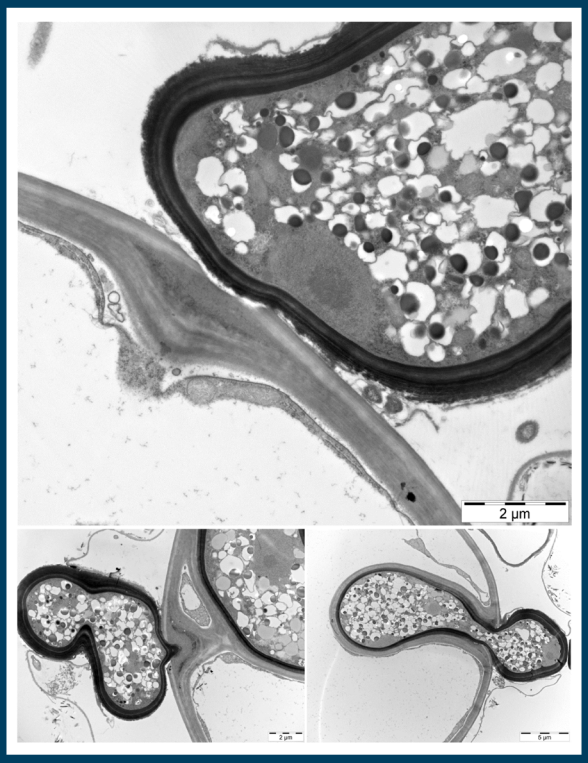
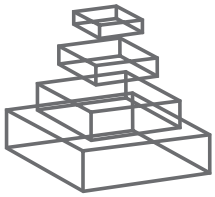
frontiers
RESEARCH TOPICS



PLANT CELL WALL IN PATHOGENESIS, PARASITISM AND SYMBIOSIS

Topic Editors
Vincenzo Lionetti and Jean-Pierre Métraux

frontiers in
PLANT SCIENCE



frontiers

ISSN 1664-8714
ISBN 978-2-88919-442-1
DOI 10.3389/978-2-88919-442-1

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

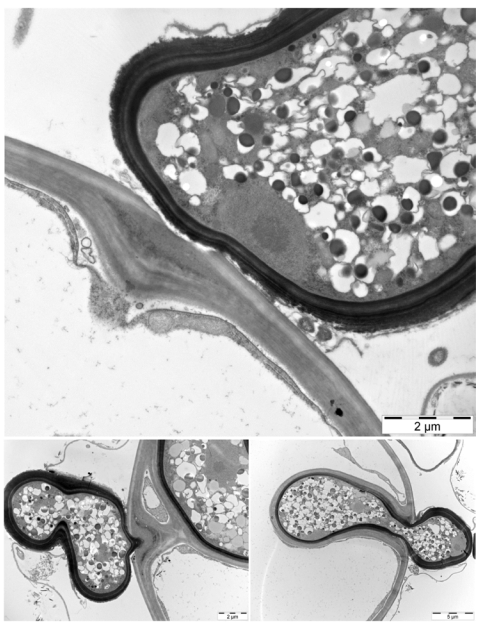
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

PLANT CELL WALL IN PATHOGENESIS, PARASITISM AND SYMBIOSIS

Topic Editors:
Vincenzo Lionetti, Sapienza Università di Roma, Italy
Jean-Pierre Métraux, Université de Fribourg, Switzerland



Serial sections showing dynamics of plant cell wall at a penetration site of Rhizophagus irregularis at the level of the hypodermis of the outer root cell layers of Petunia hybrida.

The cell wall is a complex structure mainly composed of cellulose microfibrils embedded in a cohesive hemicellulose and pectin matrix. Cell wall structural proteins, enzymes and their inhibitors are also essential components of plant cell walls. They are involved in the cross-link of cell wall polysaccharides, wall structure, and the perception and signaling of defense-related elicitors at the cell surface. In the outer part of the epidermal cells, the polysaccharides are coated by the cuticle, consisting of hydrophobic cutin, suberin and wax layers. Lignin, a macromolecule composed of highly cross-linked phenolic molecules, is a major component of the secondary cell wall. The cell wall is the first cell structure on which interactions between plants and a wide range of other organisms, including insects, nematodes, pathogenic or symbiotic micro-organisms take place. It not only represents a barrier that limits access to the cellular contents that provide a rich nutrient source for pathogens but serves as a source of elicitors of plant defense responses released upon partial enzymatic degradation of wall polysaccharides during infection. Modification of the plant cell wall can also occur at the level of plasmodesmata during virus infection as well as during abiotic stresses. The fine structure and composition of the plant cell wall as well as the regulation of its biosynthesis can thus strongly influence resistance and susceptibility to pathogens. This Research Topic provides novel insights and detailed overviews on the dynamics of the plant cell wall in plant defence, parasitism and symbiosis and describes experimental approaches to study plant cell wall modifications occurring during interaction of plants with different organisms.

