (Figure 2). Similar expression profiles were observed during infection by the non-adapted pathogen *Bgt*, suggesting that these genes are somehow involved in basal plant defence mechanisms that are not the ultimate determinants of successful penetration or resistance outcomes.

In this study, silencing and over-expression of several GT43 and GT47 candidate genes resulted in susceptibility phenotypes that might be predicted from the recent discovery that arabinoxylans are present in barley papillae (Chowdhury *et al.*, 2014). However, for some candidate genes, the predicted reciprocal resistance and susceptibility phenotypes were not obtained by up-regulation and down-regulation of their expression levels. This may be due to functional redundancy within the GT families, off target effects from the dsRNAi constructs or the need for additional enzymes to be co-expressed for normal functionality. The GT43 gene (MLOC_54026) is probably the functional homolog of the *Arabidopsis IRX9-L* gene, which is believed to be involved in xylan backbone biosynthesis (Lee *et al.*, 2010; Mortimer *et al.*, 2015b). We have found that over-expression of this GT43 gene (MLOC_54026) in combination with members of the GT47 family leads to significant increases in resistance. The highest level of resistance was obtained from combined over-expression of the GT43 (MLOC_54026) and GT47 (MLOC_14407) genes, which suggests that these two gene family members may somehow co-operate during the synthesis of the xylan backbone in effective papillae. This result is supported by reports that GT43 and GT47 enzymes might co-exist in a single xylan synthase complex and might need to interact for effective arabinoxylan biosynthesis (Zeng *et al.*, 2010). Although the current data suggest that xylan might represent a significant barrier to fungal penetration during the plant's defence responses, the direct impacts on disease resistance and the precise molecular mechanisms that would explain the effects of silencing or over-expressing theGT43 and GT47 genes have not been defined. However, silencing of specific GT43 and GT47 gene family members can significantly improve wall digestibility by cell wall degrading enzymes in grasses and eudicots (Lee *et al.*, 2009; Petersen *et al.*, 2012). These results are consistent with the data presented here, which support the hypothesis that GT43 and GT47 enzymes, through their xylan biosynthesis activity, increase cell wall rigidity against enzymatic degradation by pathogens. It should also be pointed out that the synthesis of arabinoxylans with lower degrees of substitution and/or

the removal of some of the arabinosyl residues by α-arabinofuranosidases would be expected to facilitate intermolecular binding, as would an increase in ferulic acid cross-links. In both cases one might reasonably expect that the papillae would be 'toughened' and would be more resistant to fungal penetration.

At least three candidate genes produced the expected reciprocal phenotypes when their expression levels were up- and down-regulated. Among these three genes, the GT8 (MLOC_65693) and its putative homologs in *Arabidopsis* are shown to have glucuronosyltransferase activity (Rennie *et al.*, 2012) suggesting that they may be involved in adding glucuronyl residues to the xylan backbone (Figure 1). Members of the GT8 family have also been implicated in pectin biosynthesis (Mohnen, 2008), but levels of pectic polysaccharides in the walls of barley and other grasses are generally low. As noted above for arabinosyl substitution, removal of glucuronyl substituents could also alter intermolecular interactions and enhance the strength of the walls and the papillae in such a way that the attack on cell walls by pathogens is inhibited (Mortimer *et al.*, 2010). Although a direct relationship between glucuronyl residues on the xylan backbone and disease resistance has not been demonstrated, the absence of glucuronyl residues in xylan backbone achieved through double mutation of the GUX genes (GT8 members) results in weaker stem and easier cell wall digestibility with cell wall degrading enzymes, compared with wild types (Mortimer *et al.*, 2010). This result suggests that glucuronyl residues on arabinoxylans may be required for improving the overall strength of the wall against attack by limiting enzymatic digestibility by the pathogen.

The second candidate gene that generated the expected reciprocal phenotypes was MLOC_70996, which belongs to the GT61 family. The putative functions of this gene could not be predicted because it belongs to a monocotyledon-specific phylogenetic clade that consists largely of uncharacterised genes. However, several members of the GT61 gene family

are likely to encode arabinofuranosyl transferases, which add arabinosyl residues to the (1,4)-β-xylan backbone (Anders *et al.*, 2012). In grass species, the arabinosyl residues can be covalently linked to ferulic acid residues, which may undergo oxidative dimerization reactions and become covalently attached to adjacent feruloylated arabinoxylan chains (Burr & Fry, 2009; Marcia, 2009). Modified susceptibility levels achieved by altering gene expression levels may be attributable to differential cross-linking of arabinoxylan with other polysaccharides and phenolic compounds, since previous studies showed that in grasses, arabinosyl-ferulic acid crosslinking also improved resistance against pathogen fungi by limiting enzymatic digestibility of the modified wall (Bily *et al.*, 2003; Santiago & Malvar, 2010; Lionetti *et al.*, 2015).

The third candidate gene that provided the expected phenotypes during silencing and over-expression studies was the GT31 gene (MLOC_16945). Based on the phylogenetic relationship with *Arabidopsis* genes, MLOC_16945 may be a galactosyl transferase that is involved in adding galactosyl residues from UDP-galactose onto arabinogalactan proteins (AGP) (Nguema-Ona *et al.*, 2015). AGPs have been proposed to play several roles in plant defence mechanisms, including their participation in the formation of new cell walls at the site of symbiotic interactions and through cross-linking with other polymers to provide anchor points for lignification and creating an impermeable barrier for pathogens (Berry *et al.*, 2002; Shailasree *et al.*, 2004; Nguema-Ona *et al.*, 2015). To date, the presence of AGPs in barley papillae has not been tested, but AGPs have been reported in papillae of other species, namely cotton, poinsettia and pearl millet, which form during pathogen infection (Rodriguezgalvez & Mendgen, 1995; Celio *et al.*, 2004; Shailasree *et al.*, 2004). AGPs can be attached to the plasma membrane by GPI anchors (Oxley & Bacic, 1999; Nguema-Ona *et al.*, 2013) or may be cross-linked to arabinoxylan through galactosyl residues (Tan *et al.*, 2013). Arabinoxylan is present in the inner core extending towards the outer layer of the papillae (Chowdhury *et*

al., 2014). It is therefore possible that the arabinoxylan in the papilla could be cross-linked with AGPs to provide a more rigid structure connecting the papilla to the plasma membrane.

Apart from these three top candidates, GT75 members (MLOC_64204 and MLOC_6065) also deserve our attention. Although silencing of two GT75 members had no significant effect on pathogen susceptibility levels, over-expression of these two genes led to a significant increase in resistance. A recent study has shown that both MLOC_64204 and MLOC_6065 have UDP-arabinose mutase (UAM) activity and are able to convert UDP-L-arabinopyranose to UDP-L-arabinofuranose (Hsieh *et al.*, 2015). UDP-L-Arabinofuranose is the required form of substrate arabinose for arabinosyltransferases that attach arabinosyl residues to the xylan backbone. Given that these two genes encode enzymes that are presumed to perform the same enzymatic function, it is not unexpected that silencing single members of the family had no effect. Information on silencing mutase genes and the impact on disease resistance is lacking. However, a recent study shows that in *Brachypodium*, silencing of GT75 genes increases xylanase mediated digestibility of cell wall by two-fold and is related to a significant decrease in cell wall ferulic acid and *p*-coumaric acid concentrations (Rancour *et al.*, 2015).

The central goal of the current study was to identify candidate genes involved in arabinoxylan biosynthesis during powdery mildew infection that might have significant impacts on penetration resistance. The main challenge for characterising the role of individual genes involved in arabinoxylan biosynthesis in papillae is embodied by the presence of multiple genes with overlapping function within each GT family and the associated interpretative constraints. Other members of a particular family might potentially compensate in a biosynthetic process when the candidate gene is silenced. Thus, the effects of over-expression of a single gene might be masked by altered expression patterns of related genes

or of other genes that might need to be co-expressed to form a functional multi-enzyme complex.

Despite these challenges and limitations, our results suggest that at least one member of each of the gene families associated with arabinoxylan biosynthesis play vital roles in plant-pathogen resistance mechanisms. This is presumably related to modified arabinoxylan structures and/or altered levels of arabinoxylan accumulation in papillae, both of which might provide greater mechanical strength and resistance against the physical and enzymatic mechanisms of fungal penetration. Due to technical difficulties in labelling arabinoxylan with specific antibodies in the transiently transformed cells, a direct correlation of gene expression levels and arabinoxylan biosynthesis in papillae was not possible. In order to confirm this link, further experiments will be focused on demonstrating that there is a direct link between the candidate genes described here and the biosynthesis of arabinoxylan. Stable transgenic lines expressing the selected genes of interests will allow immunohistological characterisation of papillae and cell wall arabinoxylans, to dissect their role in plant disease resistance. Expression and purification of the candidate genes in heterologous systems could allow *in vitro* biochemical analyses to be performed, again to demonstrate the synthase activity of each candidate gene. Nevertheless, data generated in the present study is consistent with a role for arabinoxylan biosynthesis genes in plant pathogenesis and indicates that manipulation of the amounts and fine structure of papillary arabinoxylan has considerable potential for the development of disease resistance lines in the future.

Supporting information

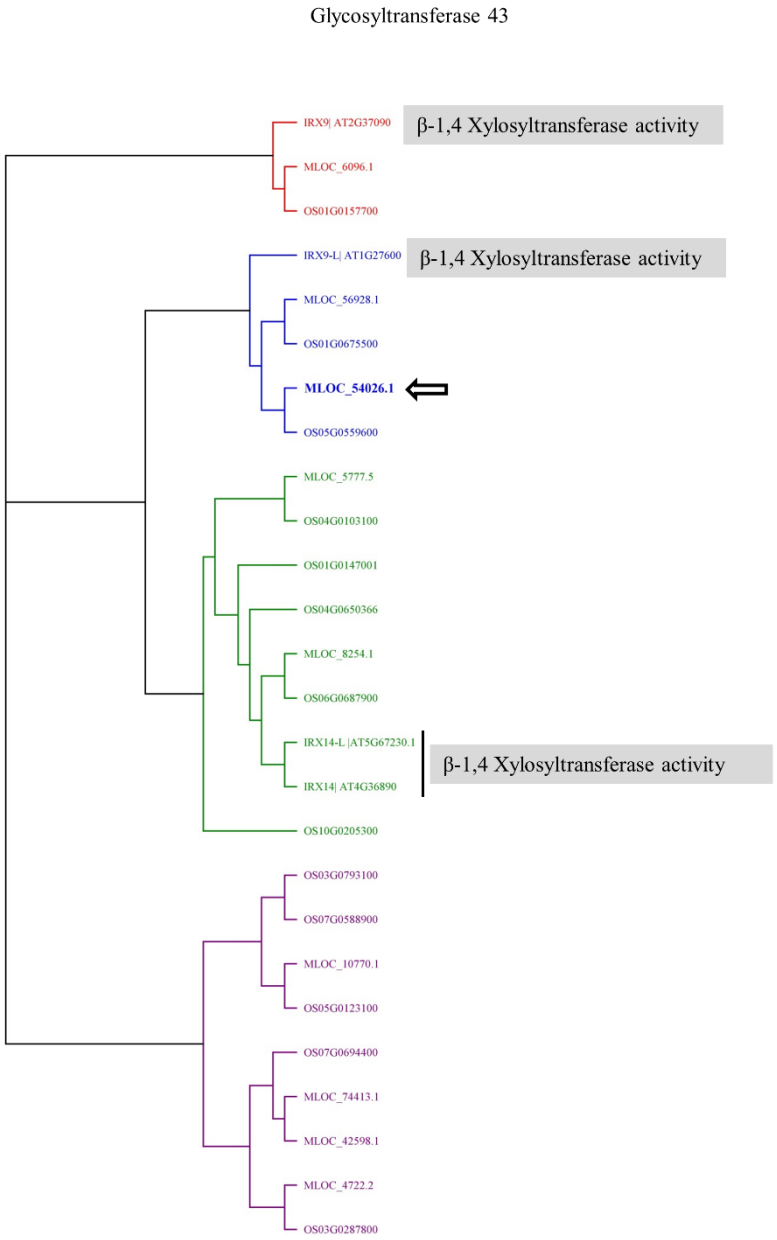

Figure S1.1. Phylogenetic relationship of GT43 members of *Arabidopsis*, barley and rice. Arrow indicates the selected candidate gene of barley.

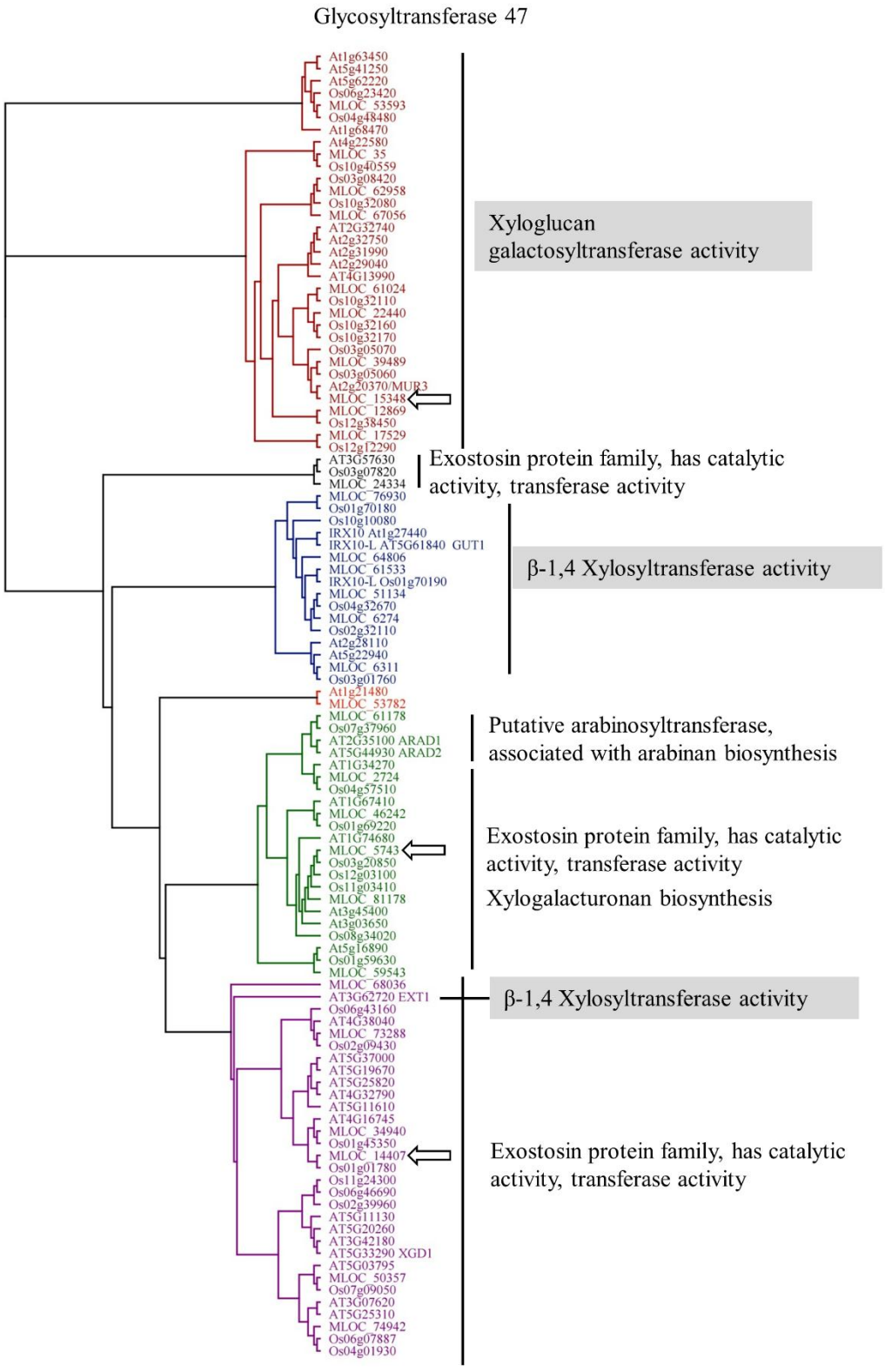

Figure S1.2. Phylogenetic relationship of GT47 family members of *Arabidopsis*, barley and rice. Arrows indicate the selected candidate genes of barley.

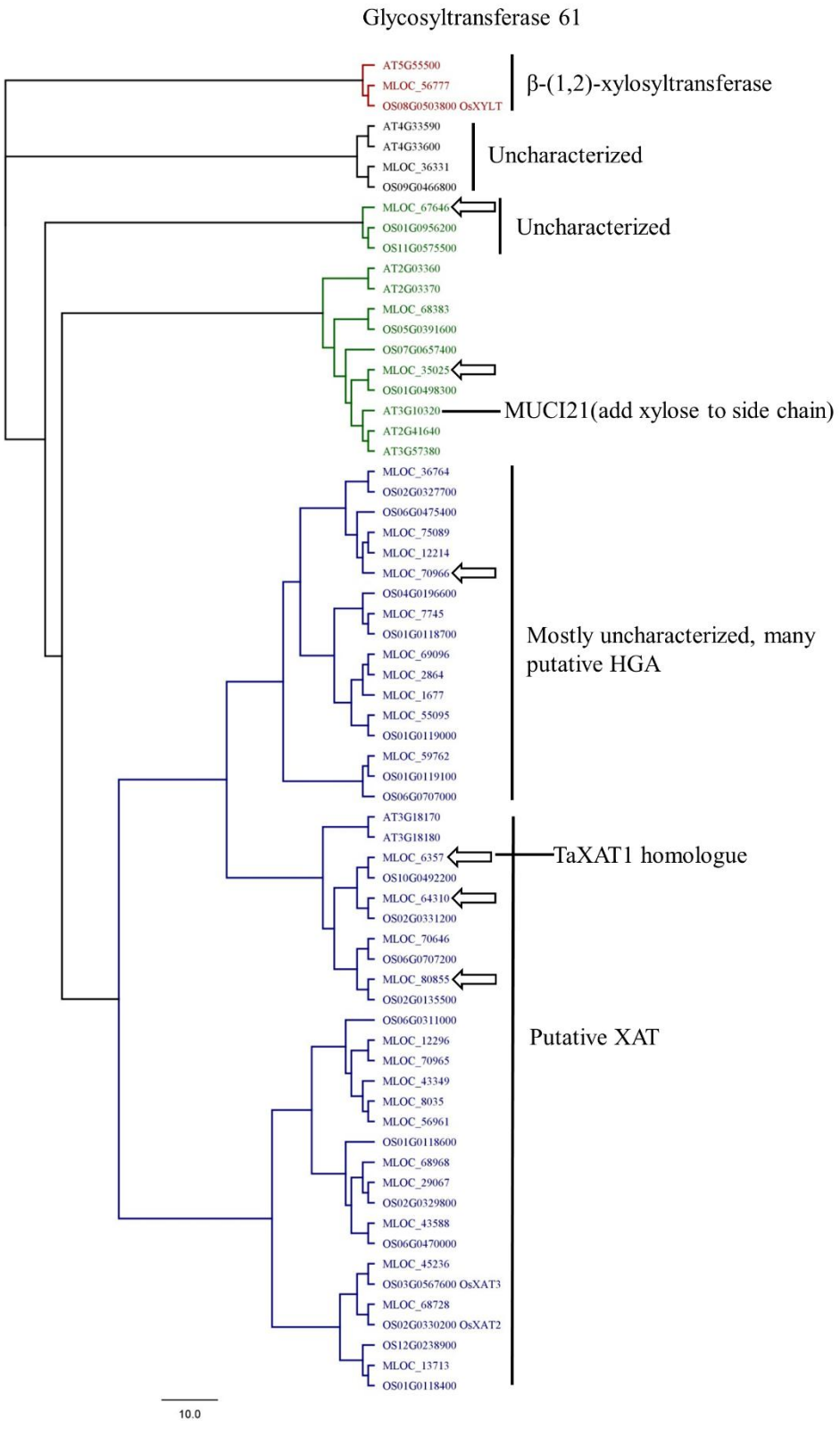

Figure S1.3. Phylogenetic relationship of GT61 family members of *Arabidopsis*, barley and rice. Arrows indicate the selected candidate genes of barley.

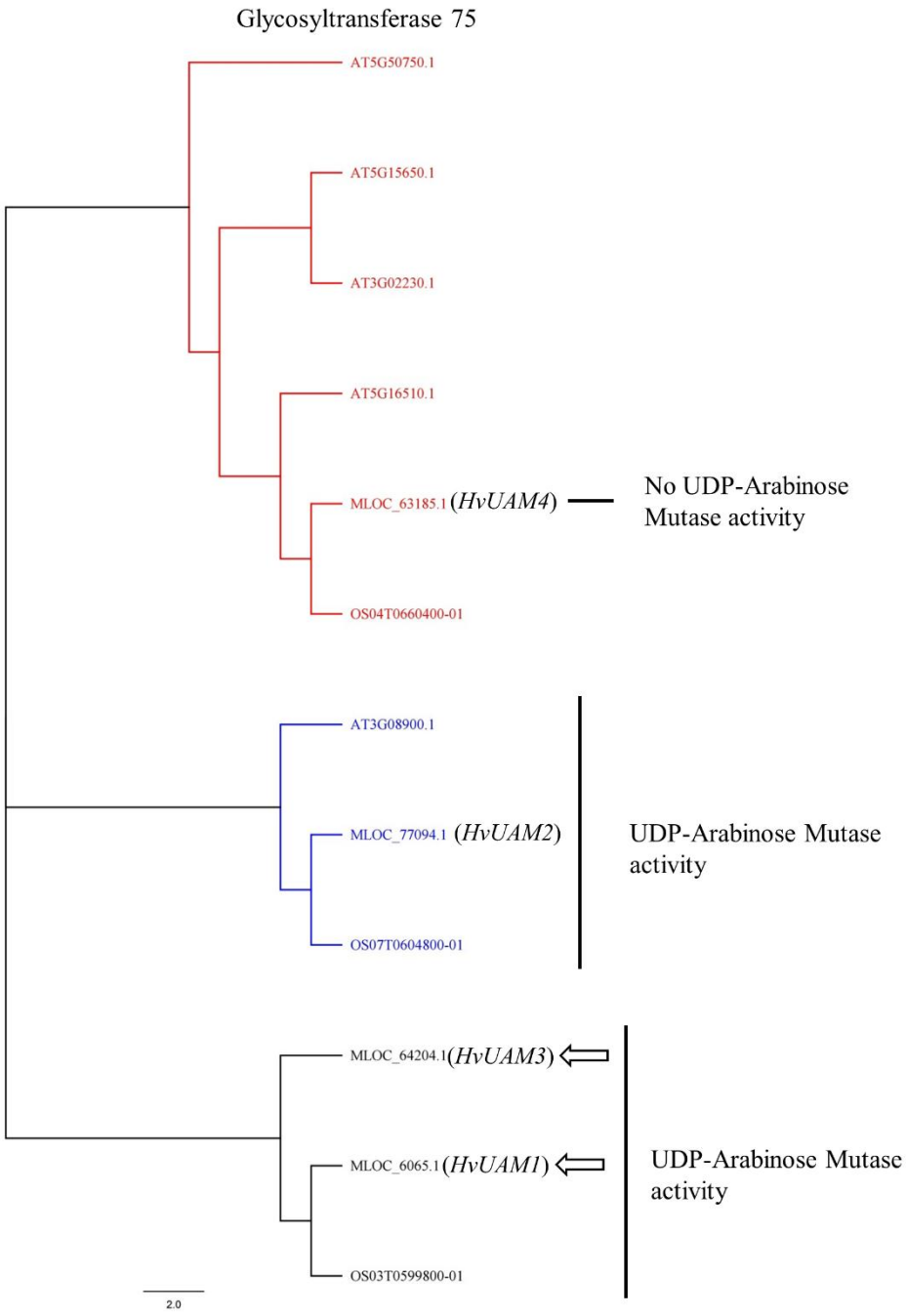

Figure S1.4. Phylogenetic relationship of GT75 family members of *Arabidopsis*, barley and rice. Arrows indicate the selected candidate genes of barley.