Table of Contents

frontiers in
PLANT SCIENCE

EDITORIAL
published: 06 November 2014
doi: 10.3389/fpls.2014.00612

Plant cell wall in pathogenesis, parasitism and symbiosis

Vincenzo Lionetti[1]* and Jean-Pierre Métraux[2]

[1] *Dipartimento di Biologia e Biotecnologie "Charles Darwin," Sapienza Università di Roma, Rome, Italy*
[2] *Department of Biology, University of Fribourg, Fribourg, Switzerland*
**Correspondence: vincenzo.lionetti@uniroma1.it*

Edited by:
Giulia De Lorenzo, Sapienza, Università di Roma, Italy

Reviewed by:
Riccardo Angelini, University Roma Tre, Italy

Keywords: plant cell wall integrity, susceptibility factors, methanol, callose, plant symbioses, cell wall degrading enzymes, plant pathogens, plant parasitic nematode

The cell wall is the foremost interface at which interactions take place between plants and a wide range of other organisms including insects, nematodes, pathogenic, or symbiotic microorganisms.

This Research Topic includes Mini Reviews, Reviews, Original Research Articles, and Perspective Articles that provide novel insights and detailed overviews on the dynamics of the plant cell wall in plant defense, parasitism, and symbiosis and describes experimental approaches to study plant cell wall modifications occurring during interaction of plants with different organisms.

The cuticle represents the first cell wall layer encountered by pathogens. Serrano and collaborators describe the influence of the cuticle components on microbial development during pathogenesis (Serrano et al., 2014). The authors highlight also the alterations of the cuticle that induce defense responses against necrotrophs. Once the microbe has penetrated, the cell wall becomes a battleground where plants and pathogens attempt to prevail. Necrotrophic fungi produce several cell wall degrading enzymes (CWDEs) to degrade plant cell wall polysaccharides that favor plant colonization. Blanco-Ulate and co-authors present a genome-wide transcriptional profiling of *Botrytis cinerea* CWDEs expressed during infection of important nutritional resources such as lettuce leaves, ripe tomato fruit, and grape berries (Blanco-Ulate et al., 2014).

Plants develop different cell wall related systems for sensing intruders. Malinovsky and co-workers provide an overview of the cell surface pattern recognition receptors (PRRs) that perceive a diverse set of microbial molecules referred to as microbial/pathogen-associated-molecular patterns (MAMPs/PAMPs) and trigger immune responses (Malinovsky et al., 2014). Bellincampi and co-authors illustrate the mechanisms of sensing the alteration of cell wall integrity (CWI) during biotic stress and explain how cell walls can be a source of the so-called damage-associated molecular patterns (DAMPs) (Bellincampi et al., 2014). Three manuscripts of this research topic deal with specific mechanisms of perception and signaling of cell wall damage and modulation of plant immunity. Emerging evidence indicates that the Arabidopsis plasma membrane-localized protein NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE 1) functions as key signaling component of the loss of membrane-cell wall adhesions during pathogen infection. The research article by Lu and co-workers reports on the identification of a citrus ortholog CsNDR1 that, when overexpressed in Arabidopsis, improves disease resistance to *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* (Lu et al., 2013). Komarova and co-authors discuss on the role of methanol as signal in plant immunity (Komarova et al., 2014). Methanol (MeOH) can be released by plant pectin methylesterases, induced by the mechanical damage of plant tissues during microbe penetration. The emission of MeOH by a wounded plant can have a priming effect, enhancing the resistance of the non-wounded, neighboring receiver plants to pathogens. Tauzin and Giardina review the emerging argument of the "sweet immunity" (Tauzin and Giardina, 2014). The authors describe the involvement of sucrose and cell wall invertases as priming agents important for triggering an appropriate defense responses during pathogen invasion.

During pathogen infection plants can repair to the loss of CWI by activating cell wall reinforcing mechanisms (Bellincampi et al., 2014). At sites of interaction with intruding microbial pathogens the cell wall is actively reinforced through the deposition of cell wall appositions, so-called papillae. Voigt present a perspective article that discusses the possible roles of the (1,3)-β-glucan callose and the other papillae components in plant defense (Voigt, 2014). Lignin is both pathogen-induced and developmentally deposited in the secondary thickened cell wall. In their review article Miedes and collaborators update us on the effect of altered lignin amount and composition on pathogen infection and spread (Miedes et al., 2014).