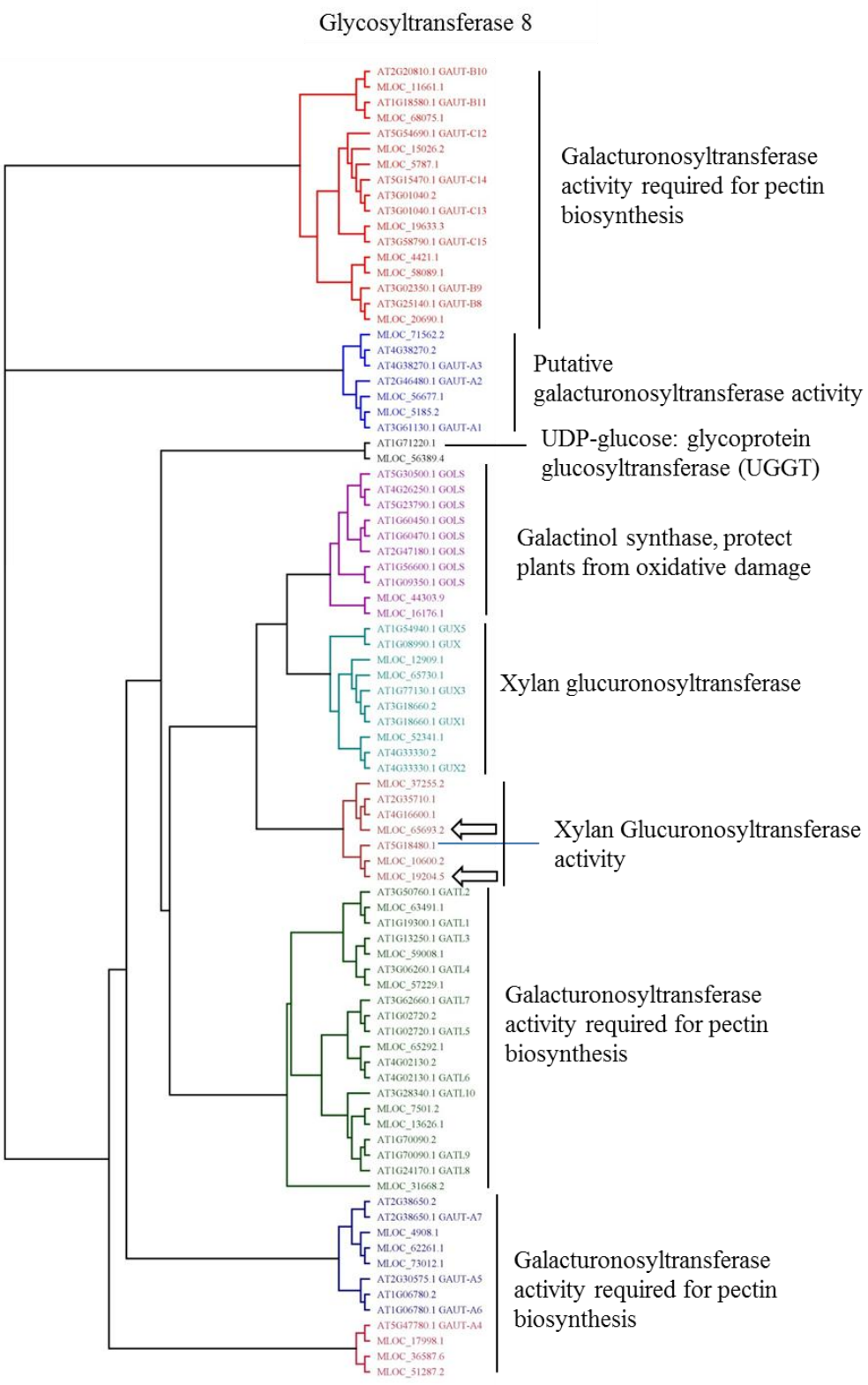

Figure S1.5. Phylogenetic relationship of GT8 family members of *Arabidopsis* and barley. Arrows indicate the selected candidate genes of barley.

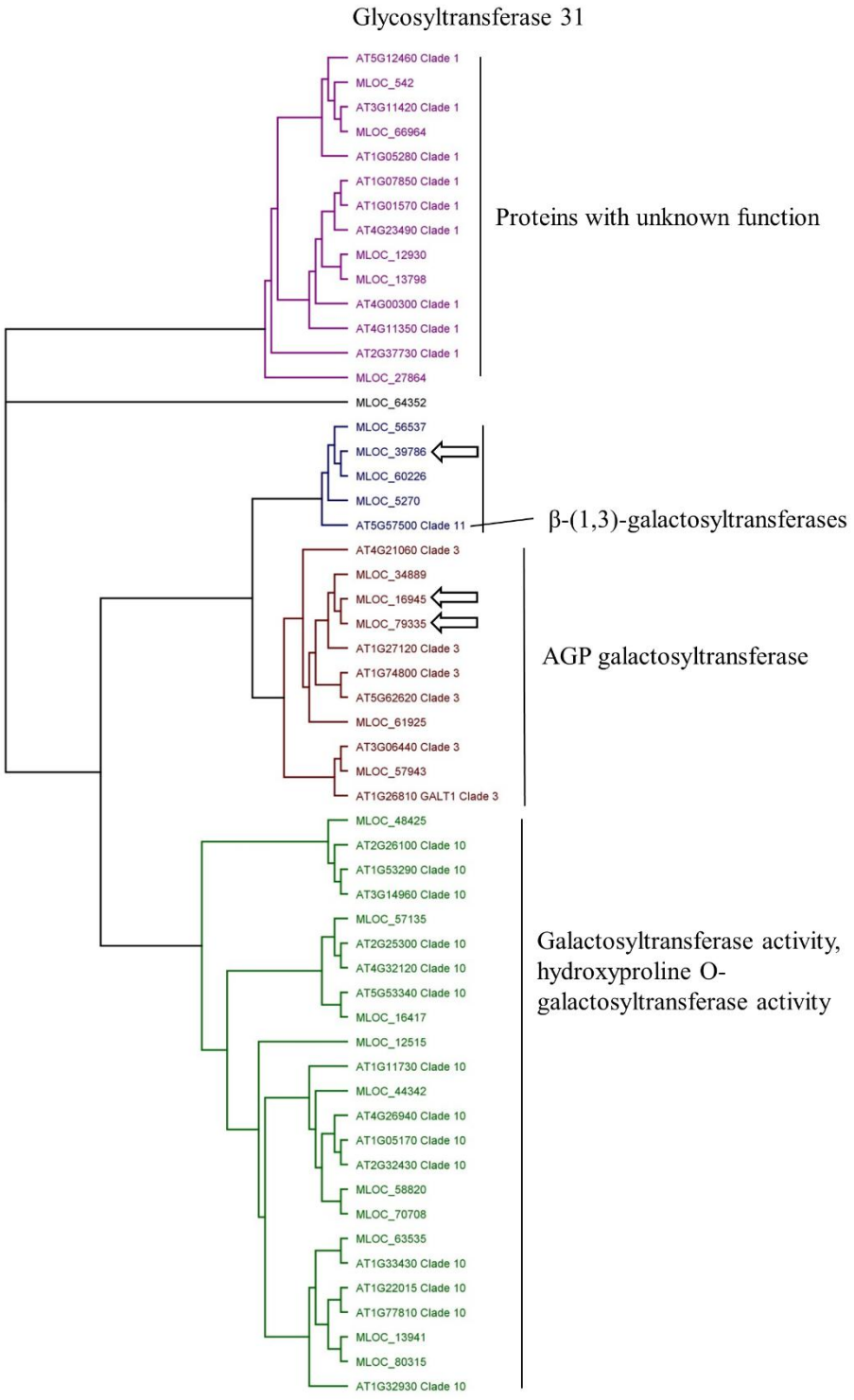

Figure S1.6. Phylogenetic relationship of GT31 family members of *Arabidopsis* and barley. Arrows indicate the selected candidate genes of barley.

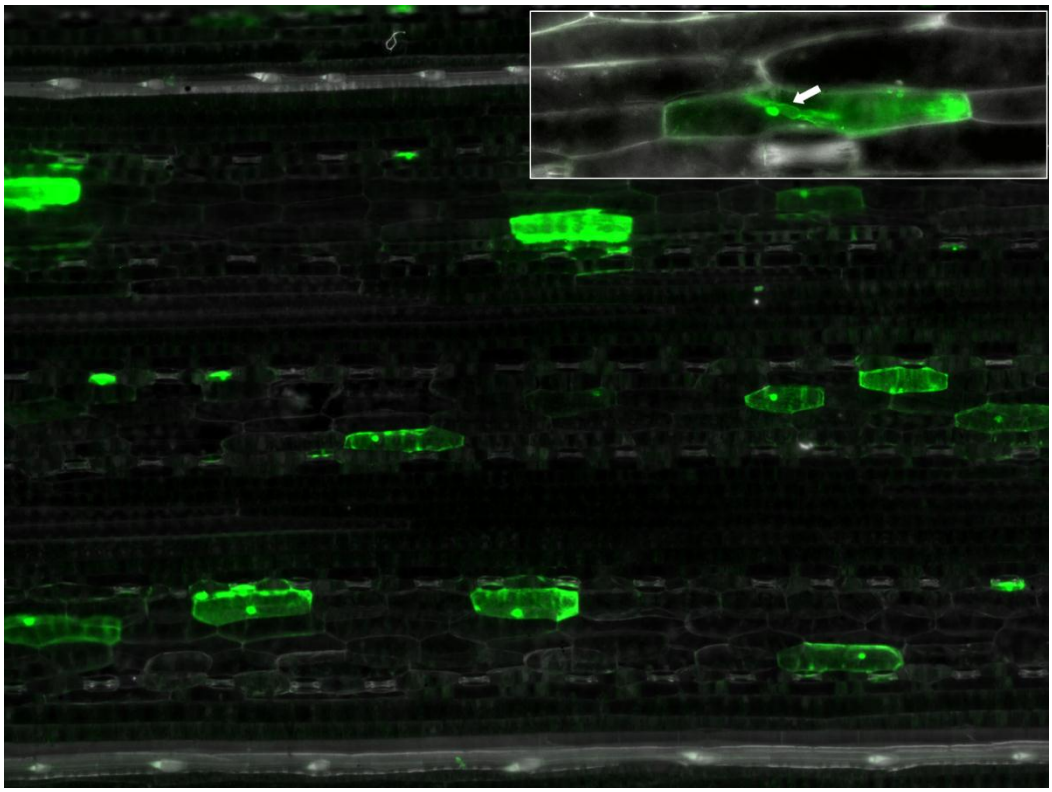

Figure S2. Transient gene expression in epidermal cells of plant leaves by Biolistic DNA Delivery. The transformed cells co-expressing GFP and the gene of interest can easily be seen by the presence of GFP fluorescence. Inset picture showing a transformed cell containing a fungal haustorium indicating a successful penetration event.

Table S1. Primers used to amplify the candidate genes for making the dsRNAi constructs used in Transiently Induced Gene Silencing experiments.

Sequence Name	Forward Primer	Reversed Primer
MLOC_5743	TAGCTCGAAAGGTGCCCTCA	TCTTGCTCCGAGAATGGAAA
MLOC_79335	GATGTGAGCATGGGCATGTG	GCCGGTATACAAGTAAGATCAGCAA
MLOC_19204	TTGGCTTGTAAAACCCGTGG	CGTGAAGGCAAAGGCAAGAG
MLOC_6065	CAAAGGCCTCATGCATCTCC	TGAGCTGACGGTCAAAAGCA
MLOC_39786	CATCCTGACGATGCCGAAG	TCCCAGGACATGACGTACCC
MLOC_65693	GCTACAAAGCGGATTGCTGG	TGGCTGCTTATACGATCCCG
MLOC_80855	TGACCAACATGGTGTTCCTCC	GTCAAGCAAAACGAGGCAGG
MLOC_6357	TATCCCCCGCTGATTACACG	GTCTCGTAGCACACCGGCTT
MLOC_67646	ATCGATCGAGCACCCTACCA	GCTACGAGAAGGCGACGAAC
MLOC_64310	ACAGCACCAACAGACGAGCA	GCAGCTCCACTACATCGGCT
MLOC_35025	CCGCATCCAACTCGCTCTT	CGGGTGTCGTTGGAGAAGTC
MLOC_70966	CGAGTGACCTGAGGCTGGAG	TCCTCTATTCCTCGGCCGTC
MLOC_64204	CAAGGAGAAGCTCGGCAAGA	CGGAAACATTAGCAGCCACA
MLOC_16945	ACCAACTGAGCCTGTCGAGC	AAGCCTCTTCTGGCCATTCC
MLOC_15348	TGTTTTCTCCAACGACGGCT	CATCTTCGTCTTTGGCAGGG
MLOC_14407	GCTCCAATCAAGAGCGTCGT	ACGCAGATGTTTCGGAAGGA
MLOC_54026	GGCTGCAAGAGAGTTGCGTT	GGCCATTCCACCACTAACCA

Table S2. Primers used to amplify the full coding region of the candidate genes used in transient over-expression experiments.

Sequence Name	Forward Primer	Reversed Primer
MLOC_5743	CCTCCACCCGCATTGCTA	CAGCTGGGATAGTTTGTTGGTC
MLOC_19204	ATCACAATAGCACCGGACAAG	CCTCGTGTAAAAATGTACAACAACA
MLOC_6065	CAGGGCTCGAGATCGAGAT	TCCAGGCCGTAACCATACACT
MLOC_39786	GCTTCCATTCCATCAAATCAAC	AGAACTAAAACAAGCGATCGATC
MLOC_65693	TAGCAGCGGCTTGTTGACC	CTGAATCAGCTGTCCATATGATGA
MLOC_80855	CAAGCAAGCAAGGGAGCTG	ATCTACCGAATCAGGGACGG
MLOC_6357	TGTGGAGGTCCTGTGCG	AAGCTCCCGCGATGATG
MLOC_67646	CGTCCATCCCGTTTCCATT	CCAACCAACCAAGCAGAGCTA
MLOC_64310	TAGCCGCACCCAGAGAGAG	CATGATCAGATAGGCCCGG
MLOC_35025	GCATCCCACTCCATCTGTCC	ACTGGGTATGGAGCATGAACAC
MLOC_70966	AGCAAGGCAGCAACGCAT	CAGTACGCAGAGGTTACGGTC
MLOC_64204	TCTGGGATCAGGGAGGAGAG	CACCGCCTGCTCAGTATGC
MLOC_16945	GAAACGGCTACTTCTCCTCGC	TCCTCTCGGACCAGACACAT
MLOC_15348	CTGCGGCTGCTGCAATG	GAGCAGCCAGTTGTGCG
MLOC_14407	AGCCCATTTCCAGTGGTTCT	CTCAAGGTGGAAGACGACTAGCT
MLOC_54026	CGACGAGAGAGCTTGAGAGAGT	CTGTGCTTCTTGTTGGGCC
HvSNAP34	GATCGATCTCGCCTCCGC	TAACAGCCCACGAAAGCAAATG

References

Anders N, Wilkinson MD, Lovegrove A, Freeman J, Tryfona T, Pellny TK, Weimar T, Mortimer JC, Stott K, Baker JM. 2012. Glycosyl transferases in family 61 mediate arabinofuranosyl transfer onto xylan in grasses. *Proceedings of the National Academy of Sciences* **109**(3): 989-993.

Andersson-Gunnerås S, Mellerowicz EJ, Love J, Segerman B, Ohmiya Y, Coutinho PM, Nilsson P, Henrissat B, Moritz T, Sundberg B. 2006. Biosynthesis of cellulose-enriched tension wood in Populus: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. *The Plant Journal* **45**(2): 144-165.

Basu D, Tian L, Wang W, Bobbs S, Herock H, Travers A, Showalter AM. 2015. A small multigene hydroxyproline-O-galactosyltransferase family functions in arabinogalactan-protein glycosylation, growth and development in Arabidopsis. *BMC Plant Biology* **15**(1): 1.

Berry AM, Rasmussen U, Bateman K, Huss-Danell K, Lindwall S, Bergman B. 2002. Arabinogalactan proteins are expressed at the symbiotic interface in root nodules of Alnus spp. *New Phytologist* **155**(3): 469-479.

Bily A, Reid L, Taylor J, Johnston D, Malouin C, Burt A, Bakan B, Regnault-Roger C, Pauls K, Arnason J. 2003. Dehydrodimers of ferulic acid in maize grain pericarp and aleurone: resistance factors to Fusarium graminearum. *Phytopathology* **93**(6): 712-719.

Boller T, Felix G 2009. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annual Review of Plant Biology*, 379-406.

Brown DM, Goubet F, Wong VW, Goodacre R, Stephens E, Dupree P, Turner SR. 2007. Comparison of five xylan synthesis mutants reveals new insight into the mechanisms of xylan synthesis. *The Plant Journal* **52**(6): 1154-1168.

Burr SJ, Fry SC. 2009. Feruloylated arabinoxylans are oxidatively cross-linked by extracellular maize peroxidase but not by horseradish peroxidase. *Molecular Plant* **2**(5): 883-892.

Burton RA, Fincher GB. 2012. Current challenges in cell wall biology in the cereals and grasses. *Front Plant Sci* **3**: 130.

Busse-Wicher M, Gomes TC, Tryfona T, Nikolovski N, Stott K, Grantham NJ, Bolam DN, Skaf MS, Dupree P. 2014. The pattern of xylan acetylation suggests xylan may

interact with cellulose microfibrils as a twofold helical screw in the secondary plant cell wall of Arabidopsis thaliana. *The Plant Journal* **79**(3): 492-506.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Research* **37**(suppl 1): D233-D238.

Carpita NC, Gibeaut DM. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **3**(1): 1-30.

Celio G, Mims C, Richardson E. 2004. Ultrastructure and immunocytochemistry of the host pathogen interface in poinsettia leaves infected with powdery mildew. *Canadian Journal of Botany* **82**(4): 421-429.

Chen X, Hedley PE, Morris J, Liu H, Niks RE, Waugh R. 2011. Combining genetical genomics and bulked segregant analysis-based differential expression: an approach to gene localization. *Theoretical and applied genetics* **122**(7): 1375-1383.

Chevalier L, Bernard S, Ramdani Y, Lamour R, Bardor M, Lerouge P, Follet-Gueye ML, Driouich A. 2010. Subcompartment localization of the side chain xyloglucan-synthesizing enzymes within Golgi stacks of tobacco suspension-cultured cells. *The Plant Journal* **64**(6): 977-989.

Chiniquy D, Sharma V, Schultink A, Baidoo EE, Rautengarten C, Cheng K, Carroll A, Ulvskov P, Harholt J, Keasling JD. 2012. XAX1 from glycosyltransferase family 61 mediates xylosyltransfer to rice xylan. *Proceedings of the National Academy of Sciences* **109**(42): 17117-17122.

Chowdhury J, Henderson M, Schweizer P, Burton RA, Fincher GB, Little A. 2014. Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated versus penetrated papillae on leaves of barley infected with Blumeria graminis f. sp. hordei. *New Phytologist* **204**(3): 650-660.

Deepak S, Shailasree S, Kini RK, Muck A, Mithöfer A, Shetty SH. 2010. Hydroxyproline-rich Glycoproteins and Plant Defence. *Journal of Phytopathology* **158**(9): 585-593.

Douchkov D, Lück S, Johrde A, Nowara D, Himmelbach A, Rajaraman J, Stein N, Sharma R, Kilian B, Schweizer P. 2014. Discovery of genes affecting resistance of barley to adapted and non-adapted powdery mildew fungi. *Genome Biology* **15**(12): 1-18.

Douchkov D, Nowara D, Zierold U, Schweizer P. 2005. A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells. *Molecular Plant-Microbe Interactions* **18**(8): 755-761.

Ebringerová A, Hromádková Z, Heinze T 2005. Hemicellulose. *Polysaccharides i*: Springer, 1-67.

Hsieh YS, Zhang Q, Yap K, Shirley NJ, Lahnstein J, Nelson CJ, Burton RA, Millar AH, Bulone V, Fincher GB. 2015. The Genetics, Transcriptional Profiles and Catalytic Properties of the UDP-Arabinose Mutase Family from Barley. *Biochemistry*.

Huckelhoven R. 2005. Powdery mildew susceptibility and biotrophic infection strategies. *Fems Microbiology Letters* **245**(1): 9-17.

Lee C, O'Neill MA, Tsumuraya Y, Darvill AG, Ye Z-H. 2007. The irregular xylem9 mutant is deficient in xylan xylosyltransferase activity. *Plant and Cell Physiology* **48**(11): 1624-1634.

Lee C, Teng Q, Huang W, Zhong R, Ye Z-H. 2009. Down-regulation of PoGT47C expression in poplar results in a reduced glucuronoxylan content and an increased wood digestibility by cellulase. *Plant and Cell Physiology* **50**(6): 1075-1089.

Lee C, Teng Q, Huang W, Zhong R, Ye Z-H. 2010. The Arabidopsis family GT43 glycosyltransferases form two functionally nonredundant groups essential for the elongation of glucuronoxylan backbone. *Plant Physiology* **153**(2): 526-541.

Li W, Guan Q, Wang Z-Y, Wang Y, Zhu J. 2013. A bi-functional xyloglucan galactosyltransferase is an indispensable salt stress tolerance determinant in Arabidopsis. *Molecular Plant* **6**(4): 1344-1354.

Lionetti V, Giancaspro A, Fabri E, Giove SL, Reem N, Zabotina OA, Blanco A, Gadaleta A, Bellincampi D. 2015. Cell wall traits as potential resources to improve resistance of durum wheat against Fusarium graminearum. *BMC Plant Biology* **15**(1): 1.

Malinovsky FG, Fangel JU, Willats WG. 2014. The role of the cell wall in plant immunity. *Plant cell wall in pathogenesis, parasitism and symbiosis* **5**: 38-49.

Marcia MdO. 2009. Feruloylation in grasses: current and future perspectives. *Molecular Plant* **2**(5): 861-872.

McCartney L, Marcus SE, Knox JP. 2005. Monoclonal antibodies to plant cell wall xylans and arabinoxylans. *Journal of Histochemistry & Cytochemistry* **53**(4): 543-546.

Mohnen D. 2008. Pectin structure and biosynthesis. *Current Opinion in Plant Biology* **11**(3): 266-277.

Mortimer JC, Faria-Blanc N, Yu X, Tryfona T, Sorieul M, Ng YZ, Zhang Z, Stott K, Anders N, Dupree P. 2015a. An unusual xylan in Arabidopsis primary cell walls is synthesised by GUX3, IRX9L, IRX10L and IRX14. *The Plant Journal* **83**(3): 413-426.

Mortimer JC, Faria-Blanc N, Yu X, Tryfona T, Sorieul M, Ng YZ, Zhang Z, Stott K, Anders N, Dupree P. 2015b. An unusual xylan in Arabidopsis primary cell walls is synthesised by GUX3, IRX9L, IRX10L and IRX14. *The Plant Journal* **83**(3): 413-426.

Mortimer JC, Miles GP, Brown DM, Zhang Z, Segura MP, Weimar T, Yu X, Seffen KA, Stephens E, Turner SR. 2010. Absence of branches from xylan in Arabidopsis gux mutants reveals potential for simplification of lignocellulosic biomass. *Proceedings of the National Academy of Sciences* **107**(40): 17409-17414.

Nguema-Ona E, Vicré-Gibouin M, Cannesan M-A, Driouich A. 2013. Arabinogalactan proteins in root–microbe interactions. *Trends in Plant Science* **18**(8): 440-449.

Nguema-Ona E, Vicré-Gibouin M, Gotté M, Plancot B, Lerouge P, Bardor M, Driouich A. 2015. Cell wall O-glycoproteins and N-glycoproteins: aspects of biosynthesis and function. *Plant Glycobiology–a sweet world of lectins, glycoproteins, glycolipids and glycans* **5**(499): 8.

Nielsen K, Olsen O, Oliver R. 1999. A transient expression system to assay putative antifungal genes on powdery mildew infected barley leaves. *Physiological and Molecular Plant Pathology* **54**(1): 1-12.

Oxley D, Bacic A. 1999. Structure of the glycosylphosphatidylinositol anchor of an arabinogalactan protein from Pyrus communis suspension-cultured cells. *Proceedings of the National Academy of Sciences* **96**(25): 14246-14251.

Panstruga R. 2004. A golden shot: how ballistic single cell transformation boosts the molecular analysis of cereal-mildew interactions. *Molecular Plant Pathology* **5**(2): 141-148.

Passardi F, Penel C, Dunand C. 2004. Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends in Plant Science* **9**(11): 534-540.

Petersen PD, Lau J, Ebert B, Yang F, Verhertbruggen Y, Kim JS, Varanasi P, Suttangkakul A, Auer M, Loqué D. 2012. Engineering of plants with improved properties as biofuels feedstocks by vessel-specific complementation of xylan biosynthesis mutants. *Biotechnology for biofuels* **5**(1): 1.

Qu Y, Egelund J, Gilson PR, Houghton F, Gleeson PA, Schultz CJ, Bacic A. 2008. Identification of a novel group of putative Arabidopsis thaliana β-(1, 3)-galactosyltransferases. *Plant Molecular Biology* **68**(1-2): 43-59.

Rancour DM, Hatfield RD, Marita JM, Rohr NA, Schmitz RJ. 2015. Cell wall composition and digestibility alterations in Brachypodium distachyon achieved

through reduced expression of the UDP-arabinopyranose mutase. *Frontiers in plant science* **6**.

Rautengarten C, Ebert B, Herter T, Petzold CJ, Ishii T, Mukhopadhyay A, Usadel B, Scheller HV. 2011. The interconversion of UDP-arabinopyranose and UDP-arabinofuranose is indispensable for plant development in Arabidopsis. *The Plant Cell* **23**(4): 1373-1390.

Rennie EA, Ebert B, Miles GP, Cahoon RE, Christiansen KM, Stonebloom S, Khatab H, Twell D, Petzold CJ, Adams PD. 2014. Identification of a sphingolipid α-glucuronosyltransferase that is essential for pollen function in Arabidopsis. *The Plant Cell* **26**(8): 3314-3325.

Rennie EA, Hansen SF, Baidoo EE, Hadi MZ, Keasling JD, Scheller HV. 2012. Three members of the Arabidopsis glycosyltransferase family 8 are xylan glucuronosyltransferases. *Plant Physiology* **159**(4): 1408-1417.

Rennie EA, Scheller HV. 2014. Xylan biosynthesis. *Current Opinion in Biotechnology* **26**: 100-107.

Rodriguezgalvez E, Mendgen K. 1995. Cell-wall synthesis in cotton roots after infection with *Fusarium-oxysporum*. *Planta* **197**(3): 535-545.

Sainsbury F, Thuenemann EC, Lomonossoff GP. 2009. pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnology Journal* **7**(7): 682-693.

Santiago R, Malvar RA. 2010. Role of dehydrodiferulates in maize resistance to pests and diseases. *International journal of molecular sciences* **11**(2): 691-703.

Schweizer P, Christoffel A, Dudler R. 1999. Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance. *The Plant Journal* **20**(5): 541-552.

Shailasree S, Kini KR, Deepak S, Kumudini B, Shetty HS. 2004. Accumulation of hydroxyproline-rich glycoproteins in pearl millet seedlings in response to Sclerospora graminicola infection. *Plant Science* **167**(6): 1227-1234.

Stone BA, Clarke AE. 1992. *Chemistry and Biology of (1→3)-β-D-Glucans*. Victoria, Australia: La Trobe University Press.

Tan L, Eberhard S, Pattathil S, Warder C, Glushka J, Yuan C, Hao Z, Zhu X, Avci U, Miller JS. 2013. An Arabidopsis cell wall proteoglycan consists of pectin and arabinoxylan covalently linked to an arabinogalactan protein. *The Plant Cell* **25**(1): 270-287.

Voiniciuc C, Günl M, Schmidt MH-W, Usadel B. 2015. Highly Branched Xylan Made by IRREGULAR XYLEM14 and MUCILAGE-RELATED21 Links Mucilage to Arabidopsis Seeds. *Plant Physiology* **169**(4): 2481-2495.

Wang Y, Zhang W-Z, Song L-F, Zou J-J, Su Z, Wu W-H. 2008. Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in Arabidopsis. *Plant Physiology* **148**(3): 1201-1211.

Wu AM, Rihouey C, Seveno M, Hörnblad E, Singh SK, Matsunaga T, Ishii T, Lerouge P, Marchant A. 2009. The Arabidopsis IRX10 and IRX10-LIKE glycosyltransferases are critical for glucuronoxylan biosynthesis during secondary cell wall formation. *The Plant Journal* **57**(4): 718-731.

Zellerhoff N, Himmelbach A, Dong W, Bieri S, Schaffrath U, Schweizer P. 2010. Nonhost resistance of barley to different fungal pathogens is associated with largely distinct, quantitative transcriptional responses. *Plant Physiology* **152**(4): 2053-2066.

Zeng W, Jiang N, Nadella R, Killen TL, Nadella V, Faik A. 2010. A glucurono (arabino) xylan synthase complex from wheat contains members of the GT43, GT47, and GT75 families and functions cooperatively. *Plant Physiology* **154**(1): 78-97.

Zeyen RJ, Carver TLW, Lyngkjaer MF. 2002. Epidermal cell papillae. *Powdery Mildews: A Comprehensive Treatise*: 107-125.

CHAPTER 6

Microscopic analysis of papillary cellulose in *Cellulose Synthase-like D2* (*HvCslD2*) dsRNAi Transgenic barley lines

Link to chapter 6

In Chapter 6 experiments are described that contribute to the RNR6 project, which was led by Dr. Patrick Schweizer, IPK, Germany. The main aim of the RNR6 project was to define the role of the *HvCslD2* gene in host and non-host resistance mechanisms of barley plants. My contribution to this project was to conduct a microscopic analysis of the papillae in *HvCslD2*_RNAi transgenic lines in order to determine whether *HvCslD2* is involved in cellulose accumulation in barley papillae. All data from the RNR6 project, including my own contribution, have been submitted for publication in the New Phytologist journal (Appendix A).

Summary

A recent study by Dr. Patrick Schweizer and his co-workers in IPK, Germany, showed that the transient down-regulation of the *HvCslD2* gene in barley resulted in increased susceptibility to both adapted and non-adapted powdery mildew pathogens (Douchkov *et al.*, 2014). This suggested that *HvCslD2* is an important factor in non-host and quantitative host resistance. New experiments, again led by Dr. Patrick Schweizer, were undertaken to further characterise the role of the plant cell wall in *HvCslD2*-associated susceptibility, using *HvCslD2* dsRNAi stable transgenic barley lines. In this project, my contribution was to evaluate if *HvCslD2* silencing in the transgenic lines led to altered levels of cellulose accumulation in the papillae that developed during powdery mildew infection. Fluorescence microscopy, in conjunction with specific cellulose-binding probes, were used for the evaluation.

A cellulose-specific probe was used to show that cellulose accumulation is significantly lower in the papillae that form during powdery mildew infection of the transgenic lines, compared with wild type barley. Furthermore, there was no significant variation in the phenolic-associated autofluorescence in papillae of the transgenic lines. Taken together, the microscope analysis suggests that the *HvCslD2*-associated penetration resistance works independently from the local accumulation of phenolic materials.

Project background

Cellulose is a major component of primary and secondary cell walls in land plants. It serves multiple functions in plant growth and development, the most obvious being to provide strength (Delmer & Amor, 1995). The presence of cellulose in the outer layer of barley papillae that develop during pathogen infection has recently been reported (Chowdhury *et al.*, 2014). The study showed that within the same barley line, the level of cellulose accumulation is higher in non-penetrated papillae compared with penetrated papillae, suggesting that host plants need to accumulate a certain level of cellulose in the papillae in order to achieve penetration resistance against fungal pathogens. To date, members of the cellulose synthase (*CesA)* family have been linked to the biosynthesis of cellulose in primary and secondary cell walls while several members of the cellulose synthase-like (*Csl)* families have been shown to be responsible for non-cellulosic polysaccharide biosynthesis (Dhugga *et al.*, 2004; Burton *et al.*, 2006). It has been shown that during infection by the host pathogen *B. graminis* f. sp. *hordei* (*Bgh*) and the non-host pathogen *B. graminis* f. sp. *tritici* (*Bgt*) none of the *CesA* members were up-regulated. However, transcripts of the cellulose synthase-like gene, *HvCslD2,* rose significantly (Douchkov *et al.*, 2014) and other studies suggest that selected members of the *CslD* gene family might also be capable of cellulose synthesis in the tip of growing cells (Doblin *et al.*, 2001; Park *et al.*, 2011).

Dr. Patrick Schweizer and his co-workers in IPK, Germany, showed that *HvCslD2* is an important factor in non-host as well as quantitative host resistance, since transient dsRNAi mediated silencing of the gene led to increased susceptibility to the non-adapted pathogen *Blumeria graminis* f. sp. *tritici* (*Bgt*) and susceptibility to an adapted pathogen, *Blumeria graminis* f. sp. *hordei* (*Bgh*) (Douchkov *et al.*, 2014). It was hypothesised that *HvCslD2* is involved in a host and non-host penetration resistance mechanism, possibly via the deposition of cellulose during infection. To further characterise the link between the *HvCslD2* gene and non-host and host penetration resistance, several *HvCslD2* RNAi stable transgenic lines were

developed at the IPK in Germany. In this investigation, my contribution involved the use of fluorescence microscopy to evaluate the cellulose content in papillae deposited in epidermal cells of the transgenic barley lines.

Materials and Methods

Plant and pathogen growth conditions and Bgh inoculation

As already described in Chapter 3, a Western Australian *Bgh* field isolate was collected from Professor Richard Oliver (The Australian Centre for Necrotrophic Fungal Pathogens, Curtin University, WA, Australia). Fungal spore production was maintained on a susceptible barley cultivar, 'Baudin' in a growth chamber at 21°C with a 16 hour photoperiod. The *HvCslD2*_RNAi stable transgenic lines E39 and E28 along with the wild type 'Golden Promise' line were also grown in a growth chamber maintained at 21°C with a 16 hour photoperiod. *Bgh* spore inoculation was carried out on the adaxial side of ten-day-old leaves following the procedure described previously (Chowdhury *et al.*, 2014).

Whole-mount tissue processing and fluorescence microscopy

Papillary cellulose was labelled with a specific probe, Pontamine Fast Scarlet 4B (PFS4B) in the whole-mount tissue following the protocol described in Chapter 3 with few modifications. In brief, eighteen hours after inoculation, leaves were cut into 3-cm pieces and decolorized in boiling ethanol (96% v/v) for 10 minutes in a water bath. The tissues were clarified overnight in saturated chloral hydrate solution (500g in 250 ml water), rinsed with Milli-Q water and washed in phosphate-buffer saline (PBS, pH7.4). Fungal structures were identified by staining with wheat germ agglutinin (WGA) conjugated with Alexa Fluor 488 (Life Technologies, Carlsbad, CA, USA), which selectively binds to chitin residues of the fungal wall (Wright, 1984). The local accumulation of cellulose in papillae was labelled with a 0.01% solution of PFS4B (Aldrich Rare Chemicals Library, St Louis, MO, USA, catalogue no. S479896) in PBS for two hours (Anderson *et al.*, 2010). Following the incubation, leaves were briefly washed with PBS and examined under a fluorescence microscope (Axio Imager M2; Carl Zeiss, Oberkochen, Germany) pre-aligned with a mercury lamp system. The PFS4B fluorescence signals were observed under the dsRED filter set at 545/25 nm excitation and 605/70 nm emission wavelengths. Autofluorescence was detected using a DAPI filter set at

365 nm excitation and 445/50 nm emission wavelengths. For quantification of fluorescence for each of the tested lines, intensity profiles of at least 50 papillae from each leaf and 5 leaves from each line were analysed using the procedure described previously (Chowdhury *et al.*, 2014).

Results and Discussion

The local accumulation of cellulose in papillae underlying fungal appressoria in epidermal cells was examined by staining with the cellulose-specific probe 'Pontamine Fast Scarlet 4B (PFS4B)'(Anderson *et al.*, 2010). The fluorescence intensity profile shows that the PFS4B labelling was significantly lower in *HvCslD2_dsRNAi* transgenic lines (E28 and E39) compared with wild type controls, which suggested a significant reduction in cellulose accumulation in these papillae (Figure 1). However, no significant differences were observed for local phenolic-associated autofluorescence in the papillae of the *HvCslD2_dsRNAi* transgenic lines compared to wild type (Figure 1). Thus, it appears likely that the function of *HvCslD2* in cell wall-based penetration resistance is independent from the accumulation of autofluorescent materials, such as the esterified phenolic acids, ferulic and *p*-coumaric acid, that are associated with wall arabinoxylans.

Thus, the increased susceptibility of the *HvCslD2*-silenced lines is associated with a reduced level of cellulose in the epidermal layer overall, which is consistent with the enhanced enzymatic digestibility of the cell walls and the reduced level of cellulose accumulation in the papillae (Figure 3 of Appendix A). High levels of autofluorescence associated with phenolic compounds were still present, but these were insufficient to stop early fungal penetration (Figure 4 of Appendix A) . The study suggests that the Hv*CslD2* gene provides a defence-related function that might be specific to barley and possibly other members of the *Poaceae* family. The fact that, at least in barley, *CslD2* is co-localizing with an important meta-QTL for disease resistance (Douchkov *et al.* 2014) opens up an opportunity to examine and exploit allelic diversity for improved yield security of the major cereal crops.

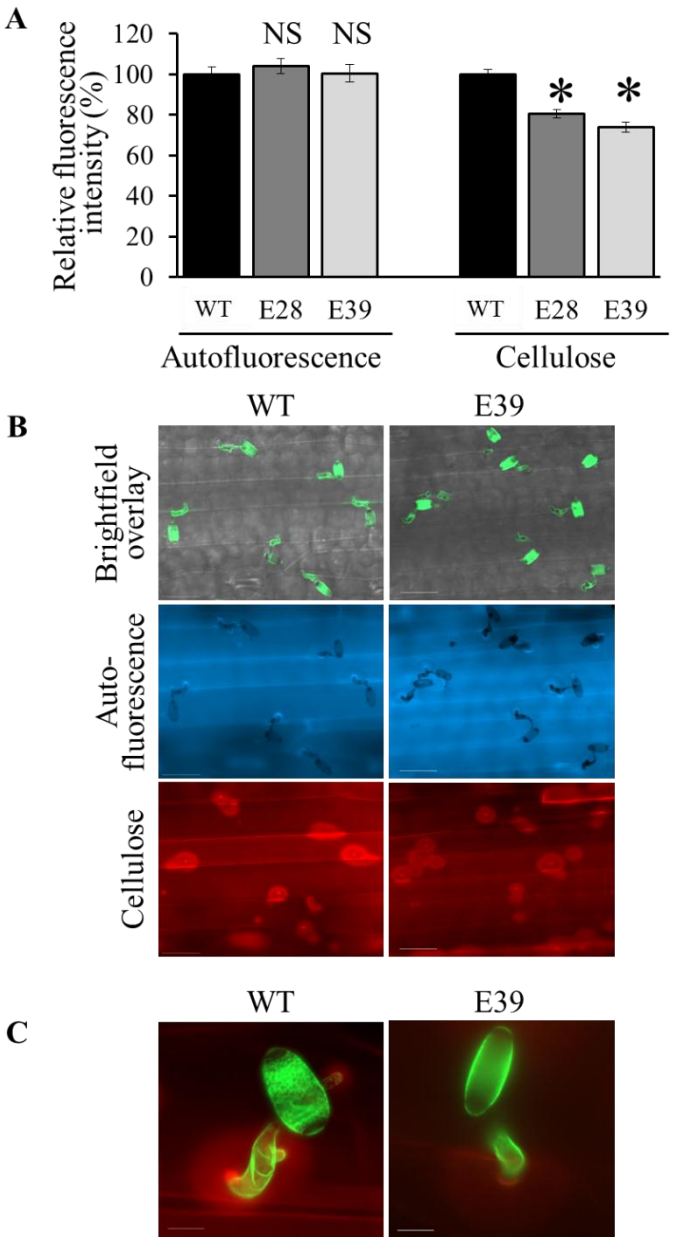

Figure 1. Reduced cellulose accumulation in the papillae of Hv*CslD2*-silenced transgenic plants. **A.** Relative fluorescence intensity of cellulose and autofluorescence in epidermal papillae formed against *B. graminis* f. sp. *hordei* at 18 hai on leaves from wildtype (wt) plants and transgenic T3 plants of E28 and E39 lines. The average maximum intensity was calculated using the maximum intensity found in the fluorescence intensity profile of 250 papillae and normalized against background tissue staining ($P < 0.05$, student T-test). In order to directly compare the different probes in one graph, the relative fluorescence intensity was calculated as a percentage of the highest maximum found for each probe. Data are Mean ± SEM

B. Labelling of cellulose and autofluorescence in epidermal papillae formed against *B. graminis* f. sp. *hordei* at 18 hai on leaves from wildtype (WT) plants and transgenic T3 plants of the E39 line. The first row shows a bright field overlay and fungal tissues are labelled with wheat germ agglutinin (WGA-AF488) (green). The second row shows autofluorescence (blue) and the third row shows cellulose labelling with Pontamine Fast Scarlet 4B (red). Scale bar = 50 µm. **C.** Labelling of cellulose (red) and fungal tissues (green) in epidermal papillae formed against *B. graminis* f. sp. *hordei* at 18 hai on leaves from wildtype (WT) plants and transgenic T3 plants of the E39 line. Scale bar = 10 µm.

References

Anderson CT, Carroll A, Akhmetova L, Somerville C. 2010. Real-Time Imaging of Cellulose Reorientation during Cell Wall Expansion in Arabidopsis Roots. *Plant Physiology* **152**(2): 787-796.

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB. 2006. Cellulose Synthase-Like CslF Genes Mediate the Synthesis of Cell Wall (1,3;1,4)-ß-d-Glucans. *Science* **311**(5769): 1940-1942.

Chowdhury J, Henderson M, Schweizer P, Burton RA, Fincher GB, Little A. 2014. Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated versus penetrated papillae on leaves of barley infected with Blumeria graminis f. sp. hordei. *New Phytologist* **204**(3): 650-660.

Delmer DP, Amor Y. 1995. Cellulose biosynthesis. *The Plant Cell* **7**(7): 987.

Dhugga KS, Barreiro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, Dolan M, Kinney AJ, Tomes D, Nichols S, Anderson P. 2004. Guar seed beta-mannan synthase is a member of the cellulose synthase super gene family. *Science* **303**(5656): 363-366.