Pathogens, parasites, and symbionts may exploit the host cell wall metabolism to support the colonization of their hosts (Bellincampi et al., 2014). An important remodeling of the plant cell wall is also exploited during plant parasitism by cyst nematodes. Bohlmann and Sobczak present a detailed overview of the cell wall degrading and modifying enzymes that nematodes produce during migration through the root, and the cell wall modifications occurring during syncytium development due to the cell wall synthetizing, modifying, and degrading proteins of the plant (Bohlmann and Sobczak, 2014). Two articles of this research topic report on the cell wall remodeling during mutualistic symbiosis of plants with different microorganisms, useful to establish an intimate interface for developmental coordination and nutrient

exchange. Rich and co-workers put the emphasis on modifications of the cell wall compartment during penetration and establishment of the symbiotic interface in root nodule symbiosis and arbuscular mycorrhizae (Rich et al., 2014). Genetic, genomic, and transcriptomic analysis related to symbiotic signaling, cell wall loosening and penetration, and the nutrition of the microbial endosymbionts are assessed. The current knowledge on the dynamics of plant and fungal walls in ectomycorrhizae and arbuscular-mycorrhizae is presented in the review article provided by Balestrini and Bonfante (2014). The cumulative evidence suggests that the plant CWDEs play a central role in the softening and remodeling of the cell wall during symbioses.

The analysis of the complex and dynamic modification of cell walls in response to pathogens is a technical challenge. Xia and co-authors summarize experimental approaches based on cell imaging, spectroscopic analyses and metabolic profiling techniques (Xia et al., 2014). The review by Delaunois and co-worker provides an insight into the modulation of the apoplastic protein patterns during pathogen infection (Delaunois et al., 2014). Super-resolution microscopy using microscopy combined with specific and efficient labeling techniques yield information on three-dimensional modifications of cell wall polymers (i.e., callose-cellulose network) at the site of attempted microbial penetration (Voigt, 2014). Carbohydrate microarrays, combining the specificity of monoclonal antibodies and carbohydrate binding modules with the multiplexed analysis capacity of microarrays, represent a promising technique to study the changes in cell wall micro-domains during plant/biotic interaction (Malinovsky et al., 2014).

These outstanding contributions reflect the considerable progress that has been made in the understanding the relationships at the cell wall interface between plants and other organisms. These publications represent our current knowledge on important physiological processes and provide food for thought for future research. As a concluding remark, we are grateful to the authors who responded enthusiastically to the call and reviewers for their valuable feedback to ensure the highest quality in the articles.

REFERENCES

Balestrini, R., and Bonfante, P. (2014). Cell wall remodeling in mycorrhizal symbiosis: a way towards biotrophism. *Front. Plant Sci.* 5:237. doi: 10.3389/fpls.2014.00237

Bellincampi, D., Cervone, F., and Lionetti, V. (2014). Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. *Front. Plant Sci.* 5:228. doi: 10.3389/fpls.2014.00228

Blanco-Ulate, B., Morales-Cruz, A., Amrine, K. C. H., Labavitch, J. M., Powell, A. L. T., and Cantu, D. (2014). Genome-wide transcriptional profiling of *Botrytis cinerea* genes targeting plant cell walls during infections of different hosts. *Front. Plant Sci.* 5:435. doi: 10.3389/fpls.2014.00435

Bohlmann, H., and Sobczak, M. (2014). The plant cell wall in the feeding sites of cyst nematodes. *Front. Plant Sci.* 5:89. doi: 10.3389/fpls.2014.00089

Delaunois, B., Jeandet, P., Clement, C., Baillieul, F., Dorey, S., and Cordelier, S. (2014). Uncovering plant-pathogen crosstalk through apoplastic proteomic studies. *Front. Plant Sci.* 5:249. doi: 10.3389/fpls.2014.00249

Komarova, T. V., Sheshukova, E. V., and Dorokhov, Y. L. (2014). Cell wall methanol as a signal in plant immunity. *Front. Plant Sci.* 5:101. doi: 10.3389/fpls.2014.00101

Lu, H., Zhang, C., Albrecht, U., Shimizu, R., Wang, G. F., and Bowman, K. D. (2013). Overexpression of a citrus NDR1 ortholog increases disease resistance in Arabidopsis. *Front. Plant Sci.* 4:157. doi: 10.3389/fpls.2013.00157

Malinovsky, F. G., Fangel, J. U., and Willats, W. G. T. (2014). The role of the cell wall in plant immunity. *Front. Plant Sci.* 5:178. doi: 10.3389/fpls.2014.00178

Miedes, E., Vanholme, R., Boerjan, W., and Molina, A. (2014). The role of the secondary cell wall in plant resistance to pathogens. *Front. Plant Sci.* 5:358. doi: 10.3389/fpls.2014.00358

Rich, M. K., Schorderet, M., and Reinhardt, D. (2014). The role of the cell wall compartment in mutualistic symbioses of plants. *Front. Plant Sci.* 5:238. doi: 10.3389/fpls.2014.00238

Serrano, M., Coluccia, F., Torres, M., L'Haridon, F., and Metraux, J. P. (2014). The cuticle and plant defense to pathogens. *Front. Plant Sci.* 5:274. doi: 10.3389/fpls.2014.00274

Tauzin, A. S., and Giardina, T. (2014). Sucrose and invertases, a part of the plant defense response to the biotic stresses. *Front. Plant Sci.* 5:293. doi: 10.3389/fpls.2014.00293

Voigt, C. A. (2014). Callose-mediated resistance to pathogenic intruders in plant defense-related papillae. *Front. Plant Sci.* 5:168. doi: 10.3389/fpls.2014.00168

Xia, Y., Petti, C., Williams, M. A., and DeBolt, S. (2014). Experimental approaches to study plant cell walls during plant-microbe interactions. *Front. Plant Sci.* 5:540. doi: 10.3389/fpls.2014.00540

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 October 2014; accepted: 20 October 2014; published online: 06 November 2014.