Doblin MS, De Melis L, Newbigin E, Bacic A, Read SM. 2001. Pollen tubes of Nicotiana alata express two genes from different β-glucan synthase families. *Plant Physiology* **125**(4): 2040-2052.

Douchkov D, Lück S, Johrde A, Nowara D, Himmelbach A, Rajaraman J, Stein N, Sharma R, Kilian B, Schweizer P. 2014. Discovery of genes affecting resistance of barley to adapted and non-adapted powdery mildew fungi. *Genome Biology* **15**(12): 1-18.

Park S, Szumlanski AL, Gu F, Guo F, Nielsen E. 2011. A role for CSLD3 during cell-wall synthesis in apical plasma membranes of tip-growing root-hair cells. *Nature cell biology* **13**(8): 973-980.

Wright CS. 1984. Structural comparison of the two distinct sugar binding sites in wheat germ agglutinin isolectin II. *Journal of Molecular Biology* **178**(1): 91-104.

CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

Understanding polysaccharide content and roles in papillae

Papillae formation at infection sites is an important aspect of a plant's pre-invasion resistance mechanism, especially against pathogens that employ biotrophic infection strategies (Bellincampi *et al.*, 2014). However, the actual function of papillae may be complex and experimentally hard to prove. A current hypothesis is that this induced cell wall modification provides both a physical and chemical barrier to halt fungal invasion or sometimes to delay the penetration process for long enough to allow other plant defence mechanisms to be initiated (Huckelhoven, 2005).

In work described in Chapter 3, the objective was to identify the polysaccharides present in barley papillae and to characterise their potential role in the penetration-resistance mechanism against the powdery mildew pathogen. Given that the papillae comprise a very small percentage of the epidermal cell wall mass, conventional polysaccharide analysis methods such as methylation analysis are difficult to use for compositional assessment, because small changes in the composition of papillae will be masked by the much larger amount of cell wall material in other cells in the epidermal layer (Pettolino *et al.*, 2012). The only unequivocal method to define the composition of papillae using chemical procedures would be to dissect out a large number of papillae for the analysis. The very small size of the papillae would make such an approach almost impossible (Malinovsky *et al.*, 2014). Therefore, a range of polysaccharide-specific probes were used to examine the compositions of the papillae *in situ*. The probes included monoclonal antibodies, carbohydrate binding modules, small molecule probes and adapted probe labelling techniques, all of which enabled the polysaccharides at the infected sites to be detected in both sectioned and whole-mounted tissues. These semi-quantitative *in situ* techniques not only allowed polysaccharide composition to be defined, but also showed the distribution of the various polysaccharides within the papillae.

The epitopes of two previously unidentified polysaccharides, namely arabinoxylan and cellulose, were shown to be present at considerable levels in barley papillae, in addition to the callose that had traditionally been assumed to be their major polysaccharide component (Chapter 3). The papillary polysaccharides were found in structured layers, with an inner core consisting of callose and arabinoxylan, with an outer encapsulating layer of arabinoxylan and cellulose. Moreover, non-penetrated papillae contain more overall of these polysaccharides compared to penetrated papillae, suggesting that they are important papillae components required for penetration resistance (Chapter 3).

Genes underlying papillary polysaccharide biosynthesis

After revealing the presence of these polysaccharides in the barley papillae, the next task was to define the key genes underlying the biosynthesis of callose, cellulose and arabinoxylan in the papillae. Identifying key genes would not only shed light on the role of each component in host penetration resistance mechanisms, but could also suggest new opportunities and strategies for generating plant resistance to a wide range of pathogens.

For the callose biosynthetic genes directly linked to papillae formation, the functional homologs of *Arabidopsis Glucan synthase-like 5* (*AtGsl5*) were sought in the barley genome, since this gene was shown to synthesise callose in papillae formed in *Arabidopsis* (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). Two barley *Gsl* genes (*HvGsl6* & *HvGsl7*) showed significant sequence homology to *AtGsl5*. However, only the transcripts of *HvGsl6* were up-regulated during *Bgh* infection (Chapter 4). The dsRNAi-mediated down-regulation of *HvGsl6*, examined using both transiently transformed cells and stable transgenic lines, led to significantly less papillary callose accumulation, compared with wild type controls. Down-regulation of *HvGsl6* in fact promoted penetration efficiency of the powdery mildew pathogen in the stable transgenic barley lines. This therefore suggested that *HvGsl6* is associated with

the accumulation of callose in barley papillae and contributes to the penetration resistance mechanism deployed against fungal pathogens.

Arabinoxylan is found in both primary and secondary walls of plants. Although the complete biosynthesis process is not fully understood, the assembly of the xylose backbone and its substitution involves many different glycosyltransferase enzymes (GTs) representing at least six families, namely GT8, GT31, GT43, GT47, GT61 and GT75. To complete the analysis of polysaccharide biosynthesis in the papillae, the transcription profiles of genes from each of the candidate arabinoxylan biosynthetic GT families were examined for any correlations with penetration resistance to *Bgh* (Chapter 5). Microarray transcript expression profiling showed that at least 17 members of the candidate GT gene families were significantly up-regulated in the barley epidermal layer in response to the host pathogen *Bgh* and non-host pathogen *Bgt* infection, compared with the uninfected control. Transient dsRNAi showed that down-regulation of some candidates significantly increased the penetration rate of the fungal pathogen compared with the control, namely MLOC_54026 (GT43), MLOC_6357 (GT61), MLOC_70966 (GT61), MLOC_65693 (GT8), MLOC_16945 (GT31) and MLOC_79335 (GT31). A transient over-expression study of the candidates was also performed to examine the possibility of enhancing penetration resistance. The data suggested that it is possible to achieve higher penetration resistance by over-expressing the following candidate genes; MLOC_39786 (GT31), MLOC_70966 (GT61), MLOC_64204 (GT75), MLOC_19204 (GT8), MLOC_64310 (GT61), MLOC_16945 (GT31), MLOC_14407 (GT47), MLOC_65693 (GT8) and MLOC_6065 (GT75). For a few candidate genes, down-regulation and up-regulation did not lead to reciprocal phenotypes, suggesting that they may require ancillary factors for normal activity. This effect was mainly seen for members of the GT43 and GT47 families. Over-expression of a particular GT43 gene (MLOC_54026) in combination with members of the GT47 family led to significant increases in resistance, which was supported by evidence that they have been claimed to be present with GT61 proteins in

an arabinoxylan synthesis complex in wheat (Zeng *et al.*, 2010; Jiang *et al.*, 2016). Due to a number of experimental constraints it was not possible to confidently correlate altered expression levels of specific candidate genes directly with enhanced arabinoxylan accumulation in papillae of the transformed cells. However, the results from these experiments demonstrated that some of the candidate genes are important in the papillae-based penetration resistance mechanism, presumably through the biosynthesis of arabinoxylan in barley papilla, although their direct role in this process is yet to be proven (Chapter 5).

To identify putative candidate genes involved in the biosynthesis of cellulose in papillae, a collaborative research project was conducted with Dr Patrick Schweizer at IPK, Germany (Chapter 6). The transcripts of the barley *HvCslD2* gene were considerably up-regulated during powdery mildew infection and a transient dsRNAi-mediated screen confirmed that *HvCslD2* is an important factor in non-host as well as quantitative host resistance (Douchkov *et al.*, 2014). While various other factors were characterised in the *HvCslD2* project, my contribution to this investigation was to evaluate the cellulose content within the transgenic lines, using fluorescence microscopy. Significantly less cellulose accumulated in the papillae of *HvCslD2*_RNAi stable transgenic lines when compared with wild type plants. Together with the increased penetration efficiency of the powdery mildew pathogen in transgenic barley lines, these data suggested that *HvCslD2* is an important factor of both host and non-host resistance of barley plants, probably through the accumulation of cellulose in the epidermal layer papillae. A hypothetical model depicting papillary and cell wall dynamics during barley-powdery mildew interactions is presented in Figure 1.

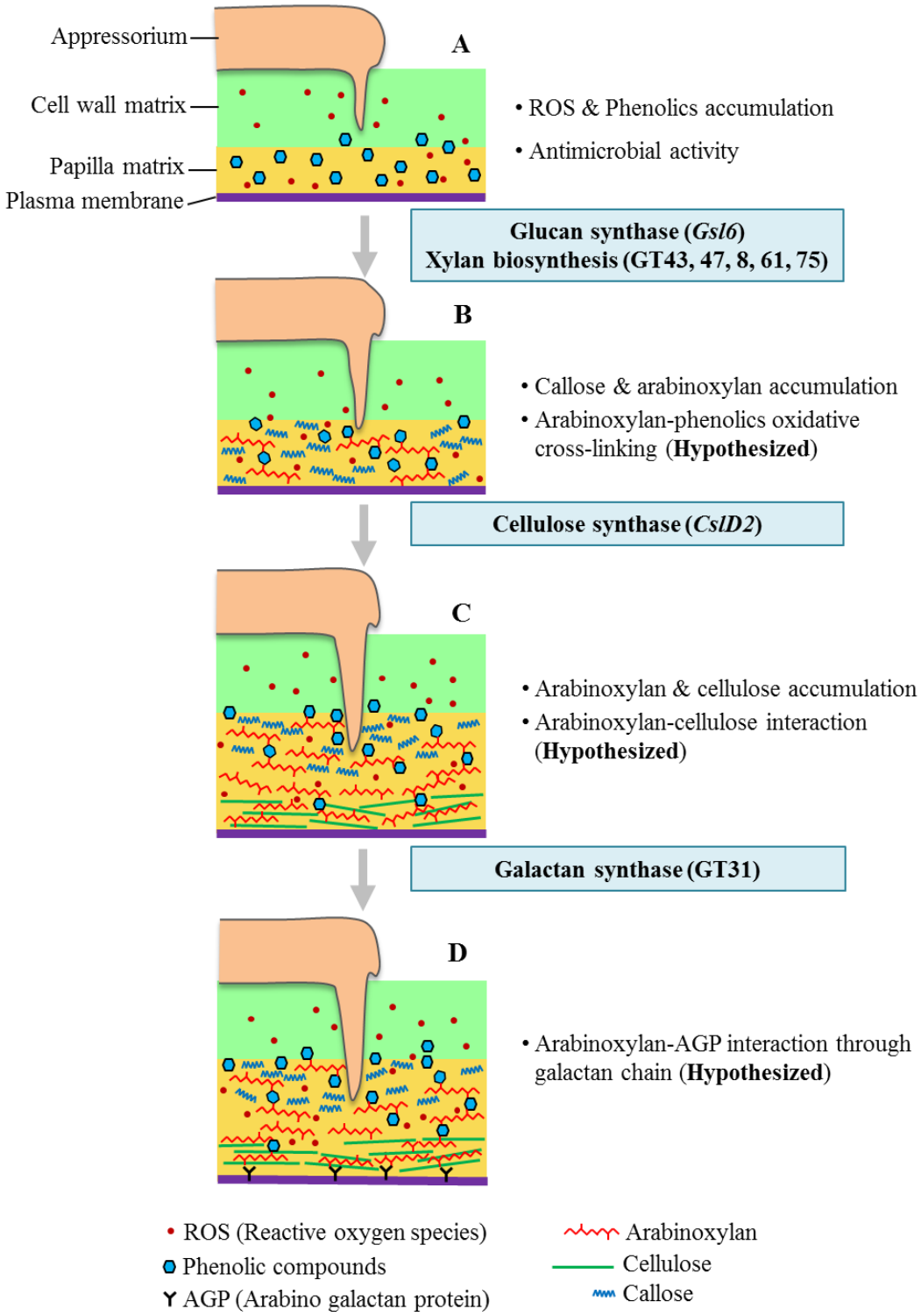

Figure 1. A hypothetical model of papillary dynamics during barley-powdery mildew interactions. The model depicts a situation where the fungal penetration peg is halted by an effective papillary matrix. For simplicity, the dynamics of wall proteins, lipids and inorganic

molecules are not presented in this model. **A.** The reactive oxygen species (ROS) can be detected at the very early stage of plant-pathogen interactions. Apart from its own antimicrobial activity to slow-down the penetration process, ROS act as secondary signalling molecules to trigger stress related gene expression and may also be required for oxidative cross-linking of wall polymers. **B.** Callose and arabinoxylan start accumulating in the papilla matrix and create a physical barrier through the activity of glucan and arabinoxylan synthases. Further reinforcement may be achieved by oxidative cross-linking of arabinoxylans via their phenolic acid constituents. **C.** Along with arabinoxylan, cellulose is deposited at the later stage of papillae development, presumably by the activity of a cellulose synthase. Arabinoxylan can potentially interact with cellulose and other polysaccharides to provide reinforcement for the papillae and also to increase the resistance of cellulose to physical and enzymatic attack. **D.** In the final stages, arabinoxylan may be cross-linked with galactan residues of the membrane bound AGP (Tan *et al.*, 2013)to provide a more rigid structure by connecting the papilla to the plasma membrane.

Future Directions

This study has provided new insights into the papillae-based penetration resistance mechanism against biotrophic fungal pathogens through characterising the papillae polysaccharides and the key genes that are likely to be responsible for their biosynthesis. However, the knowledge gained from this study has generated new questions which need to be addressed in order to better understand how pre-invasion resistance mechanisms are mediated though effective papillae. Some key questions are listed below.

Are the three papillae polysaccharides inter-linked and/or further cross-linked by phenolic compounds?

Oxidative cross-linking is an important aspect of cell wall reinforcement, by providing additional strength against mechanical pressure and enzymatic degradation exerted by the pathogens (Bradley *et al.*, 1992; Brisson *et al.*, 1994). Arabinoxylan, via the arabinose substituents, may be ester-linked to ferulic acid residues, which can be covalently cross-linked to another arabinoxylan chain (Marcia, 2009). Xylan chains may also interact with the surface of cellulose microfibrils, through extensive intermolecular hydrogen bonding and hydrophobic interactions (Busse-Wicher *et al.*, 2014). The frequent presence of H_2O_2 near effective papillae may indicate that ROS play an important role in cell wall cross-linking (Huckelhoven *et al.*, 1999; Burr & Fry, 2009). However, the use of traditional biochemical methods to examine oxidative cross-linking in the papillae are currently very challenging because of the difficulty in dissecting out sufficient papillary material for analysis. A polyclonal antibody raised against ferulic acid that is ester linked to arabinoxylans (Philippe *et al.*, 2007) could be a more useful option to examine both the location and extent of the cross-linking.

Is the polysaccharide content observed in the barley papillae also present in papillae that form against non-adapted pathogens or in papillae of other cereal crop species?

Cell wall polysaccharide compositions vary across different plant types, especially between monocot and eudicot species. In eudicots, cellulose fibres are embedded in a matrix

rich in xyloglucan and pectic polysaccharides, whilst in monocots, cellulose fibres are embedded in a polysaccharide matrix consisting predominantly of (1,3;1,4)-β-glucan and arabinoxylan (Vogel, 2008). It is assumed that xyloglucan and pectin in eudicot species play the same role as (1,3;1,4)-β-glucan and arabinoxylan in monocots, but we are far from understanding how linkages between the different polymer combinations are functionally equivalent eg., how sets of different polymers perform the same role. Similarly, it is clear that papillae polysaccharide composition also differs between monocot and eudicot species. For example, xyloglucan has been detected in the papillae of some eudicot species (Celio *et al.*, 2004), but this polysaccharide is not generally found at significant levels in the monocot wall or in monocot papillae. Cellulose, which is an important component of barley papillae, was not detected in the papillae of *Arabidopsis*, and was thus used as a useful control for differentiating between the epidermal cell wall and the papillae polysaccharides (Eggert *et al.*, 2014). It is therefore likely that the different polysaccharides present in monocot and eudicot species may play a similar role in building papillae that are effective against pathogens.

To examine whether callose, arabinoxylan and cellulose are present in the papillae of various monocot species during pathogen infections, the probe labelling techniques described in Chapter 3 (Chowdhury *et al.*, 2014) could be used to provide spatial information *in situ*. If this is paired with recently developed metabolomics profiling techniques such as high throughput carbohydrate microarray techniques, in combination with monoclonal antibodies (Moller *et al.*, 2007), papillary polysaccharide composition across various species could be screened in a relatively short amount of time. The same techniques could be used to examine the composition of papillae formed in dicotyledonous plants.

Is the deposition of papillary polysaccharides differentially regulated in various papillae-based resistant genotypes?

Since this present study suggests that earlier and heavier deposition of polysaccharides is most likely to improve penetration resistance, it is now important to characterise the polysaccharide

deposition into papillae with respect to the timing, amount and patterns of deposition, and to compare deposition between susceptible and papillae-based resistant genotypes. Along with the techniques in microscopy that have been used in the present study, such studies may also require techniques that allow quantitative analyses of epidermal papillary polysaccharides, in order to compare samples from various genotypes collected at different time points of papillary development (8h to 16 h). The high throughput carbohydrate microarrays combined with the specificity of monoclonal antibodies and carbohydrate hydrate binding modules hold considerable promise to serve this purpose (Moller *et al.*, 2007). Compared with conventional biochemical techniques such as methylation and linkage analysis, carbohydrate microarrays require smaller amounts of cell wall materials (0.5 to 10 mg) and can be used to rapidly analyse large numbers of samples (Malinovsky *et al.*, 2014). If the polysaccharides are differentially deposited between the susceptible and resistance genotypes, further analyses need to be performed to define the genetic factors behind this difference, in both host and pathogen. One possible variable is the fungal virulence effectors that may interfere with the biosynthesis and transportation mechanisms of papillary materials in susceptible hosts, but not in resistant ones. Another possibility is that the hydrolases of virulent pathogens are specifically adapted to more efficiently degrade the polysaccharides in susceptible host cell walls. Global gene expression profiling of host and pathogen, combined with apoplastic proteomic analysis (Gupta *et al.*, 2015) during fungal interactions, may unlock valuable information with which to address these questions. In addition, a recently developed laser microdissection technique adapted for down-stream proteome and carbohydrate analysis of the epidermal layer papillae could be extremely useful in this regard (Falter *et al.*, 2015).

Can the polysaccharide biosynthetic genes be utilized to improve host penetration resistance against pathogens?

Testing stable transgenic lines over-expressing polysaccharide biosynthetic genes against a range of pathogens would help to answer this question. For example, higher and

earlier accumulation of papillary callose through the over-expression of the *AtGsl5* gene leads to complete penetration resistance against powdery mildew pathogens (Ellinger *et al.*, 2013). However, a constitutive modification of the cell wall may negatively affect the normal growth and development of the host plant. Balancing cell wall modification with disease resistance and agronomic performance is an important consideration, as is obtaining widespread community acceptance of crop plants that have been genetically modified to improve disease resistance. In order to limit unwanted effects on plant growth and yield, the use of constitutive promoters could be avoided. Instead, pathogen induced promoters may provide a much better choice, since gene expression will only be triggered in the presence of the pathogen. For example, the use of the barley germin-like GER4c promoter enabled strong transgene expression only during powdery mildew pathogen infection in barley and wheat leaf epidermis (Himmelbach *et al.*, 2010). Furthermore, synthetic plant promoters containing defined regulatory elements that are induced during various pathogen attacks and wound infections could also be considered (Rushton *et al.*, 2002).

Concluding remarks

Pre-invasion resistance through localised cell wall modification is an important mechanism of the plant immune system. Enhanced penetration resistance by means of genetic engineering of the plant cell wall holds great promise for durable non-host resistance to a broad range of pathogens. This study demonstrates important dynamic features of cell wall polysaccharides in assisting against pathogen invasion. However, defining the full picture of the cell wall modifications taking place at plant-pathogen interaction sites may prove to be very challenging. To get a better idea of the events underpinning pre-invasion resistance, more studies are needed to unravel the interactions between wall polysaccharides and the other components of a plant-pathogen system. While the microscopy and genomic approaches used in this study can be very useful, we should also take the advantage of a combined computational, genomics, metabolomics and proteomics approach, with additional insights provided by the study of the co-evolutionary aspects of plant-pathogen interactions.

References:

Bellincampi D, Cervone F, Lionetti V. 2014. Plant cell wall dynamics and wall-related susceptibility in plant–pathogen interactions. *Plant cell wall in pathogenesis, parasitism and symbiosis* **5**: 30-37.

Bradley DJ, Kjellbom P, Lamb CJ. 1992. Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* **70**(1): 21-30.

Brisson LF, Tenhaken R, Lamb C. 1994. Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. *The Plant Cell* **6**(12): 1703-1712.

Burr SJ, Fry SC. 2009. Feruloylated arabinoxylans are oxidatively cross-linked by extracellular maize peroxidase but not by horseradish peroxidase. *Molecular Plant* **2**(5): 883-892.

Busse-Wicher M, Gomes TC, Tryfona T, Nikolovski N, Stott K, Grantham NJ, Bolam DN, Skaf MS, Dupree P. 2014. The pattern of xylan acetylation suggests xylan may interact with cellulose microfibrils as a twofold helical screw in the secondary plant cell wall of Arabidopsis thaliana. *The Plant Journal* **79**(3): 492-506.

Celio G, Mims C, Richardson E. 2004. Ultrastructure and immunocytochemistry of the host pathogen interface in poinsettia leaves infected with powdery mildew. *Canadian Journal of Botany* **82**(4): 421-429.

Chowdhury J, Henderson M, Schweizer P, Burton RA, Fincher GB, Little A. 2014. Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated versus penetrated papillae on leaves of barley infected with Blumeria graminis f. sp. hordei. *New Phytologist* **204**(3): 650-660.

Douchkov D, Lück S, Johrde A, Nowara D, Himmelbach A, Rajaraman J, Stein N, Sharma R, Kilian B, Schweizer P. 2014. Discovery of genes affecting resistance of barley to adapted and non-adapted powdery mildew fungi. *Genome Biology* **15**(12): 1-18.

Eggert D, Naumann M, Reimer R, Voigt CA. 2014. Nanoscale glucan polymer network causes pathogen resistance. *Scientific reports* **4**.

Ellinger D, Naumann M, Falter C, Zwikowics C, Jamrow T, Manisseri C, Somerville SC, Voigt CA. 2013. Elevated early callose deposition results in complete penetration resistance to powdery mildew in Arabidopsis. *Plant Physiology* **161**(3): 1433-1444.

Falter C, Ellinger D, Von Hülsen B, Heim R, Voigt CA. 2015. Simple preparation of plant epidermal tissue for laser microdissection and downstream quantitative proteome and carbohydrate analysis. *Frontiers in plant science* **6**.

Gupta R, Lee SE, Agrawal GK, Rakwal R, Park S, Wang Y, Kim ST. 2015. Understanding the plant-pathogen interactions in the context of proteomics-generated apoplastic proteins inventory. *Frontiers in plant science* **6**.

Himmelbach A, Liu L, Zierold U, Altschmied L, Maucher H, Beier F, Müller D, Hensel G, Heise A, Schützendübel A. 2010. Promoters of the barley germin-like GER4 gene cluster enable strong transgene expression in response to pathogen attack. *The Plant Cell* **22**(3): 937-952.

Huckelhoven R. 2005. Powdery mildew susceptibility and biotrophic infection strategies. *Fems Microbiology Letters* **245**(1): 9-17.

Huckelhoven R, Fodor J, Preis C, Kogel KH. 1999. Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiology* **119**(4): 1251-1260.

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB. 2003. An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* **15**(11): 2503-2513.

Jiang N, Wiemels RE, Soya A, Whitley R, Held M, Faik A. 2016. Composition, assembly, and trafficking of a wheat xylan synthase complex (XSC). *Plant Physiology*: DOI:10.1104/pp.1115.01777.

Malinovsky FG, Fangel JU, Willats WG. 2014. The role of the cell wall in plant immunity. *Plant cell wall in pathogenesis, parasitism and symbiosis* **5**: 38-49.

Marcia MdO. 2009. Feruloylation in grasses: current and future perspectives. *Molecular Plant* **2**(5): 861-872.

Moller I, Sørensen I, Bernal AJ, Blaukopf C, Lee K, Øbro J, Pettolino F, Roberts A, Mikkelsen JD, Knox JP. 2007. High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *The Plant Journal* **50**(6): 1118-1128.

Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC. 2003. Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* **301**(5635): 969-972.

Pettolino FA, Walsh C, Fincher GB, Bacic A. 2012. Determining the polysaccharide composition of plant cell walls. *Nature Protocols* **7**(9): 1590-1607.

Philippe S, Tranquet O, Utille J-P, Saulnier L, Guillon F. 2007. Investigation of ferulate deposition in endosperm cell walls of mature and developing wheat grains by using a polyclonal antibody. *Planta* **225**(5): 1287-1299.

Rushton PJ, Reinstädler A, Lipka V, Lippok B, Somssich IE. 2002. Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen-and wound-induced signaling. *The Plant Cell* **14**(4): 749-762.

Tan L, Eberhard S, Pattathil S, Warder C, Glushka J, Yuan C, Hao Z, Zhu X, Avci U, Miller JS. 2013. An Arabidopsis cell wall proteoglycan consists of pectin and arabinoxylan covalently linked to an arabinogalactan protein. *The Plant Cell* **25**(1): 270-287.

Vogel J. 2008. Unique aspects of the grass cell wall. *Current Opinion in Plant Biology* **11**(3): 301-307.

Zeng W, Jiang N, Nadella R, Killen TL, Nadella V, Faik A. 2010. A glucurono (arabino) xylan synthase complex from wheat contains members of the GT43, GT47, and GT75 families and functions cooperatively. *Plant Physiology* **154**(1): 78-97.

Appendix-A

The cellulose synthase-like D2 gene mediates nonhost- and quantitative host resistance in barley

Journal:	*New Phytologist*
Manuscript ID	Draft
Manuscript Type:	MS - Regular Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	Douchkov, Dimitar; Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Breeding Research Lueck, Stefanie; Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Breeding Research Hensel, Goetz; Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Molecular Plant Physiology Kumlehn, Jochen; Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Molecular Plant Physiology Rajaraman, Jeyaraman; Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Breeding Research Johrde, Annika; Syngenta Seeds GmbH, Global cereals & DFC crop coordinator NSPP Dublin, Monika; University of Melbourne, School of Botany Beahan, Cherie; ARC Centreof Excellence in Plant Cell Walls, School of Botany, Kopischke, Michaela; The Sainsbury Laboratory, Norwich Research Park Fuchs, Rene; Staatliches Weinbauinstitut, Versuchs- und Forschungsanstalt für Weinbau und Weinbehandlung Lipka, Volker; Georg-August-University Goettingen, Dep of Plant Cell Biology Niks, Rients; Wageningen University, Labratory of Plant Breeding Bulone, Vincent; KTH Royal Institute of Technology, School of Biotechnology Little, Alan; University of Adelaide, ARC Centre of Excellence in Plant Cell Walls Chowdhury, Md Jamil; University of Adelaide, ARC Centre of Excellence in Plant Cell Walls, School of Agriculture, Food and Wine Burton, Rachel; University of Adelaide, ARC Centre of Excellence in Plant Cell Walls Bacic, Antony; University of Melbourne , School of Botany Fincher, Geoff; University of Adelaide, ARC Centre of Excellence in Plant Cell Walls, School of Agriculture, Food and Wine Schweizer, Patrick; Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Breeding Research
Key Words:	Cellulose synthase like, CSL, Blumeria graminis, cell wall, RNAi, transgenic

The *cellulose synthase-like D2* gene mediates nonhost- and quantitative host resistance in barley

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Total word count main body of the text: 4924
Word count Introduction: 822

For Peer Review

Word count Materials and Methods:	923
Word count Results:	1886
Word count Discussion:	1223
Word count Acknowledgements:	70
Figures: (Fig.1-4 in colour)	4
Supporting information files:	16

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SUMMARY

- Cell walls and cellular turgor pressure shape and suspend the bodies of all vascular plants. In response to attack by fungal and oomycete pathogens, which usually breach their host's cell walls by mechanical force or by secreted lytic enzymes, plants often form local cell-wall appositions as an important first line of defense. The involvement of cell-wall biosynthetic enzymes in the formation of cell-wall appositions is still poorly understood, especially in grasses including cereal crops.
- In order to validate a cell-wall-related candidate gene encoding cellulose synthase-like D2 (HvCslD2), which was discovered in an RNAi-based screening as important factor of nonhost- as well as quantitative host resistance, we generated and characterized transgenic barley (*Hordeum vulgare*) plants silenced in Hv*CslD2*.
- Barley plants silenced in Hv*CslD2* showed no growth defects but were more successfully penetrated by non-adapted and virulent isolates of the powdery mildew fungus *Blumeria graminis*, respectively. Penetration was associated with a lower cellulose:glucuronoarabinoxylan ratio in epidermal cell walls and increased penetration of local cell-wall appositions despite the local accumulation of autofluorescent, resistance-related phenolic compounds.
- The results suggest HvCslD2-mediated cell-wall changes as an important defense reaction against fungal penetration, possibly acting in parallel to the local accumulation of lignin-like materials.

KEYWORDS

Cellulose synthase like, CSL, *Blumeria graminis*, cell wall, RNAi, transgenic plants

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INTRODUCTION

Fungal and oomycete pathogens usually have to breach the cell walls of their host plants via penetration pegs that emerge from appressoria. This is achieved by mechanical force driven by either high turgor pressure or by releasing cell wall degrading enzymes, or both (Pryce-Jones *et al.*, 1999; Huckelhoven & Panstruga, 2011). In response, plants have evolved a first line of defense consisting of local cell wall appositions, or papillae, that contain different glycans, phenolic compounds and antimicrobial substances (Kwon *et al.*, 2008). An accumulation of phenolic compounds has been described in cell-wall appositions of the cereal species wheat and barley, and a defense-related role has been shown for enzymes of the shikimate- and phenylpropanoid pathways leading to such compounds and for enzymes of lignification reactions (Stadnik & Buchenauer, 1999; Kruger *et al.*, 2002; Bhuiyan *et al.*, 2009; Hu *et al.*, 2009; Douchkov *et al.*, 2014). The polysaccharide composition of papillae was traditionally believed to be mainly (1,3)-β-glucans (callose), but it has been shown recently that besides callose, heteroxylans and cellulose are found in effective papillae of barley leaves challenged with the powdery mildew fungus *Blumeria graminis* f.sp. *hordei (Bgh)* (Chowdhury *et al.*, 2014). The role of callose, a widely occurring and readily detectable marker of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in plants (Hardham *et al.*, 2007), appears to be more complex because defense- but also susceptibility-related effects of callose accumulation have been described in powdery mildew-attacked *Arabidopsis thaliana* (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003; Wawrzynska *et al.*, 2010). Recently, constitutive overexpression of the putative callose synthase At*GSL5* was found to cause immunity, suggesting that timely accumulation or constitutive presence of callose does have a strong protective effect (Ellinger *et al.*, 2013). Both in mono- and di-cotyledons, pectin (like) material has been found to be an important barrier against pathogen attack and plant-encoded polygalacturonase inhibitors as well as pectinmethylesterase inhibitors were shown to affect the outcome of the corresponding interactions (Volpi *et al.*, 2011; Ferrari *et al.*, 2012). On the other hand, very little is known about a possible defense-related role of regulators or enzymes for other non-cellulosic wall polysaccharides. These include the xyloglucans from dicotyledonous plants and the heteroxylans and (1,3;1,4)-β-D-glucans from the *Poaceae* family of monocotyledons, which includes commercially important cereals and grasses (Carpita, 1996). Indeed, there has been a dramatic shift in the composition of non-cellulosic wall polysaccharides in the *Poaceae*. In dicotyledons and many non-commelinoid monocotyledons, xyloglucans and pectins are the major non-cellulosic polysaccharides, with lower levels of heteroxylans and heteromannans but, in the *Poaceae*, levels of these xyloglucans and pectic polysaccharides are much lower and the heteroxylans appear to form the 'core' non-cellulosic

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polysaccharides of the wall. In addition, (1,3;1,4)-β-glucans are often found in walls of the *Poaceae* but are not widely distributed in other higher plants (Scheller & Ulvskov, 2010; Burton & Fincher, 2012; Burton & Fincher, 2014).

Cellulose synthase (CesA) and cellulose synthase-like (Csl) proteins are encoded by a multigene family in vascular plants and are classified in the GT2 family of glycosyl transferases (Richmond & Somerville, 2000; Cantarel *et al.*, 2009; Lombard *et al.*, 2014). The *Csl* genes fall into nine subfamilies (*A*-*J*), out of which *CslA, CslC, CslD* and *CslE* are widely distributed in all plants, whereas *CslF, CslH* and *CslJ* are limited largely to the *Poaceae* family and *CslB* and *CslG* genes are present only in dicotyledons. The *Csl* genes are believed to mediate the synthesis of least some of non-cellulosic wall polysaccharides, including the backbones of heteromannans (*CslA* clade), xyloglucans (*CslC* clade) and (1,3;1,4)-β-glucans (*CslF* and *CslH* clades); the *CslD* genes might participate in cellulose synthesis in tip-growing cells (Doblin *et al.*, 2001; Park *et al.*, 2011; Dhugga, 2012). While the catalytic activities and functions of several Csl's in plant development or during periods of abiotic stress have been determined (Zhu *et al.*, 2010), their role in biotic stress and the resistance of mono- or di-cotyledons to pathogen attack has not been defined in detail. Given that cell wall-to-cell wall interactions are likely to be early events in the pathogen's attempts to penetrate a plant host, it would not be surprising if changes occur in the expression levels of genes encoding wall polysaccharide synthases. In two functional screenings by transient-induced gene silencing (TIGS) for genes that are required for nonhost resistance (HNR) or that affect quantitative host resistance (QR) against powdery mildew fungi we tested a total of 1144 barley genes. This led to the discovery of 10 *Rnr* (for *required for nonhost resistance*) candidate genes plus to 90 candidate genes affecting QR (Douchkov et al. 2014).

Here we present structural and functional data to validate *Rnr6* encoding HvCslD2, a protein with high sequence similarity to rice (*Oryza sativa*) OsCslD2 and *Arabidopsis thaliana* AtCslD2 and AtCslD3 proteins, respectively. The results suggest a grass-specific functional diversification of the D clade of Csl proteins from cell-wall biosynthesis in tip-growing or dividing cells to include local cell-wall reinforcement upon pathogenic penetration attempts.

MATERIALS AND METHODS

Plant and fungal material

For TIGS, 7-day-old seedlings of spring barley cv. Maythorpe were used. This genotype was well suited for TIGS experiments in host- as well as nonhost interactions with powdery mildew fungi because it was fully resistant to the wheat powdery mildew *B. graminis* f.sp. *tritici* (*Bgt*) while exhibiting a moderate level of QR to *Bgh*. Seedlings were grown in a plant

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incubator (Panasonic Healthcare Co. Ltd, Ōizumi, Gunma, Japan) at 20°C constant temperature, 55% rel. humidity and 16 h illumination (intensity level 5) by fluorescent tubes (OSRAM L36W/840). For the generation of stable transgenic barley cv. Golden Promise, a γ-irradiated mutant of cv. Maythorpe, was used. Bombarded leaf segments or transgenic plants were inoculated with Swiss *Bgt* field isolate FAL 92315, Swiss *Bgh* field isolate CH4.8, or several adapted as well as non-adapted rust (*Puccinia* sp.) isolates as specified in Supporting Information Table S2. For inoculation of *A. thaliana Col*-0 wildtype plants or mutants derived from the same accession, *Bgt* isolate FAL 92315 or *Erysiphe cichoracearum* isolate UCSC1 was used.

TIGS

TIGS constructs of selected target genes were generated and transferred into barley leaf epidermal cells by particle bombardment as described (Douchkov *et al.*, 2005). For NHR and for QR experiments, leaf segments were inoculated three days after the bombardment with Swiss *Bgt* field isolate FAL 92315 and Swiss *Bgh* field isolate CH4.8, respectively, at a density of 150-200 conidia mm^{-2}. Transformed GUS-stained epidermal cells as well as haustoria-containing transformed (susceptible) cells were counted 48 h after inoculation, and TIGS effects were statistically analysed according to (Douchkov et al., 2014).

Transgenic plants

The binary RNAi construct pIPKb009_CslD2 (Supporting Information Figure S10) was generated by using the RNAi-vector pIPKb009 (Himmelbach *et al.*, 2007). The LR-reaction with entry clone pIPKTA38_CslD2 that contained 984 bp spanning Hv*CslD2* cDNA sequence from position 1016-1192 of the encoded protein plus 450 bp of 3'UTR was done as described (Douchkov *et al.*, 2005). Immature barley embryos (cv. Golden Promise) were transformed with the binary RNAi-vector described above using the *Agrobacterium tumefaciens* strain AGL1 as described (Hensel *et al.*, 2008). The resulting plantlets were selected on medium containing hygromycin (50 mg L^{-1}). For plant phenotyping, segregating T1 populations were used first. Two events (E28 and E39) were selected for further experiments carried out in segregating or homozygous T2 lines (as indicated), and in homozygous T3 lines. An azygous control line AZ39 was also established from one azygous T2 individual of event E39 and used as control, where indicated.

Inoculation of transgenic plants

Test plants were grown in 54-pot trays in a growth chamber at 15°C (dark period 8h) and 20°C (light period, 16 h, provided by fluorescent tubes (Philips TL-D). Approximately 10-20 seedlings at the 2-3-leaf stage of selected primary transgenic lines were used per inoculation

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experiment with *Bgh* or *Bgt*. Infection by *Bgh* was visually scored 7 dai according to (Altpeter *et al.*, 2005). For the microscopic determination of *Bgt* infection, detached segments of second leaves were inoculated with approximately 20-50 spores/mm and incubated as described (Altpeter *et al.*, 2005). After Coomassie-staining of *Bgt* hyphae at 48 hai, total numbers of colonies per leaf segment were counted by bright-field microscopy at 100 x magnification and related to the total area per detached leaf segment (Seiffert & Schweizer, 2005).

For linkage analysis of cell-wall components, the ethanol-insoluble residues fraction was extracted from the abaxial epidermis and the remaining partially peeled first leaves of eight- to nine-day-old seedlings of the T3 generation (Pettolino *et al.*, 2012).

RNAi rescue

The RNAi rescue construct pIPKTA9_HvCslD2_rescue was made by replacing a 1,267 bp fragment of Hv*CslD2* flanked by *Sph*I sites in the transient-expression vector pIPKTA9_HvCslD2 with a 552 bp synthetic sequence (Eurofins MWG Operon, Ebersberg, Germany) thus replacing the C-terminal part of the coding sequence corresponding to 175 amino acids of the HvCslD2 protein by a mutagenized sequence containing a high number of silent point mutations (Supporting Information Figure S2). *In silico* testing of the synthetic gene by the si-Fi software for silencing by the used Hv*CslD2* RNAi constructs construct did not reveal any 21-mer oligonucleotide as potential target. Therefore, we assume this rescue construct to be fully insensitive against Hv*CslD2* silencing.

RNA blots

Total RNA from first leaves of 2-week-old seedlings was isolated with TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), separated by agarose gel electrophoresis and blotted onto Amersham Hybond-XL membrane (GE Healthcare, Little Chalfont, United Kingdom) by capillary transfer using standard protocols. The RNA was hybridized with a ^{32}P random-prime labelled HvCSLD2 PCR fragment of 1,023 bp using standard methods. The labeling was visualized on phosphoimager FLA-3000 (Fujifilm, Tokyo, Japan) after 16 h exposure time.

Cell-wall analysis

Epidermal cell walls of wildtype and transgenic barley plants were tested for altered resistance to digestion by fungal cell-wall degrading enzymes as described (Schweizer, 2008). Briefly, leaves were attached to glass slides at the abaxial side using double-sided adhesive tape, followed by careful removal of the mesophyll tissue and incubation under a drop of buffer solution containing fungal enzyme preparations. At the times indicated, the dissolution of cross(short) epidermal cell walls was quantified under the light microscope.

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The local accumulation of cellulose in cell-wall appositions was quantified at 18 hai by Pontamine Fast Scarlet 4B staining of whole-mount leaf material as described in (Chowdhury et al., 2014). Local autofluorescence at 18 hai by Bgh was detected using a DAPI filter set at 365 nm excitation and 445/50 nm emission wavelengths. Autofluorescence at 48 hai by *Bgt* was detected using Zeiss filter set 38 (BP 470/40 nm; FT 495 nm; BP 525/50 nm).

RESULTS

Sequence analysis of Rnr6

Based on EST-sequence information of barley, two bacterial artificial chromosome (BAC) clones containing Hv*CslD2* sequences from a *H. vulgare* cv. Morex library (Yu *et al.*, 2000) were identified, shotgun-sequenced and assembled. This resulted in a contig of 27.5 kbp containing the entire Hv*CslD2* structural gene plus 14 kbp upstream and 9 kbp downstream sequence (GenBank Acc. KP122993). Figure 1A shows that the predicted HvCslD2 protein contains conserved domains of the Csl family including three glycosyltransferase motifs with the 'D, D, D, QXXRW' motif (where X is any amino acid) and eight transmembrane helices (Delmer, 1999). In addition, similar to CesA proteins, we found two putative zinc fingers near the NH_2-terminus, the second of which perfectly matched the consensus CXXC-$(X)_{11}$-CXXC. Proteins encoded by the *CslD*-clade are the only family members that contain putative zinc fingers, which are proposed to regulate rosette formation and protein turnover of the cellulose synthase complex localized at the plasmalemma (Kurek *et al.*, 2002). Alternatively, the zinc fingers might be involved in the binding of the CslD protein to another protein.

Target-gene specificity of Hv*CslD2 silencing*

To confirm Hv*CslD2* as the true RNAi target, we performed a transient RNAi-rescue experiment as described for the silencing of a barley *Polyubiquitin* and several *Bgh* effector candidate genes (Dong *et al.*, 2006; Nowara *et al.*, 2010); Pliego *et al.*, 2013). In this type of experiment, the RNAi construct is co-expressed with a synthetic gene that encodes the target protein but is insensitive to the RNAi effect due to a high density of silent point mutations at codon wobble base positions. The predicted outcome of the experiment will be a functional complementation only of true RNAi target effects (Figure 1B and Supporting Information Figure S2). As shown in Figure 1C, TIGS of Hv*CslD2* induced hyper-susceptibility of barley epidermal cells to *Bgh*, which was complemented by the co-bombarded RNAi-rescue construct confirming Hv*CslD2* as the functional RNAi target. In addition to enhanced susceptibility triggered by Hv*CslD2* silencing, we found enhanced resistance to *Bgh* by transiently over-expressing Hv*CslD2*, suggesting that the encoded protein is a limiting factor for quantitative resistance (QR), at least in the barley cv

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253 "Maythorpe" that was used for the over-expression experiment. TIGS of Hv*CslD4*, another D-
254 clade member of barley that we found in an EST collection, did not affect haustorium
255 formation (Table 1).
256
257 *Silencing of HvCslD2 in transgenic plants*
258 Transgenic barley plants that carry an RNAi construct against Hv*CslD2* were generated and
259 characterized with respect to plant growth and resistance to adapted virulent as well as non-
260 adapted isolates of *B. graminis*. The level of QR and NHR was tested in segregating T_1
261 populations established from a number of transgenic events, and average data per
262 population were analysed (Figure 2A). Several events caused strongly enhanced nonhost
263 susceptibility to *Bgt*, and often the same events were also more susceptible to the adapted
264 host pathogen *Bgh* (Pearson's r=0.716, p=0.0018). None of the events showed obvious
265 growth abnormalities suggesting that Hv*CslD2* silencing had no pleiotropic effects in barley.
266 The events E28 and E39 were selected for further work in T2 and T3 generations because
267 they contained individuals with strongly enhanced nonhost susceptibility (Figure 2F-G) and
268 markedly reduced Hv*CslD2* mRNA levels (Figure 2B) together with good seed set (Figure
269 2C-E). On Hv*CslD2*-silenced plants, the wheat pathogen *Bgt* was able to fulfil its asexual life
270 cycle and produce conidiospores (Figure 2F-H). Susceptibility of E28 and E39 to *Bgt* co-
271 segregated with the presence of the *hpt* selectable marker gene (Supporting Information
272 Table S1). The slightly enhanced *Bgt* susceptibility of the pool of azygous, segregating plants
273 derived from both events was likely due to individuals within this group that were scored as
274 hygromycin-sensitive *(i.e.* azygous) although they contained the transgene locus. This
275 assumption is based on the observation that approximately 25% of hygromycin-sensitive
276 individuals contained the *hpt* gene based on genomic PCR data but apparently did not
277 accumulate enough marker protein (data not shown). Transient RNAi rescue of Hv*CslD2* was
278 also performed in transgenic, homozygous T3 plants of event E39 to test if nonhost
279 susceptibility could be complemented. As shown in Figure 1D, epidermal cells transiently
280 expressing the *CslD2* RNAi rescue construct regained normal nonhost immunity to *Bgt*,
281 thereby demonstrating that nonhost susceptibility was caused by Hv*CslD2* silencing. This
282 result also indicates that the constitutively reduced Hv*CslD2* mRNA levels in the transgenic
283 plants did not irreversibly change the cell-wall properties with respect to powdery-mildew
284 resistance.
285 In addition to *Bgt* we tested progeny of the two selected transgenic events for
286 enhanced susceptibility to the adapted and non-adapted rust isolates *Puccinia hordei*, *P.*
287 *hordei-secalini, P. hordei-murini,* and *P. triticina*, respectively. As shown in Supporting
288 Information Table S2 Hv*CslD2* silencing appeared to have no effect on QR or NHR to any of
289 the tested adapted or non-adapted rust pathogens. This suggests, together with the fact that

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cereal rust fungi do not penetrate the epidermis but enter via stomata, that Hv*CslD2* might have an epidermis-specific or powdery mildew-specific function.