*Citation: Lionetti V and Métraux J-P (2014) Plant cell wall in pathogenesis, parasitism and symbiosis. Front. Plant Sci. **5**:612. doi: 10.3389/fpls.2014.00612*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 13 June 2014
doi: 10.3389/fpls.2014.00274

The cuticle and plant defense to pathogens

Mario Serrano, Fania Coluccia, Martha Torres, Floriane L'Haridon and Jean-Pierre Métraux*

Department of Biology, University of Fribourg, Fribourg, Switzerland

Edited by:
Vincenzo Lionetti, "Sapienza" Università di Roma, Italy

Reviewed by:
Prashant Singh, Iowa State University, USA
José Juan Reina-Pinto, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora" – Consejo Superior de Investigaciones Científicas – University of Málaga, Spain

***Correspondence:**
Jean-Pierre Métraux, Department of Biology, University of Fribourg, Chemin du Musée 10, 1700 Fribourg, Switzerland
e-mail: jean-pierre.metraux@unifr.ch

The cuticle provides a physical barrier against water loss and protects against irradiation, xenobiotics, and pathogens. Components of the cuticle are perceived by invading fungi and activate developmental processes during pathogenesis. In addition, cuticle alterations of various types induce a syndrome of reactions that often results in resistance to necrotrophs. This article reviews the current knowledge on the role of the cuticle in relation to the perception of pathogens and activation of defenses.

Keywords: *Arabidopsis*, innate immunity, *Botrytis cinerea*, resistance, cuticle, cutin monomers, wax, ROS

INTRODUCTION

The cuticle is a barrier coating the outer surface of epidermal cells of organs of the aerial parts of the plants. It protects against water loss, various abiotic and biotic stress. The structure and properties of the cuticle has received increased attention in the past years and a number of detailed reviews have been published (Martin, 1964; Kolattukudy, 1985; Goodwin and Jenks, 2005; Muller and Riederer, 2005; Reina-Pinto and Yephremov, 2009a; Schreiber, 2010; Domínguez et al., 2011a,b; Nawrath et al., 2013; Yeats and Rose, 2013). The cuticle is structurally diverse among species but exhibits the organization of a composite material consisting in cutin, a polyester that is partly covered and interspersed with waxes (epicuticular and intracuticular waxes). The epicuticular waxes and the cuticle with intracuticular waxes are referred to as the cuticle proper. The cuticle proper lies above a so-called cuticular layer made of cutin and polysaccharides that is closely associated with the cell wall of the underlying epidermis cell. The cutin polymer is typically made of esterified ω- and mid-chain hydroxy and epoxy C16 and C18 fatty acids and some glycerol (Heredia, 2003). This polymer can be cleaved by esterases and yields various cutin monomers. The cuticular wax, is a complex mixture of very long-chain fatty acids (C20–C40) and their derivatives that include alkanes, aldehydes, primary and secondary alcohols, ketones, and esters. Depending on the species, secondary metabolites, such as flavonoids and triterpenoids are also found among the wax components (Samuels et al., 2008). An increasing number of genes involved in the biosynthesis of the cuticle have been identified mainly in *Arabidopsis thaliana* and help to understand its biosynthesis (Pollard et al., 2008; Kunst and Samuels, 2009; Beisson et al., 2012; Bernard and Joubès, 2013; Lee and Suh, 2013). The overall picture of cutin synthesis whereby precursors are assembled in the cell and exported to the cell wall can now be completed but many details still remain unanswered; for example, the nature of the exported cutin or wax precursors, the process of extracellular assembly or the elements involved general control of this complex developmental process. Highlights of the advances in this area comprise the identification of an ABC transporter ABCG32/PEC1 involved in cuticle assembly (Bessire et al., 2011), the description of several classes of transcription factors involved in cutin and wax biosynthesis (Javelle et al., 2010; Seo et al., 2011; Nadakuduti et al., 2012) or post-transcriptional regulation of cuticle biosynthesis by the zinc-finger protein SERRATE (Voisin et al., 2009). The involvement of protein monoubiquitination in the regulation of cuticle biosynthesis was recently documented as several genes of cutin and wax biosynthetic pathway were found to be targets for histone H2B monoubiquitination (Ménard et al., 2014).