Cell-wall alterations

By using an assay for susceptibility of epidermal cell-walls to degradation by a mixture of fungal cellulase, hemicellulases and pectinases (Schweizer, 2008), we found that the walls of transgenic lines were digested more rapidly (Figure 3A). Thus, reduced expression of Hv*CslD2* changed degradation-related cell-wall properties in the absence of fungal attack, which is probably related to its observed basal level of expression. The enhanced hydrolytic digestibility was paralleled by a significantly reduced relative abundance of cellulose and by an enhanced glucuronoarabinoxylan content in epidermal cell walls from non-attacked transgenic plants, as revealed by linkage analysis of the polysaccharides in isolated cell walls (Figure 3B). In contrast, no changes in relative cellulose abundance were found in whole leaf samples, and other cell-wall components were not affected by Hv*CslD2* silencing in either tissue preparations (Supporting Information Table S3). Furthermore, we looked into the local accumulation of cellulose in cell-wall appositions underlying Bgh appressoria by staining with Pontamine Fast Scarlet 4B and found a significant decrease of local cellulose accumulation in both events E28 and E39 at 18 hai at the stage of haustorial initial formation, while the accumulation of autofluorescent, phenolic compounds was unaffected by Hv*CslD2* silencing (Figure 3C-E). Taken together, Hv*CslD2* silencing reduced cellulose abundance in epidermal cell walls prior to and following *Bgh* attack, in line with its basal level of expression that is increased after inoculation, possibly leading to local HvCslD2 protein accumulation underneath or nearby appressoria.

Local cell-wall autofluorescence excited in barley and other grass species by near-UV or blue light is known to reflect the accumulation of phenolic compounds including esterified phenolic acids, which are derived from the pathogen-inducible phenylpropanoid pathway (Huckelhoven, 2007). The accumulation of phenolic compounds at 18 to 36 hai has been functionally linked to fungal penetration resistance in barley and wheat (Stadnik & Buchenauer, 1999; Kruger *et al.*, 2002; Bhuiyan *et al.*, 2007). This is in line with our observation that autofluorescence in non-penetrated cell-wall appositions of non-transgenic plants was high, but was usually low in the few inefficient sites that were successfully penetrated by *Bgt* (Figure 4A and B). By contrast in Hv*CslD2*-silenced plants, we found strong autofluorescence in penetrated cell-wall appositions that were associated with established primary haustoria and the formation of elongating secondary hyphae, thus indicating that the function of Hv*CslD2* for cell wall-based penetration resistance was independent from phenolic compound accumulation. It appears likely that the autofluorescing materials corresponded to esterified phenolic acids such as ferulic acid and *p*-coumaric acid,

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which increased in these papillae, similar to results in *Bgt*-attacked wheat (Stadnik & Buchenauer, 2000). Taken together, we found reduced levels of cellulose in Hv*CslD2*-silenced plants, which was associated with the enhanced enzymatic digestibility of the cell walls and an enhanced fraction of penetrated cell-wall appositions despite high levels of autofluorescing phenolic compounds that were insufficient to stop early fungal development.

Regulation and function of the CslD family in barley

HvCslD2 is one member of the *CesA/Csl* multigene family in barley with a total of 22 identified Csl-family members according to the annotation of a custom 44K oligonucleotide array of Agilent (Table 1). We addressed transcript regulation and defense-related functions of these other *Csl* family members by applying array-based transcript profiling and TIGS. This resulted in the discovery of five family members being significantly regulated by *Bgh* or *Bgt* attack in RNA samples from epidermal peels (Supporting Information Figure S3). Remarkably, all were down-regulated except Hv*CslD2* that stood out as the only up-regulated transcript. For a majority of the identified Hv*Csl* genes we successfully generated TIGS constructs from EST collections and used these for bombardment of cvs Maythorpe and Golden Promise. The silencing of seven genes (Hv*CslA2*, Hv*CslA11,* Hv*CslC10,* Hv*CslD1,* Hv*CslE6,* Hv*CslF3* and Hv*CslH1*) resulted in significantly reduced relative SI upon *Bgh* challenge in at least one of the two tested genotypes (Table 1). In most cases where relative SI was significantly reduced in one genotype a trend for reduction was observed in the second genotype, too. Most robust resistance-enhancing TIGS effects were found for Hv*CslA2* and Hv*CslC10*. While Hv*CslC10* is not represented on the 44K oligonucleotide array, Hv*CslA2* was significantly down regulated upon powdery mildew attack. The conflicting TIGS data of Hv*CslA11* between Maythorpe and Golden Promise suggest a more complex and genotype-dependent role of this family member. In summary, transcript regulation- as well as TIGS data might indicate opposite functions of Hv*CslD2* and other *Csl* family members with respect to defense-related cell-wall modifications.

Comparison of CslD genes in barley and Arabidopsis thaliana

No phenotypic data from rice mutants of the most closely related gene *OsCslD2* have been reported to date thereby preventing functional comparisons between the encoded proteins of the two grass species. In order to compare the function of Hv*CslD2* homologs in a more distantly-related plant species, we tested a homozygous double T-DNA insertion mutant of *A. thaliana* carrying mutated alleles of *AtCslD2* (AT5G16910) and *AtCslD3* (AT3G03050), which represent the closest homologs to *CslD2* of barley (Bernal *et al.*, 2008). Both, nonhost immunity to *Bgt* and host basal resistance to *Erysiphe cichoracearum* appeared to be unaffected, whereas double *pen1/pen2*- and triple *pen2/pad4/sag101* mutant lines showed

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the expected enhanced susceptibility phenotypes (Lipka *et al.*, 2005; Supporting Information Figure S4). Because the regulation of transcript levels often provides clues to potential biological functions of genes, we compared publicly available expression profiles of Hv*CslD2,* *AtCslD2* and *AtCslD3*. In both barley and *A. thaliana, CslD2/3* transcripts were found to be enhanced in cultured cells or protoplasts (Supporting Information Figure S5). On the other hand, Hv*CslD2* was only expressed at low levels in barley root tissues but was up-regulated in powdery mildew-attacked leaves, including leaf epidermis, whereas in *A. thaliana, AtCslD2* and *AtCslD3* were highly expressed in roots but not induced by *G. orontii* infection (Supporting Information Figures S3, and S5-7). In conclusion, the transcript profiling data are consistent with different functions of corresponding *CslD* homologs in the two species. Additional support for a role of Hv*CslD2* in pathogen defense of barley comes from the genetic mapping of the gene to a sub-telomeric position on chromosome 7H that overlaps a meta-QTL for quantitative resistance to *Bgh* (Schweizer & Stein, 2011) (Supporting Information Figure S8). In *A. thaliana*, the mapped QTL for powdery mildew or downy mildew resistance do not co-localize with At*CslD2* or At*CslD3*, except for one downy-mildew QTL on chromosome 5 (Schiff *et al.*, 2001; Wilson *et al.*, 2001; Nemri *et al.*, 2010).

DISCUSSION

Csl proteins are encoded by multigene families in vascular plants. Genes of the of *CslD* clade are required for normal plant development in *A. thaliana*, rice and maize (Hunter *et al.*, 2012). The D-clade members most closely related to Hv*CslD2* including *AtCslD3* (*Kojak*) and *OsCslD1* are implicated in root-hair elongation, which might indicate a role in primary-wall synthesis of rapidly dividing, tip-growing cells (Kim *et al.*, 2007; Bernal *et al.*, 2008; Penning *et al.*, 2009; Park *et al.*, 2011; Yin *et al.*, 2011). However, we could not detect any defective root hair or general growth phenotypes in transgenic barley plants expressing Hv*CslD2* RNAi constructs under the control of the ubiquitously active CaMV 35S promoter, which also caused strong Hv*CslD2* silencing in roots (Supporting Information Figure S9). The transgenic barley lines showed a tendency for enhanced grain yield in the greenhouse, although this effect was only statistically significant in pooled data from several transgenic lines (Supporting Information Table S4). This could indicate a certain physiological cost to express the defense-related Hv*CslD2* gene, similar to, for example, the expression of the resistance-related *Lr34* allele causing leaf-tip necrosis in wheat (Krattinger *et al.*, 2009). Instead of developmental phenotypes, we found an effect on biotic stress tolerance, insofar as Hv*CslD2* contributed to NHR and QR against the powdery mildew fungi *Bgt* and *Bgh*, respectively. Because we did not observe a clear effect of Hv*CslD2* silencing on either host or nonhost resistance to the tested rust pathogens, we suggest that the HvCslD2 protein might be

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specifically directed against adapted and non-adapted powdery mildew fungi that, due to their high epidemic potential and genetic flexibility, pose a constant and serious threat – and selection pressure - on their hosts (McDonald & Linde, 2002). In accordance with this suggestion, the Hv*CslD2* transcript appears not to be regulated in rust-attacked leaves (PlexDB, experiment BB61).

Silencing and mutation of *CslD2/3* genes in barley and *A. thaliana,* respectively, had different phenotypic effects and thus indicate distinct roles in the two species: While the encoded proteins appear to be involved in primary-wall elongation of rapidly tip-growing root-hair cells in *A. thaliana* their closest homologue in barley, Hv*CslD2,* is shown here to be related to defense against pathogens. One possible explanation for this functional diversification is the rapid evolution of the distinct grass cell wall after separation of the *Poales* from other Commelinid orders, which might have locked Hv*CslD2* into the new function of rapid cell-wall fortification at cell-wall appositions underneath pathogen appressoria, away from rapid tip growth that is seen in extant root hairs and (possibly) pollen tubes of other species. Both would imply high catalytic activity and/or processivity of the encoded proteins for the formation of cell-wall matrix material or fibers. On the other hand, it was reported that *OsCslD1* and *ZmCslD5* are required for root hair formation demonstrating that the *CslD* clade also contains family members with evolutionarily more conserved functions across monocot and dicots species (Kim *et al.*, 2007; Penning *et al.*, 2009; Yuo *et al.*, 2011). Attempts to localize HvCslD2 in transiently expressing barley epidermal cells were unsuccessful because N- and C-terminal fusion proteins with GFP did not produce detectable fluorescence signals (data not shown).

Biochemical studies of *csld1*, *csld3* (*kojak*) and *csld4* mutants of *A. thaliana* suggested that AtCSLD-clade proteins mediate (1,4)-β-glucan biosynthesis (Park *et al.*, 2011; Yin *et al.*, 2011). We addressed this question by expressing wildtype- as well as codon-optimized versions of the gene in *Saccharomyces cerevisiae,* but proteins lost enzymatic activity during purification (data not shown). As an alternative approach, site-directed nuclease-based Hv*CslD2* mutagenesis is being performed for similar complementation studies in the future. Several CslD proteins appear to affect cell-wall composition in a complex manner, resulting in reduced xylan- and cellulose- but increased arabinosyl-residue contents in rice loss-of-function mutants of *OsCslD1* and *OsCslD4* (Li *et al.*, 2009; Luan *et al.*, 2011). Also, altered patchy distributions of both cellulose and xyloglucan in root-hair walls of *A. thaliana csld3* mutants were reported and this supported the involvement of pleiotropic cell wall-related effects (Galway *et al.*, 2011). One possible explanation for such complex, presumably indirect effects on cell-wall structure or composition may be that the CslD2-protein participates in multiprotein complexes of cell-wall biosynthetic enzymes. This would be consistent with the observed zinc finger motifs in the

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HvCslD2 protein. In this context it has been shown that a bacterial cellulose synthase interacts with a second protein that is required for activity (Morgan *et al.*, 2013). Furthermore, the formation of Golgi-localized heterocomplexes of the (1,4)-β-glucan synthase AtCSLC4 and the XXT5 xyloglucan xylosyltransferase was recently described, which again suggests cooperative activities between Csl proteins and other cell-wall biosynthetic enzymes (Chou *et al.*, 2012). In the light of these findings, the observed reduced cellulose content of cell-walls and cell-wall appositions of Hv*CslD2*-silenced barley lines might either directly reflect cellulose biosynthetic activity of the protein or its interaction with a cellulose-synthesizing multiprotein complex.

Clues for the activity of HvCslD2 protein might also be derived from the set of cell-wall degrading enzymes encoded by the *Bgh* genome, although its complexity may be lower compared with other phytopathogenic fungi (Bindschedler *et al.*, 2011) (Spanu *et al.*, 2010). The identified *Bgh* genes include a number of putative (1,3;1,4)-β-glucanases (GH16) and (1,3)-β-glucanases (GH17) that would degrade (1,3;1,4)-β-glucans and callose, respectively, at least one cellulase (GH5) and two members of the family GH61 oxidoreductases that catalyze oxidative cleavage of cellulose (Beeson *et al.*, 2012; Supporting Information Table S5). At least some of the genes encoding predicted cell-wall degrading enzymes appear to be functional because their expression patterns are supported by proteomic data (Bindschedler *et al.*, 2011). The predicted enzymatic functions of these fungal-encoded proteins are in line with the recent observation that penetration resistance of barley cell-wall appositions was correlated with enhanced local accumulation of cellulose and callose (Chowdhury *et al.*, 2014). Host-induced silencing of cell-wall degrading enzymes in *Bgh* may be an interesting approach to narrow down most critical host or nonhost cell-wall components for future targeted approaches to regulators and catalytic barley proteins (Nowara *et al.*, 2010; Pliego *et al.*, 2013).

In summary, a phenotype-driven RNAi screen revealed Hv*CslD2* as a novel component of cell-wall based pre-penetration resistance to powdery mildew in barley but not in *A. thaliana*. The closest homologs of Hv*CslD2* in *A. thaliana* (AtCslD2/3) as well as other members of the D-clade in grasses are implicated in the execution of plant developmental programs and body architecture in root, shoot or flower parts. Plant development therefore is the likely initial function of *CslD* genes before individual members such as Hv*CslD2* became locked into a new defense-related role by a neo-functionalization process driven by plant-pathogen co-evolutionary pressure, perhaps together with rapid grass cell-wall evolution determining the biochemical framework in which the HvCslD2 protein is likely to act. The Hv*CslD2* gene described here provides a case of an evolutionarily highly conserved plant protein with a defense-related function that might be specific to barley and possibly other members of the *Poaceae* family. The fact that, at least in barley, *CslD2* is co-localizing with

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an important meta-QTL for disease resistance opens up an opportunity to examine and exploit allelic diversity for improved yield security of the major cereal crops. More work will be required to clarify the defense-related function of HvCslD2 protein, also in the context of other Csl family members that appear also relevant for barley-powdery mildew interactions.

ACKNOWLEDGEMENTS

We would like to thank Gabi Brantin, Manuela Knauft, and Cornelia Marthe for excellent technical assistance. This work was supported by the German Ministry for Research and Education (BMBF projects GABI-nonhost, GABI-phenome and BARLEY-fortress to P.S. and J.K.), by BASF Plant Science GmbH (to P.S.), by EU-FP6 project Bioexploit (to P.S.), and by the Australian Research Council (ARC Centre of Excellence in Plant Cell Walls to G.B.F., R.A.B. and A.B.).

REFERENCES

Altpeter F, Varshney A, Abderhalden O, Douchkov D, Sautter C, Kumlehn J, Dudler R, Schweizer P. 2005. Stable expression of a defense-related gene in wheat epidermis under transcriptional control of a novel promoter confers pathogen resistance. *Plant Molecular Biology* **57**(2): 271-283.

Beeson WT, Phillips CM, Cate JHD, Marletta MA. 2012. Oxidative Cleavage of Cellulose by Fungal Copper-Dependent Polysaccharide Monooxygenases. *Journal of the American Chemical Society* **134**(2): 890-892.

Bernal AJ, Yoo CM, Mutwil M, Jensen JK, Hou G, Blaukopf C, Sorensen I, Blancaflor EB, Scheller HV, Willats WGT. 2008. Functional Analysis of the Cellulose Synthase-Like Genes CSLD1, CSLD2, and CSLD4 in Tip-Growing Arabidopsis Cells. *Plant Physiology* **148**(3): 1238-1253.

Bhuiyan NH, Liu WP, Liu GS, Selvaraj G, Wei YD, King J. 2007. Transcriptional regulation of genes involved in the pathways of biosynthesis and supply of methyl units in response to powdery mildew attack and abiotic stresses in wheat. *Plant Molecular Biology* **64**(3): 305-318.

Bhuiyan NH, Selvaraj G, Wei YD, King J. 2009. Gene expression profiling and silencing reveal that monolignol biosynthesis plays a critical role in penetration defence in wheat against powdery mildew invasion. *Journal of Experimental Botany* **60**(2): 509-521.

For Peer Review

510 **Bindschedler LV, McGuffin LJ, Burgis TA, Spanu PD, Cramer R. 2011.** Proteogenomics
511 and in silico structural and functional annotation of the barley powdery mildew
512 Blumeria graminis f. sp hordei. *Methods* **54**(4): 432-441.
513 **Burton RA, Fincher GB. 2012.** Current challenges in cell wall biology in the cereals and
514 grasses. *Frontiers in Plant Science* **3**.
515 **Burton RA, Fincher GB. 2014.** Evolution and development of cell walls in cereal grains.
516 *Front Plant Sci* **5**: 456.
517 **Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. 2009.** The
518 Carbohydrate-Active EnZymes database (CAZy): an expert resource for
519 Glycogenomics. *Nucleic Acids Research* **37**: D233-D238.
520 **Carpita NC. 1996.** Structure and biogenesis of the cell walls of grasses. *Annual Review of*
521 *Plant Physiology and Plant Molecular Biology* **47**: 445-476.
522 **Chou YH, Pogorelko G, Zabotina OA. 2012.** Xyloglucan Xylosyltransferases XXT1, XXT2,
523 and XXT5 and the Glucan Synthase CSLC4 Form Golgi-Localized Multiprotein
524 Complexes. *Plant Physiology* **159**(4): 1355-1366.
525 **Chowdhury J, Henderson H, Schweizer P, Burton RA, Fincher GB, Little A. 2014.**
526 Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated
527 versus penetrated papillae on leaves of barley infected with Blumeria graminisf.
528 sp.hordei. *New Phytologist* **204**: 650-660.
529 **Delmer DP. 1999.** Cellulose biosynthesis: Exciting times for a difficult field of study. *Annual*
530 *Review of Plant Physiology and Plant Molecular Biology* **50**: 245-276.
531 **Dhugga KS. 2012.** Biosynthesis of non-cellulosic polysaccharides of plant cell walls.
532 *Phytochemistry* **74**: 8-19.
533 **Doblin MS, De Melis L, Newbigin E, Bacic A, Read SM. 2001.** Pollen tubes of Nicotiana
534 alata express two genes from different beta-glucan synthase families. *Plant*
535 *Physiology* **125**(4): 2040-2052.
536 **Dong WB, Nowara D, Schweizer P. 2006.** Protein polyubiquitination plays a role in basal
537 host resistance of barley. *Plant Cell* **18**(11): 3321-3331.
538 **Douchkov D, Lück S, Johrde A, Nowara D, Himmelbach A, Rajaraman J, Stein N,**
539 **Sharma R, Kilian B, Schweizer P. 2014.** Discovery of genes for affecting resistance
540 of barley to adapted and non-adapted powdery mildew fungi. *Genome Biology* **15**:
541 518.
542 **Douchkov D, Nowara D, Zierold U, Schweizer P. 2005.** A high-throughput gene-silencing
543 system for the functional assessment of defense-related genes in barley epidermal
544 cells. *Molecular Plant-Microbe Interactions* **18**(8): 755-761.

For Peer Review

Ellinger D, Naumann M, Falter C, Zwikowics C, Jamrow T, Manisseri C, Somerville SC, Voigt CA. 2013. Elevated Early Callose Deposition Results in Complete Penetration Resistance to Powdery Mildew in Arabidopsis. *Plant Physiology* **161**(3): 1433-1444.

Ferrari S, Sella L, Janni M, De Lorenzo G, Favaron F, D'Ovidio R. 2012. Transgenic expression of polygalacturonase-inhibiting proteins in Arabidopsis and wheat increases resistance to the flower pathogen Fusarium graminearum. *Plant Biology* **14**: 31-38.

Galway ME, Eng RC, Schiefelbein JW, Wasteneys GO. 2011. Root hair-specific disruption of cellulose and xyloglucan in AtCSLD3 mutants, and factors affecting the post-rupture resumption of mutant root hair growth. *Planta* **233**(5): 985-999.

Hardham AR, Jones DA, Takemoto D. 2007. Cytoskeleton and cell wall function in penetration resistance. *Current Opinion in Plant Biology* **10**(4): 342-348.

Hensel G, Valkov V, Middlefell-Williams J, Kumlehn J. 2008. Efficient generation of transgenic barley: The way forward to modulate plant-microbe interactions. *Journal of Plant Physiology* **165**(1): 71-82.

Himmelbach A, Zierold U, Hensel G, Riechen J, Douchkov D, Schweizer P, Kumlehn J. 2007. A set of modular binary vectors for transformation of cereals. *Plant Physiology* **145**(4): 1192-1200.