Here we will focus on the function of the plant cuticle in relation to the interaction with leaf pathogens.

THE CUTICLE AS A SOURCE OF SIGNALS

A number of recent reviews have been published that describe various aspects of the biological functions of the cuticle in relation to their physical and biochemical properties (Muller and Riederer, 2005; Reina-Pinto and Yephremov, 2009a). The focus of this chapter will be dedicated to the hypothesis that the cuticle might constitute a potential source of signals for the pathogens or for the plant itself.

PERCEPTION OF CUTICLE COMPONENTS BY FUNGI

Cutin hydrolysates were shown early on to induce the activity of an extracellular cutinase in *Fusarium solani pv. pisi*. Fractionation of the cutin hydrolysates established that the ω-hydroxy fatty acid fraction contained most of the activity. The optimal length of the aliphatic chain is 16 carbons, the activity mostly depends on a hydroxyl group at the ω carbon whereas the presence of the carboxyl group had no significant effect (Lin and Kolattukudy, 1978). Chemically synthesized cuticle monomers

also activate fungal development (Ahmed et al., 2003). Kolattukudy (1985) proposed that cuticle-degrading pathogens sense plant surfaces by cutin monomers that activate fungal cutinolytic activity. Cutin monomers are initially generated by basal cutinase activity in fungal spores landing on plant surfaces. Sensing of cutin monomers would then induce high levels of cutinase required for penetration. The induction of cutinase in *F. oxysporum* results from a transcriptional activation (Woloshuk and Kolattukudy, 1986). Furthermore, a transcription factor CTF1 was identified that binds to a G-rich palindromic binding site of the cutinase promoter (Kamper et al., 1994). Cuticular components can also induce other aspects of fungal developmental. For example, cutin monomers induce the germination and appressorium in the rice blast fungus *Magnaporthe grisea* (Gilbert et al., 1996); and appressorial tube formation in *Erysiphe graminis* (Francis et al., 1996). Cutin monomers also induce a protein kinase, LIPK (lipid-induced protein kinase) in *Colletotrichum trifolii*, the causal agent of alfalfa anthracnose. LIPK is essential for triggering infection structure formation in the fungus (Dickman et al., 2003). Besides cutin monomers, surface waxes also activate development processes in fungi. For instance, surface waxes of avocado, including terpenoid components, induce germination and appressorium formation in *C. gloeosporioides*, a pathogen of avocado, while waxes from other plants were not effective (Podila et al., 1993; Kolattukudy et al., 1995). Chloroform extracts of wax from wheat leaf surfaces induce appressorium in *Puccinia graminis* f.sp. *tritici* (Reisige et al., 2006). Appressorium formation in the rice pathogen *M. grisea* is induced by leaf wax of rice or other plants or synthetic n-C22 fatty acid, fatty alcohol or alkane (Hegde and Kolattukudy, 1997). Recently, it was shown that the prepenetration processes of the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* is stimulated by very-long-chain aldehydes that are wax constituents of the cuticle (Ringelmann et al., 2009; Hansjakob et al., 2010, 2011). For example, during the formation of the primary germ tube in *Blumerica graminis* f.sp. *hordei*, very-long-chain aldehydes (typical components of surface waxes) can stimulate the migration of the nucleus inside the conidia toward the site of primary germ tube emergence (Hansjakob et al., 2012).

Taken together, these observations document the perception of cuticular components by fungi. In the next section, we will show that the plant itself can also detect and react to components of the cuticle.

THE CUTICLE AND THE PERCEPTION OF ITS PRODUCTS BY THE PLANT

The action of fungal cutinase and related enzymes during the early stages of fungal contact with plant surfaces prepares the infection site both for adhesion and penetration (Deising et al., 1992; Nielsen et al., 2000). Cuticle breakdown products constitute potential signals perceived by the plant that are among the first elicitors to be generated during infection. While it is difficult to determine the nature and concentration of cutin monomers at the infection court, the hypothesis that such monomers could be perceived by the plant was tested in barley and rice treated by ectopic treatments with synthetic analogs (Schweizer et al., 1994, 1996b). Two monomers of the C18 family were effective in protecting barley against *E. graminis* and rice against *M. grisea*, most likely by acting on the plant since these molecules have no direct fungicidal effect. Treatment of suspension-cultured potato cells with cutin monomers induces medium alkalinization, production of ethylene (ET) and accumulation of defense-related genes (Schweizer et al., 1996a). The most active compound was n,16-hydroxypalmitic acid ($n = 8, 9,$ or 10), a predominant component of the potato cuticle. When etiolated cucumber hypocotyls are gently abraded, cutin monomers from hydrolysates of cucumber, apple, and tomato cutin induce the production of H_2O_2 (Fauth et al., 1998). The gentle abrasion was proposed to reproduce the action of cutinase released by a potential pathogen allowing the plant to perceive and respond to cutin monomers that can readily diffuse through the permeabilized cuticle. A surprising observation of the action of cutinase was made by the addition of purified cutinase from *Venturia inaequalis* or from *F. solani* directly to spores of *Rhizoctonia solani* prior to inoculation of bean leaves. A decrease in symptoms was observed in inoculation droplets containing spores together with cutinase compared to spores with water. The effect of cutinase depends on its lipolytic esterase activity. Pathogenesis-related (PR) protein genes were not associated with cutinase-induced resistance responses of bean leaves in response to cutinase action (Parker and Koller, 1998). This intriguing observation was pursued further by directly expressing a fungal cutinase gene in the cell wall of plants. To this purpose, a cutinase gene from *F. solani pv. pisi* was expressed in *Arabidopsis thaliana* under the control of the CaMV35S promoter and targeted to the cell wall (Sieber et al., 2000). A normal layer of wax, but a partly absent cuticle, characterizes cutinase-expressing plants that exhibit enhanced permeability to solutes. A subsequent study provided a detailed assessment to the reaction toward pathogens (Chassot et al., 2007). No difference was observed between cutinase-expressing plants (so-called CUTE plants) and wild types after infection with the biotrophs *E. cichoracearum*, *Hyaloperonospora parasitica,* and *Phytophthora brassicae* or the non-host *Blumeria graminis*. Importantly, CUTE plants displayed almost complete immunity toward the necrotrophic fungus *Botrytis cinerea*. The protection requires the enzymatic activity of the protein, since transformants with a cutinase gene mutated in the active site of the enzyme are not protected. Ectopic application of *Fusarium* cutinase to *Arabidopsis thaliana* leaves also protects against *Botrytis cinerea* and is not the result of a direct action of the cutinase on *Botrytis cinerea,* in agreement with the overexpression experiments (Chassot et al., 2007). Expression of the lipase A gene of *Botrytis cinerea* also provides full protection, confirming the importance of the cutinolytic activity for protection (Chassot et al., 2007). To some extent this is reminiscent of the experiments of Parker and Koller (1998) where active cutinase mixed to spores of *R. solani* led to protection in bean leaves. There was no correlation between the expression of marker genes for the salicylic acid (SA), ET, or jasmonic acid (JA) pathways and expression of the cutinase gene of *F. solani* in *Arabidopsis thaliana* mutants of the SA (*pad4, sid2*), ET (*etr1, ein2, pad2*) and of the JA (*jar1*) pathways clearly show fully independence of cutinase-induced protection on SA, ET, and JA. A number of genes identified from microarray experiments showed an earlier and stronger expression after inoculation with *Botrytis cinerea* of CUTE plants compared

to wild types. Fifteen genes were selected and overexpressed in *Arabidopsis thaliana* and eight of these provided increased tolerance to *Botrytis cinerea*. These genes included members of the lipid transfer protein (LTP), the peroxidase (PO), and the protein inhibitor (PI) gene families. Members of the LTPs, PER, and PIs could each contribute in part to the observed resistance induced by *Botrytis cinerea* in CUTE plants (see discussion in Chassot et al., 2007, 2008). Resistance in CUTE plants was also proposed to result from the rapid diffusion of a potential fungitoxic metabolite through the permeable cuticular layer into the inoculation droplet. A fungitoxic activity was observed in the inoculation droplets of *Botrytis cinerea* spore suspension placed on CUTE but not on wild type plants (Chassot et al., 2007) but the chemical nature of the leaf diffusate has not yet been characterized.

A number of studies have reported on *Arabidopsis thaliana* mutants impaired in various aspects of the biosynthesis of the cuticle or that have otherwise an increase in cuticular permeability. An intriguing observation is that several but not all cuticle mutants have an altered permeability and an increased resistance to *Botrytis cinerea* (**Table 1**).