Hu PS, Meng Y, Wise RP. 2009. Functional Contribution of Chorismate Synthase, Anthranilate Synthase, and Chorismate Mutase to Penetration Resistance in Barley-Powdery Mildew Interactions. *Molecular Plant-Microbe Interactions* **22**(3): 311-320.

Huckelhoven R. 2007. Cell wall - Associated mechanisms of disease resistance and susceptibility. *Annual Review of Phytopathology* **45**: 101-127.

Huckelhoven R, Panstruga R. 2011. Cell biology of the plant-powdery mildew interaction. *Current Opinion in Plant Biology* **14**(6): 738-746.

Hunter CT, Kirienko DH, Sylvester AW, Peter GF, McCarty DR, Koch KE. 2012. Cellulose Synthase-Like D1 Is Integral to Normal Cell Division, Expansion, and Leaf Development in Maize. *Plant Physiology* **158**(2): 708-724.

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB. 2003. An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* **15**(11): 2503-2513.

Kim CM, Park SH, Je BI, Park SH, Park SJ, Piao HL, Eun MY, Dolan L, Han CD. 2007. OsCSLD1, a cellulose synthase-like D1 gene, is required for root hair morphogenesis in rice. *Plant Physiol* **143**(3): 1220-1230.

Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter LL, Keller B. 2009. A Putative ABC Transporter Confers Durable Resistance to Multiple Fungal Pathogens in Wheat. *Science* **323**(5919): 1360-1363.

For Peer Review

582 **Kruger WM, Carver TLW, Zeyen RJ. 2002.** Effects of inhibiting phenolic biosynthesis on
583 penetration resistance of barley isolines containing seven powdery mildew resistance
584 genes or alleles. *Physiological and Molecular Plant Pathology* **61**(1): 41-51.
585 **Kurek I, Kawagoe Y, Jacob-Wilk D, Doblin M, Delmer D. 2002.** Dimerization of cotton fiber
586 cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains.
587 *Proceedings of the National Academy of Sciences of the United States of America*
588 **99**(17): 11109-11114.
589 **Kwon C, Bednarek P, Schulze-Lefert P. 2008.** Secretory pathways in plant immune
590 responses. *Plant Physiology* **147**(4): 1575-1583.
591 **Li M, Xiong GY, Li R, Cui JJ, Tang D, Zhang BC, Pauly M, Cheng ZK, Zhou YH. 2009.**
592 Rice cellulose synthase-like D4 is essential for normal cell-wall biosynthesis and plant
593 growth. *Plant Journal* **60**(6): 1055-1069.
594 **Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W,**
595 **Rosahl S, Scheel D, et al. 2005.** Pre- and postinvasion defenses both contribute to
596 nonhost resistance in Arabidopsis. *Science* **310**(5751): 1180-1183.
597 **Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B. 2014.** The carbohydrate-
598 active enzymes database (CAZy) in 2013. *Nucleic Acids Research* **42**(D1): D490-
599 D495.
600 **Luan WJ, Liu YQ, Zhang FX, Song YL, Wang ZY, Peng YK, Sun ZX. 2011.** OsCD1
601 encodes a putative member of the cellulose synthase-like D sub-family and is
602 essential for rice plant architecture and growth. *Plant Biotechnology Journal* **9**(4):
603 513-524.
604 **McDonald BA, Linde C. 2002.** The population genetics of plant pathogens and breeding
605 strategies for durable resistance. *Euphytica* **124**(2): 163-180.
606 **Morgan JLW, Strumillo J, Zimmer J. 2013.** Crystallographic snapshot of cellulose
607 synthesis and membrane translocation. *Nature* **493**(7431): 181-U192.
608 **Nemri A, Atwell S, Tarone AM, Huang YS, Zhao K, Studholme DJ, Nordborg M, Jones**
609 **JDG. 2010.** Genome-wide survey of Arabidopsis natural variation in downy mildew
610 resistance using combined association and linkage mapping. *Proceedings of the*
611 *National Academy of Sciences of the United States of America* **107**(22): 10302-
612 10307.
613 **Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC. 2003.** Loss of a
614 callose synthase results in salicylic acid-dependent disease resistance. *Science*
615 **301**(5635): 969-972.
616 **Nowara D, Gay A, Lacomme C, Shaw J, Ridout C, Douchkov D, Hensel G, Kumlehn J,**
617 **Schweizer P. 2010.** HIGS: Host-Induced Gene Silencing in the Obligate Biotrophic
618 Fungal Pathogen Blumeria graminis. *Plant Cell* **22**(9): 3130-3141.

For Peer Review

619 **Park S, Szumlanski AL, Gu FW, Guo F, Nielsen E. 2011.** A role for CSLD3 during cell-wall
620 synthesis in apical plasma membranes of tip-growing root-hair cells. *Nature Cell*
621 *Biology* **13**(8): 973-U227.
622 **Penning BW, Hunter CT, Tayengwa R, Eveland AL, Dugard CK, Olek AT, Vermerris W,**
623 **Koch KE, McCarty DR, Davis MF, et al. 2009.** Genetic Resources for Maize Cell
624 Wall Biology. *Plant Physiology* **151**(4): 1703-1728.
625 **Pettolino FA, Walsh C, Fincher GB, Bacic A. 2012.** Determining the polysaccharide
626 composition of plant cell walls. *Nature Protocols* **7**(9): 1590-1607.
627 **Pliego C, Nowara D, Bonciani G, Gheorghe DM, Xu R, Surana P, Whigham E, Nettleton**
628 **D, Bogdanove AJ, Wise RP, et al. 2013.** Host-Induced Gene Silencing in Barley
629 Powdery Mildew Reveals a Class of Ribonuclease-Like Effectors. *Molecular Plant-*
630 *Microbe Interactions* **26**(6): 633-642.
631 **Pryce-Jones E, Carver T, Gurr SJ. 1999.** The roles of cellulase enzymes and mechanical
632 force in host penetration by Erysiphe graminis f.sp hordei. *Physiological and*
633 *Molecular Plant Pathology* **55**(3): 175-182.
634 **Richmond TA, Somerville CR. 2000.** The cellulose synthase superfamily. *Plant Physiology*
635 **124**(2): 495-498.
636 **Scheller HV, Ulvskov P. 2010.** Hemicelluloses. *Annual Review of Plant Biology, Vol 61* **61**:
637 263-289.
638 **Schiff CL, Wilson IW, Somerville SC. 2001.** Polygenic powdery mildew disease resistance
639 in Arabidopsis thaliana: quantitative trait analysis of the accession Warschau-1. *Plant*
640 *Pathology* **50**(6): 690-701.
641 **Schweizer P. 2008.** Tissue-specific expression of a defence-related peroxidase in transgenic
642 wheat potentiates cell death in pathogen-attacked leaf epidermis. *Molecular Plant*
643 *Pathology* **9**(1): 45-57.
644 **Schweizer P, Stein N. 2011.** Large-Scale Data Integration Reveals Colocalization of Gene
645 Functional Groups with Meta-QTL for Multiple Disease Resistance in Barley.
646 *Molecular Plant-Microbe Interactions* **24**(12): 1492-1501.
647 **Seiffert U, Schweizer P. 2005.** A pattern recognition tool for quantitative analysis of in planta
648 hyphal growth of powdery mildew fungi. *Molecular Plant-Microbe Interactions* **18**(9):
649 906-912.
650 **Spanu PD, Abbott JC, Amselem J, Burgis TA, Soanes DM, Stuber K, van Themaat EVL,**
651 **Brown JKM, Butcher SA, Gurr SJ, et al. 2010.** Genome Expansion and Gene Loss
652 in Powdery Mildew Fungi Reveal Tradeoffs in Extreme Parasitism. *Science*
653 **330**(6010): 1543-1546.
654 **Stadnik MJ, Buchenauer H. 1999.** Accumulation of autofluorogenic compounds at the
655 penetration site of Blumeria graminis f. sp tritici is associated with both

For Peer Review

benzothiadiazole-induced and quantitative resistance of wheat. *Journal of Phytopathology-Phytopathologische Zeitschrift* **147**(10): 615-622.

Stadnik MJ, Buchenauer H. 2000. Inhibition of phenylalanine ammonia-lyase suppresses the resistance induced by benzothiadiazole in wheat to Blumeria graminis f. sp tritici. *Physiological and Molecular Plant Pathology* **57**(1): 25-34.

Volpi C, Janni M, Lionetti V, Bellincampi D, Favaron F, D'Ovidio R. 2011. The Ectopic Expression of a Pectin Methyl Esterase Inhibitor Increases Pectin Methyl Esterification and Limits Fungal Diseases in Wheat. *Molecular Plant-Microbe Interactions* **24**(9): 1012-1019.

Wawrzynska A, Rodibaugh NL, Innes RW. 2010. Synergistic Activation of Defense Responses in Arabidopsis by Simultaneous Loss of the GSL5 Callose Synthase and the EDR1 Protein Kinase. *Molecular Plant-Microbe Interactions* **23**(5): 578-584.

Wilson IW, Schiff CL, Hughes DE, Somerville SC. 2001. Quantitative trait loci analysis of powdery mildew disease resistance in the Arabidopsis thaliana accession Kashmir-1. *Genetics* **158**(3): 1301-1309.

Yin L, Verhertbruggen Y, Oikawa A, Manisseri C, Knierim B, Prak L, Jensen JK, Knox JP, Auer M, Willats WGT, et al. 2011. The Cooperative Activities of CSLD2, CSLD3, and CSLD5 Are Required for Normal Arabidopsis Development. *Molecular Plant* **4**(6): 1024-1037.

Yu Y, Tomkins JP, Waugh R, Frisch DA, Kudrna D, Kleinhofs A, Brueggeman RS, Muehlbauer GJ, Wise RP, Wing RA. 2000. A bacterial artificial chromosome library for barley (Hordeum vulgare L.) and the identification of clones containing putative resistance genes. *Theoretical and Applied Genetics* **101**(7): 1093-1099.

Yuo T, Shiotani K, Shitsukawa N, Miyao A, Hirochika H, Ichii M, Taketa S. 2011. Root hairless 2 (rth2) mutant represents a loss-of-function allele of the cellulose synthase-like gene OsCSLD1 in rice (Oryza sativa L.). *Breeding Science* **61**(3): 225-233.

Zhu JH, Lee BH, Dellinger M, Cui XP, Zhang CQ, Wu S, Nothnagel EA, Zhu JK. 2010. A cellulose synthase-like protein is required for osmotic stress tolerance in Arabidopsis. *Plant Journal* **63**(1): 128-140.

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FIGURE LEGENDS

Figure 1: The cellulose synthase-like protein HvCslD2 mediates NHR- and QR in barley.
(A) Domains and motifs of the encoded barley protein HvCslD2. (B) Generation of a RNAi-rescue construct of Hv*CslD2* in order to complement RNAi effects. 35S, Cauliflower mosaic virus *35S* promoter; E1-3, first to third exon; I1-2, introns 1 and 2; T, *35S* terminator; mut, saturated by silent point mutations; wt, wildtype. (C) Complementation of QR by the RNAi-rescue construct pIPKTA09_HvCslD2_rescue of *CslD2* in leaf epidermal cells with transiently silenced Hv*CslD2*. In addition, wildtype Hv*CslD2* cDNA was transiently expressed in the absence of an RNAi construct. Mean ± SEM of the log(2)-transformed relative susceptibility index (SI) compared to the empty vector control (n=5 independent bombardments). Two and three asterisks indicate significant construct effect with $p<0.01$ and $p<0.001$, respectively. (D) Complementation of nonhost resistance by the Hv*CslD2* RNAi rescue construct in bombarded epidermal cells of Hv*CslD2*-silenced transgenic plants (E39). Mean ± SEM from 3-7 bombardments in two experimental series. Different letters above columns indicate significant group mean values with $p<0.05$ (1-way ANOVA).

Figure 2: Silencing of Hv*CslD2* in transgenic plants specifically enhanced disease susceptibility.
(A) Reduced nonhost- and quantitative host resistance in segregating populations of transgenic T1 plants. Penetration efficiency (PE) of *B. graminis* f.sp. *tritici* (*Bgt*) on the second leaf was determined under the microscope 48 h after inoculation. Transgenic plants expressing *GFP* or a *GFP*-silencing construct were used as additional controls. Infection severity of *B. graminis* f.sp. *hordei* (*Bgh*) was scored macroscopically on third to fifth leaf 7 days after inoculation and related to average infection values of wildtype plants. The red bars indicate the two selected events RNR6_RNAi_E28 (E28) and RNR6_RNAi_E39 (E39) used in all subsequent experiments.
(B) Silencing of Hv*CslD2* in leaves of E28 and E39 as shown by RNA-blot hybridization. RNA was isolated from single transgenic plants of E28 or E39, from pools of azygous individuals from the segregating T2 lines used, or from wildtype plants. Ethidium bromide-stained gel loading and blotting controls are shown below. (C) Normal growth of homozygous T2 plants of E28 and E39, compared to wildtype or *GUS*-expressing transgenic control plants.
(D and E) Normal formation of root hairs by a transgenic plant of E39.
(F and G) Nonhost immunity of wildtype plants (F) and abundant hyphal growth on a leaf of a transgenic T2 plant of E28 (G) at 6 d after inoculation. Bars, 50 µM.

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(H) Sporulation (arrow) of *Bgt* on a transgenic T2 plant of E28 at 5 d after inoculation. Bar, 100 µM.

Figure 3: Altered cell-wall characteristics of Hv*CslD2*-silenced, transgenic plants.
(A) Increased susceptibility of cell walls to fungal cell-wall degrading enzymes. Epidermal cell layers of first leaves were fixed onto glass slides and exposed to a mixture of cellulase, pectinase and hemicellulase enzymes, followed by microscopic assessment of the degree of dissolution of cross cell walls under the microscope at the times indicated. Mean ± SEM from 3 digestion experiments. wt, wildtype plants; azygous, hygromycin-sensitive individuals from segregating T2 lines of E28 and E39. Mean values were compared by 1-way ANOVA; “ab”, significantly different from azygous control; “b”, different from azygous and wildtype (wt) controls.
(B) Altered cellulose-to-(glucurono)arabinoxylan ratio in leaf epidermal cell walls of non-inoculated barley transgenic lines. Plants of T3 lines were used for cell-wall preparations, followed by methylation analysis of major cell-wall components. wt, Golden Promise wildtype plants.
(C) Relative fluorescence intensity of cellulose and autofluorescence in epidermal papillae formed against *B. graminis* f.sp. *hordei* at 18 hai on leaves from wildtype (wt) plants and transgenic T3 plants of E28 and E39 lines. The average maximum intensity was calculated using the maximum intensity found in the fluorescence intensity profile of 250 papillae and normalized against background tissue staining. In order to directly compare the different probes in one graph the relative fluorescence intensity was calculated as a percentage of the highest maximum found for each probe. Mean ± SEM
(D) Labelling of cellulose and autofluorescence in epidermal papillae formed against *B. graminis* f.sp. *hordei* at 18 hai on leaves from wildtype (wt) plants and transgenic T3 plants of the E39 line. The first row shows a bright field overlay and fungal tissues are labelled with wheat germ agglutinin (WGA-AF488) (green). The second row shows autofluorescence (blue) and the third row shows cellulose labelling with Pontamine Fast Scarlet 4B (red). Scale bar = 50 µm.
(E) Labelling of cellulose (red) and fungal tissues (green) in epidermal papillae formed against *B. graminis* f.sp. *hordei* at 18 hai on leaves from wildtype (wt) plants and transgenic T3 plants of the E39 line. Scale bar = 10 µm.

Figure 4: Autofluorescing phenolic compounds at cell-wall appositions of transgenic, Hv*CslD2*-silenced barley plants attacked by *Bgt*.
(A) Plants of the azygous control line AZ39 showed a lower percentage of penetrated cell-wall appositions (in total 17 observed sites) with detectable autofluorescence, compared to

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non-penetrated ones (in total 493 observed sites). In Hv*CslD2*-silenced T3 plants this difference in autofluorescence intensity between penetrated and non-penetrated call-wall appositions was weaker and statistically not significant. Quantitative microscopic examination of non-penetrated and penetrated cell-wall appositions of wildtype and transgenic lines was done 48 h after inoculation.

(B) Autofluorescence of phenolic compounds at sites of attempted penetration in wildtype (wt) and Hv*CslD2*-silenced plants. Bar = 20 µm.

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TABLES

Table 1: Regulation and TIGS effects of Hv*Csl* multigene family members.

Contig Nr. (HarvEST U35)[a]	Barley gene	Homologous rice gene	Probe name Agilent 44K oligonucleotide array	Regul PM[b] versus Ctr	Regul PM host versus NH[c]	cv. Maythorpe		cv. Golden Promise	
						Rel. SI[d] (log2)	p[e] rel. SI	Rel. SI (log2)	p rel. SI
18522	HvCslA01	LOC_Os02g09930	CUST_10688_PI390587928	NS[f]	NS	-0.14	0.3042	-0.88	0.0527
8056	HvCslA02	LOC_Os10g26630	CUST_8464_PI390587928	DOWN	NS	-1.08	**0.0076**[g]	-0.69	**0.0077**
32868	HvCslA03	LOC_Os06g12460	CUST_17205_PI390587928	NS	NS	n.a.[h]	n.a.	n.a.	n.a.
12348	HvCslA04	LOC_Os03g07350	CUST_38987_PI390587928	NS	NS	n.a.	n.a.	n.a.	n.a.
5565	HvCslA07	LOC_Os07g43710	CUST_13694_PI390587928	NS	NS	-0.67	0.1898	-0.32	0.1701
36995	HvCslA09	LOC_Os06g12460	CUST_23487_PI390587928	NS	NS	n.a.	n.a.	n.a.	n.a.
6978	HvCslA11	LOC_Os08g33740	CUST_9190_PI390587928	DOWN	YES	0.51	**0.0032**	-0.79	**0.0329**
38464	HvCslC03	LOC_Os08g15420	CUST_26346_PI390587928	NS	NS	-0.45	0.3604	-0.37	0.1904
15154	HvCslC07	LOC_Os05g43530	CUST_22686_PI390587928	NS	NS	n.a.	n.a.	n.a.	n.a.
15157	HvCslC09	LOC_Os03g56060	CUST_22683_PI390587928	NS	NS	n.a.	n.a.	-0.06	0.3205
HDP12I23[i]	HvCslC10	LOC_Os07g03260	Not present	n.a.	n.a.	-1.29	**0.0103**	-0.76	**0.0210**
HDP39E18[i]	HvCslD01	LOC_Os10g42750	Not present	n.a.	n.a.	n.a.	n.a.	-0.91	**0.0476**
17157	HvCslD02	LOC_Os06g02180	CUST_35946_PI390587928	UP	YES	1.08	**0.0003**	0.29	**0.0290**

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29568	HvCslD04	LOC_Os12g36890	CUST_26918_PI390587928	DOWN	YES	0.39	0.1374	n.a.	n.a.
42209	HvCslE01	LOC_Os09g30130	CUST_1561_PI390587928	NS	NS	n.a.	n.a.	n.a.	n.a.
37339	HvCslE02	LOC_Os02g49332	CUST_39365_PI390587928	NS	NS	-0.22	0.0990	-0.66	0.0670
2913	HvCslE06	LOC_Os09g30130	CUST_17766_PI390587928	NS	NS	-0.82	0.0886	-0.75	**0.0008**
21134	HvCslF03	LOC_Os07g36750	CUST_1168_PI390587928	NS	NS	-0.01	0.1071	-0.22	**0.0188**
1914	HvCslF06	LOC_Os08g06380	CUST_36245_PI390587928	DOWN	YES	-0.39	0.4541	-0.03	0.4445
10863	HvCslF08	LOC_Os07g36630	CUST_2795_PI390587928	NS	NS	n.a.	n.a.	n.a.	n.a.
22777	HvCslF09	LOC_Os07g36610	CUST_22096_PI390587928	NS	NS	-0.73	0.2920	n.a.	n.a.
MLOC_13463.1[i]	HvCslF10	Not present	Not present	n.a.	n.a.	n.a.	n.a.	-0.54	0.0701
3097	HvCslH01	LOC_Os04g35030	CUST_39045_PI390587928	NS	NS	-1.11	**0.0357**	-0.47	0.0862

[a]Unigene number in assembly 35 of HarvEST database of expressed sequence tags from barley (http://harvest.ucr.edu/).

[b]PM, powdery mildew.

[c]NH, nonhost.

[d]Log(2)-transformed susceptibility index, normalized to the empty vector control pIPKTA30.

[e]p-value from 1-sample t-test against null-hypothetical value “0”.

[f]NS, non-significant based on corresponding statistical test.

[g]Significant TIGS effects are highlighted in bold.

[h]Not analyzed.

[i]EST or barley high-confidence gene ID (NCBI) because no HarvEST unigene available.

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SUPPORTING INFORMATION

Table S1:

Co-segregation of initial colony growth of *Bgt* with the presence of the *hpt* selectable marker gene in transgenic barley.

Table S2:

Summary of rust interaction phenotypes of RNAi events silenced in Hv*CslD2* expression.

Table S3:

Summary of cell-wall composition of non-inoculated plants of RNAi events silenced in Hv*CslD2* expression.

Table S4:

Grain yield in the greenhouse of RNAi events silenced in Hv*CslD2* expression.

Table S5:

Predicted cell-wall degrading enzymes encoded by the *Bgh* genome.

Figure S1:

Phylogenetic tree of Csl protein sequences from barley, rice and D-clade members of *A. thaliana*.

Figure S2:

Alignment of DNA and protein sequences at the C-terminal part of Hv*CslD2* wildtype cDNA and the RNAi rescue construct (pIPKTA09_HvCslD2_rescue).

Figure S3:

Transcript profiles of significantly *B. graminis*-regulated Hv*Csl* family members in barley epidermal peels.

Figure S4:

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Mutagenesis of *AtCslD2* and *AtCslD3* (*Kojak*) has no effect on NHR and QR in *A. thaliana*.

Figure S5:

Tissue-panel expression data of selected members of the *CslD* clade in *A. thaliana*, rice and barley, derived from the Genevestigator database.

Figure S6:

Significant transcript regulation of Hv*CslD2* (Affymetrix "Barley1" chip, probeset rbasd15h01_s_at) in barley leaves inoculated with *Bgh*, data derived from PlexDB database).

Figure S7:

No significant regulation of *AtCslD2* (Affymetrix ATH1 chip Contig246460_at) and *AtCslD3* (Contig258850_at) in *Erysiphe orontii*-attacked *A. thaliana* leaves, data derived from PlexDB database).

Figure S8:

Co-localization of Hv*CslD2* map position with a Meta-QTL for resistance to *Bgh*.

Figure S9: Silencing of the Hv*CslD2* target gene in leaves and also in roots of transgenic plants.

Figure S10:

Schematic map of recombinant binary Gateway-compatible vector pIPKb009_HvCslD2 used for the generation of Hv*CslD2*-silenced transgenic barley (Himmelbach *et al.*, 2007).

Notes S1:

Multiple FastA file of all the spliced-genomic or cDNA sequences of Hv*Csl* genes that were used for the construction of the phylogenetic tree shown in Figure S1.

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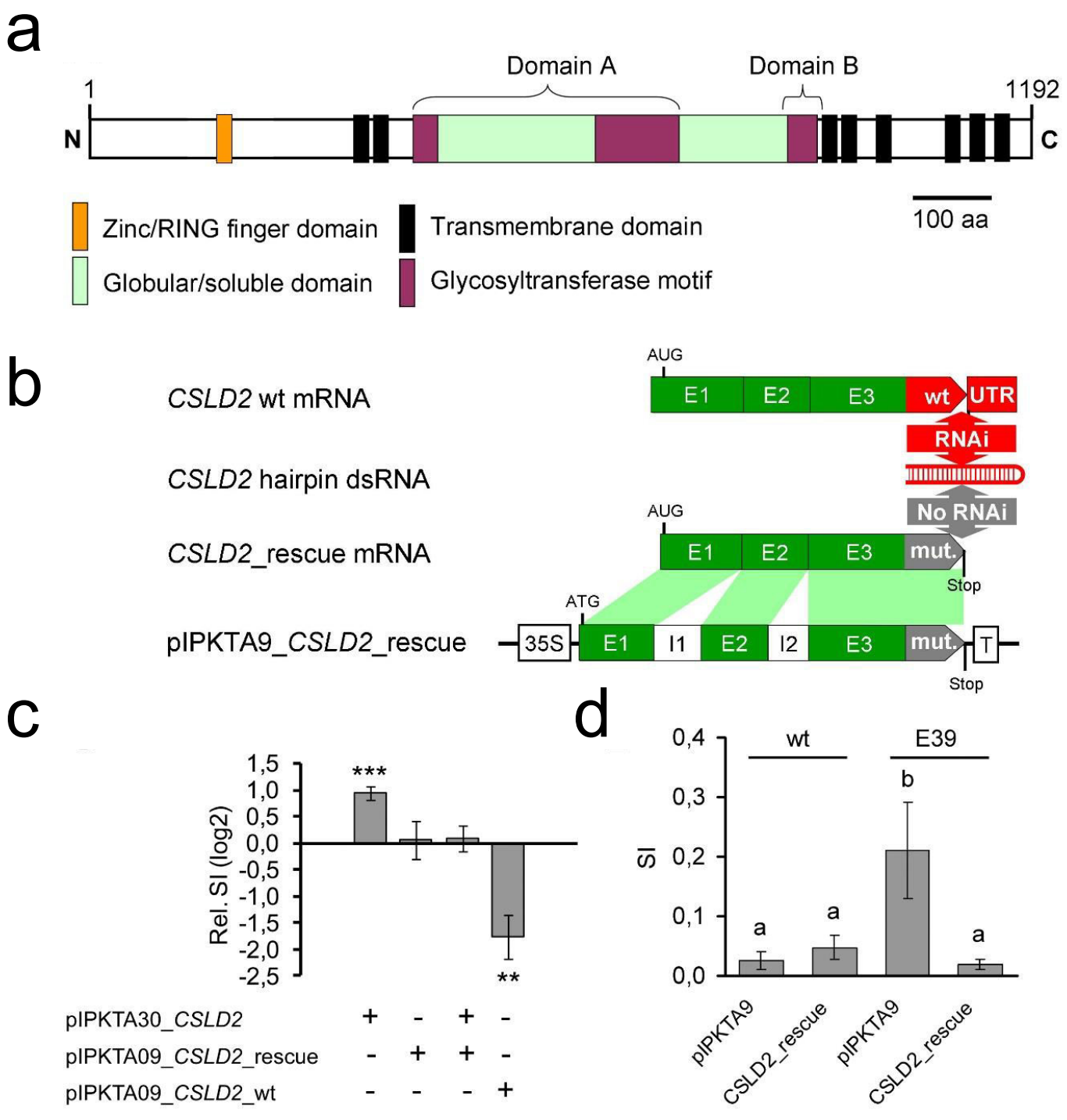
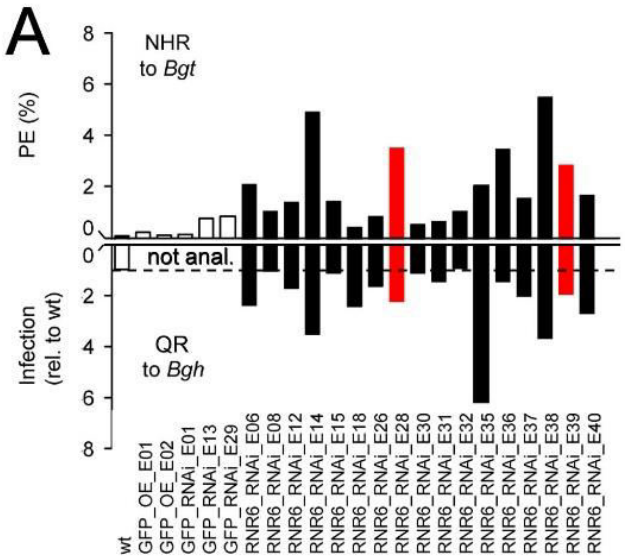

a
Domain A
Domain B
1
1192
N
C
100 aa
Zinc/RING finger domain
Transmembrane domain
Globular/soluble domain
Glycosyltransferase motif
b
CSLD2 wt mRNA
CSLD2 hairpin dsRNA
CSLD2_rescue mRNA
pIPKTA9_CSLD2_rescue
AUG
E1
E2
E3
wt
UTR
RNAi
No RNAi
mut.
Stop
ATG
35S
I1
I2
T
c
Rel. SI (log2)
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
***
**
pIPKTA30_CSLD2
pIPKTA09_CSLD2_rescue
pIPKTA09_CSLD2_wt
d
SI
0,4
0,3
0,2
0,1
0,0
wt
E39
a
b
pIPKTA9
CSLD2_rescue


A
NHR
to Bgt
PE (%)
not anal.
Infection
(rel. to wt)
QR
to Bgh
wt
GFP_OE_E01
GFP_OE_E02
GFP_RNAi_E01
GFP_RNAi_E13
GFP_RNAi_E29
RNR6_RNAi_E06
RNR6_RNAi_E08
RNR6_RNAi_E12
RNR6_RNAi_E14
RNR6_RNAi_E15
RNR6_RNAi_E18
RNR6_RNAi_E26
RNR6_RNAi_E28
RNR6_RNAi_E30
RNR6_RNAi_E31
RNR6_RNAi_E32
RNR6_RNAi_E35
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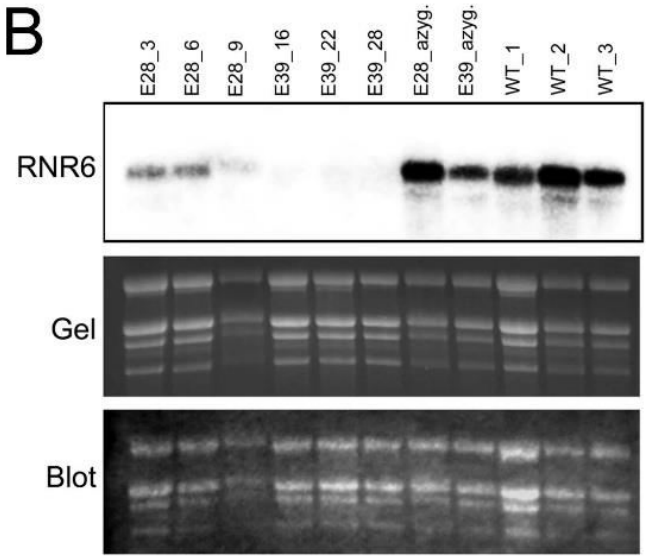
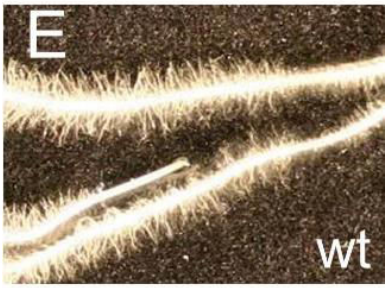
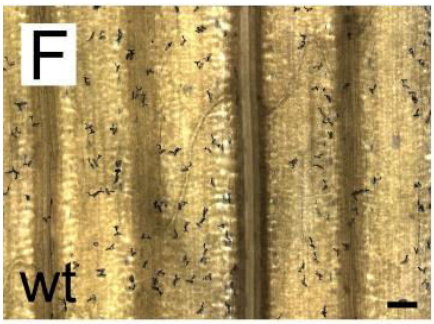
RNR6_RNAi_E39
RNR6_RNAi_E40

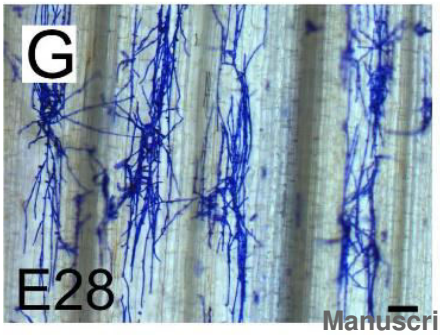

B
E28_3
E28_6
E28_9
E39_16
E39_22
E39_28
E28_azyg.
E39_azyg.
WT_1
WT_2
WT_3
RNR6
Gel
Blot


C
50 cm
wt
GUS
E28
E39

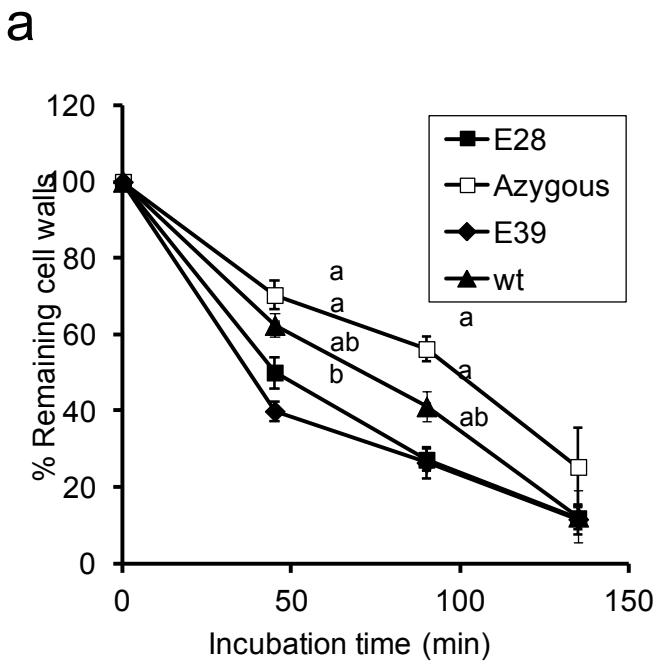

D
E39

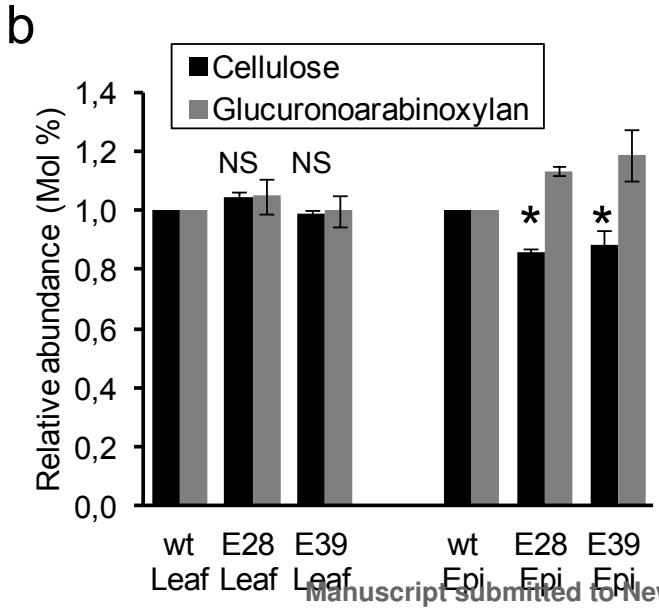

E
wt

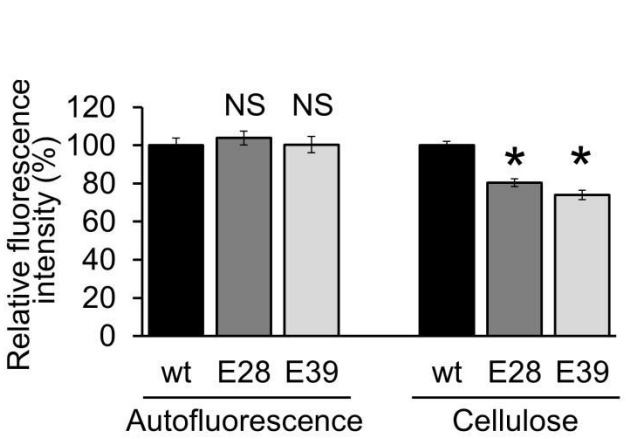

F
wt

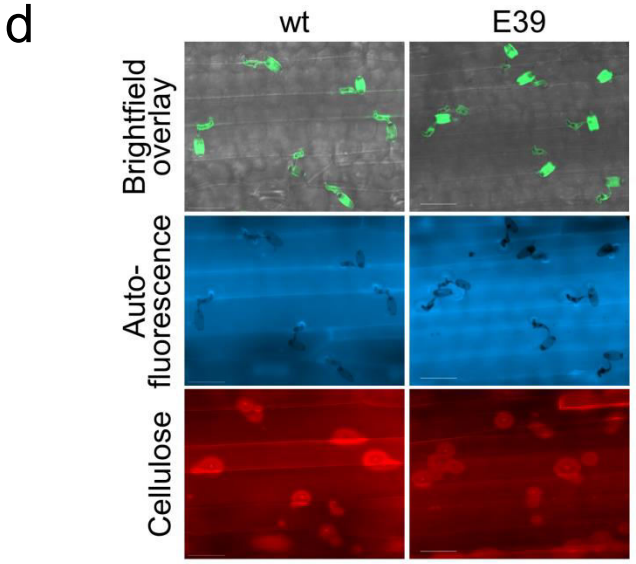

G
E28

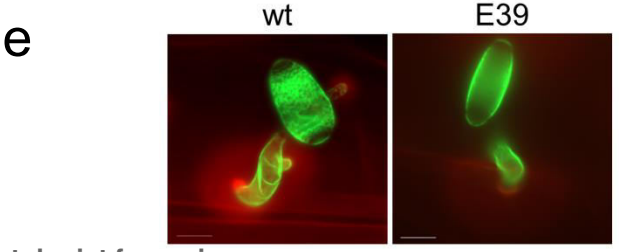

H
E28


a
% Remaining cell walls
Incubation time (min)
E28
Azygous
E39
wt
a
a
ab
b
a
a
ab

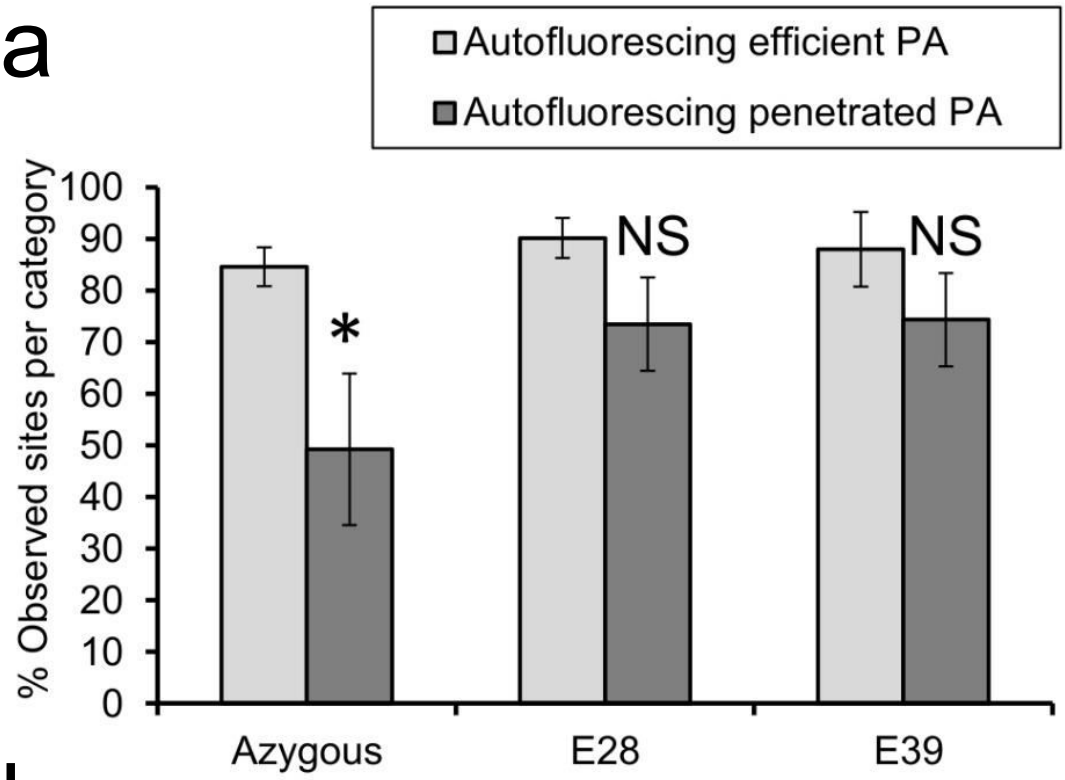

b
Relative abundance (Mol %)
Cellulose
Glucuronoarabinoxylan
NS
NS
*
*
wt Leaf
E28 Leaf
E39 Leaf
wt Epi
E28 Epi
E39 Epi


c
Relative fluorescence intensity (%)
NS
NS
*
*
wt
E28
E39
wt
E28
E39
Autofluorescence
Cellulose

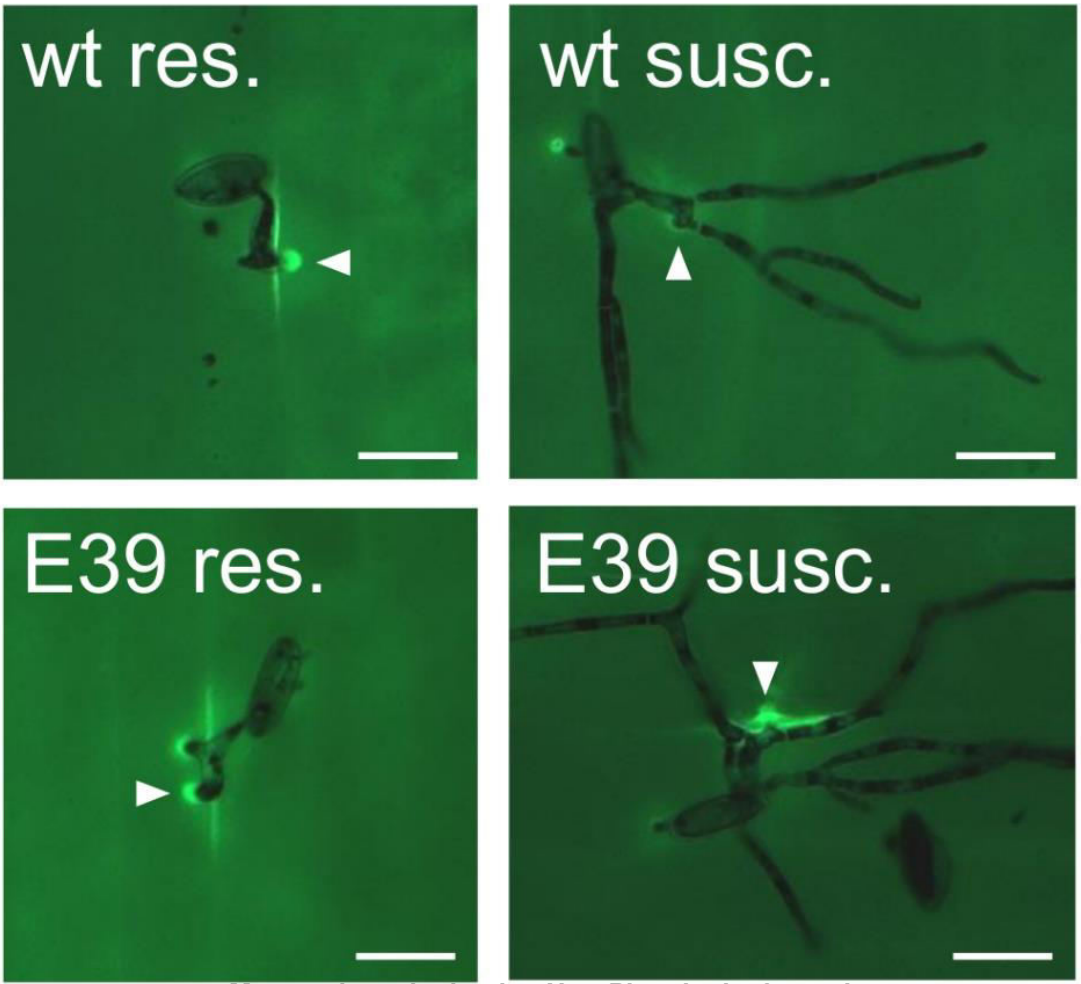

d
wt
E39
Brightfield overlay
Auto-fluorescence
Cellulose


e
wt
E39

a


Autofluorescing efficient PA
Autofluorescing penetrated PA
% Observed sites per category
100
90
80
70
60
50
40
30
20
10
0
*
NS
NS
Azygous
E28
E39

b


wt res.
wt susc.
E39 res.
E39 susc.