The *lcr (lacerata*) mutant is impaired in a gene coding for a cytochrome P450 monooxygenase involved in the formation of ω-hydroxy fatty acids in yeast and could be involved in cutin biosynthesis (Wellesen et al., 2001). Reduced levels of the major constituents of cuticular polyesters and cutin were observed in the *hth* (allelic to *ace*/*hth, adhesion of calyx edges*/*hothead*) mutant that is characterized by a deficient fatty acid ω-alcohol dehydrogenase activity (Kurdyukov et al., 2006a). Increased accumulation of cell-wall-bound lipids and epicuticular waxes occurs in *bdg* (*bodyguard*) mutants compared to WT plants (Kurdyukov et al., 2006b). The cuticle of *lacs2* (*long-chain acyl-CoA synthetase*; Schnurr et al., 2004) an identical mutant as *bre1* (*Botrytis resistant*; Bessire et al., 2007) is thinner than that of WT plants and contains reduced amounts of dicarboxylic acid monomers in the cutin polyester. The *sma4* (*symptoms to multiple avr genotypes4*) is allelic to *lacs2* (Tang et al., 2007). The *fdh* (*fiddlehead*) is mutated in a gene encoding a protein involved in the synthesis of long-chain lipids (Yephremov et al., 1999; Pruitt et al., 2000; Voisin et al., 2009). The *pec1* (*permeable cuticle 1*) is characterized by a knockout of ATP BINDING CASSETTEG32 (ABCG32), an ABC transporter localized at the plasma membrane of epidermal cells; available evidence suggests that ABCG32 exports cutin precursors for the synthesis of the cuticular layer in the epidermal cell (Bessire et al., 2011). Abscisic acid (ABA) deficiency causes an increase cuticular permeability and resistance to *Botrytis cinerea* as observed in the *sitiens* as well as the *abi2* and *abi3* mutants of tomato and *Arabidopsis thaliana* respectively (Curvers et al., 2010; L'Haridon et al., 2011). An enhanced cuticular permeability and resistance to *Botrytis cinerea* was also observed in the *myb96-1* (*MYB96-deficient*) mutant characterized by downregulated ABA-dependent wax biosynthetic genes (Seo et al., 2011). In tomato, overexpression of SlSHINE3, a transcription factor expressed predominantly in the epidermis, leads to leaves with increased permeability, an increase in cutin monomer content and resistance to *Botrytis cinerea* and *Xanthomonas campestris pv. vesicatoria* (Buxdorf et al., 2014). An increase in resistance to *Botrytis cinerea* was observed when cutin monomers extracted from WT- and SlSHINE3-overexpressing leaves are applied to tomato leaves. Details on the amounts and quality of the cutin monomers or on their mode of action (direct versus indirect) that could explain this result are not known. In the same article, the authors show that only cutin monomers of SlSHINE3-overexpressing leaves induced the expression of defense genes in tomato (Buxdorf et al., 2014). But, not all mutants affected in the cuticle structure show an enhanced resistance to necrotrophic pathogens. The *cer1* mutant of *Arabidopsis thaliana* is affected in an enzyme predicted to be involved in alkane biosynthesis (Bourdenx et al., 2011). *CER1* shows the same expression pattern and localization as other enzymes expressed in the epidermis of aerial organs. Overexpression of *CER1* results in plants with a reduced permeability associated with an improved resistance to water deficient soils. Such plants showed a increased susceptibility to *Pseudomonas syringae pv. tomato* and to the necrotrophic *Sclerotinia sclerotiorum*. The *gl1* mutation affects cuticle formation, but is still susceptible to *Botrytis cinerea* (Xia et al., 2010; Benikhlef et al., 2013). The *rst1* (*RESURRECTION1*) mutant exhibits enhanced susceptibility to the biotrophic fungal pathogen *E. cichoracearum* but enhanced resistance to the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola*. RST1 is plasma membrane protein and is possibly involved in suppressing the biosynthesis of cuticle lipids; the increased levels of cutin monomers and cuticular waxes in *rst1* suggest this. Despite this, *rst1*shows a clear departure from the behavior of other mutants since the permeability of the cuticle is normal (Mang et al., 2009). Another intriguing observation was made with *Arabidopsis thaliana acp4* mutants defective in acyl carrier protein (ACP4). The *acp4* mutants were tested in the context of systemic acquired resistance (SAR); they are able to generate a mobile SAR signal from lower leaves inoculated with bacteria but unable to perceive it in the upper leaf. The *acp4* also display cuticular defects with reduced levels of fatty acids, alkanes and primary alcohols compared to WT plants associated with ultrastructural changes and an increased cuticular permeability (Xia et al., 2009). When wild type Col-0 plants were abraded to remove the cuticle in the upper leaves, SAR was also compromised. It was concluded that an intact cuticle is required for the onset of SAR. It remains difficult to explain how defects in the cuticle impart SAR. Abraded plants are not perfect mimics for the cuticle-defective *acp4* mutants and possibly other compensatory mechanisms might take place differently in both types of plants. It remains now to be shown how an intact cuticular layer can influence SAR. Soft mechanical stress (SMS) applied to leaves was shown to increase resistance to *Botrytis cinerea* and lead to the production of reactive oxygen species (ROS; Benikhlef et al., 2013). SMS resembles the delicate mechanical abrasion of the cuticle used by Xia et al. (2009) and it would now be interesting to know if abraded plants show increased resistance to *Botrytis cinerea*.

Considering the mutants listed in **Table 1**, modifications in cuticular structure associated with enhanced permeability are correlated with enhanced resistance to *Botrytis cinerea*. In addition to resistance, many of these mutants spontaneously accumulate ROS. For instance, the cuticular mutants *bdg* and *lacs2* constitutively produce a green fluorescence upon staining with 5-(and 6)-carboxy-29,79-dichloro dihydrofluorescein diacetate (DCF-DA) a

Table 1 | Mutants displaying alterations in the cuticle structure or in permeability.

Mutant	Plant	Function of wild type gene product	Properties			
			Resistance to *B. cinerea*	Cuticle permeability	Fungitoxic diffusate	
lcr, lacerata	*A.t.*	CYP86AB catalyzes w-hydroxylation of fatty acids ranging from C12 to C18:1	+	+	nt	Wellesen et al. (2001), Bessire et al. (2007)
Hth, hothead; allelic to adhesion of calyx edges (ace)	*A.t.*	Protein with sequence similarity to long-chain FA w-alcohol dehydrogenases	±	±	nt	Lolle et al. (1998), Kurdyukov et al. (2006a), Bessire et al. (2007)
Bdg, bodyguard	*A.t.*	Member of the a/b-hydrolase fold protein superfamily	+	+	+	Kurdyukov et al. (2006b), Chassot et al. (2007)
lacs2.3, long-chain acyl-CoA synthetase	*A.t.*	Long-chain acyl-CoA synthetase	+	+	+	Bessire et al. (2007)
sma4, symptoms to multiple avr genotypes4	*A.t.*	Long-chain acyl-CoA synthetase2	+	+	+	Tang et al. (2007)
fdh, fiddlehead	*A.t.*	Likely to be involved in the synthesis of long chain fatty acids	+	nt	nt	Yephremov et al. (1999), Pruitt et al. (2000), Voisin et al. (2009)
pec1, permeable cuticle1	*A.t.*	ATP binding cassette 32 (ABCG32) transporter	+	+	+	Bessire et al. (2011)
myb96, myeloblastosis transcription factor 96	*A.t.*	ABA-responsive R2R3 type transcription factor	+	+	nt	Seo et al. (2011), Benikhlef et al. (2013)
sitiens	*S.l.*	Abscisic aldehyde oxidase	+	+	nt	Curvers et al. (2010)
aba2, ABA biosynthesis	*A.t.*	Short-chain alcohol dehydrogenase	+	+	nt	Cheng et al. (2002), L'Haridon et al. (2011)
aba3, ABA biosynthesis	*A.t.*	MoCo sulfurase	+	+	nt	Bittner et al. (2001), Xiong et al. (2001), L'Haridon et al. (2011)

A.t., Arabidopsis thaliana; S.l., Solanum lycopersicum; nt, not tested.

fluorescent probe for ROS (L'Haridon et al., 2011; Benikhlef et al., 2013). Treatment of wild type leaf surfaces with fungal cutinase also results in ROS accumulation (L'Haridon et al., 2011). ROS has a multifaceted mode of action and can reach toxic levels acting directly as an antimicrobial or participate in various steps during the activation of defense responses such as modification of the cell wall, signal transduction pathways, programmed cell death, or post-translational regulation (De Tullio, 2010; Torres, 2010; Mittler et al., 2011). At this point, it is not well known why ROS are made in *bdg* and *lacs2* or in cutinase-treated leaves. Presumably, cutin monomers or other compounds accumulating in developmental mutants of the cuticle might be perceived by the plant and result in the production of ROS. A possible early event preceding ROS accumulation might be a Ca^{2+} burst as was shown after wounding or SMS (Beneloujaephajri et al., 2013; Benikhlef et al., 2013). ROS are produced earlier and in higher amounts after inoculation with *Botrytis cinerea* in the *aba2* and *aba3* mutants of ABA biosynthesis as well as in the wax biosynthesis mutant *myb96-1* and these plants were also shown to have an increased cuticular permeability (L'Haridon et al., 2011). All these examples offer the interesting possibility to find out how ROS are produced in relation to the cuticular properties.

CUTE, *lcr, hth, bdg, lacs2/bre1, sma4,* and *pec1* displayed increased resistance to *Botrytis cinerea* and the presence of a fungitoxic activity in leaf diffusates that correlated with an increased permeability of the cuticle (Bessire et al., 2007, 2011; Chassot et al., 2007). Thus, the presence of a fungitoxic activity appears to be mostly associated with an increase in cuticular permeability. The question now arises on the nature of the fungitoxic compound present in the leaf diffusates. At this point, it is tacitly assumed that in all cases the same compound is involved; a chemical characterization will eventually clarify this point. Another intriguing possibility is that phylloplane microbes might contribute directly or indirectly to this activity. For instance, the presence of distinct patterns of microbial communities was observed on the surface of different *Arabidopsis thaliana eceriferum* wax mutants (*cer1, cer6, cer9, cer16*) compared to the corresponding wild type ecotype *Ler* (Reisberg et al., 2013). This interesting observation shows that plant cuticular wax composition can affect the community composition of phyllosphere bacteria. Likely, it is possible that other changes in the composition of the plant surface might also affect bacterial communities. The extent to which such microbes contribute to the fungitoxic activities in leaf diffusates or even to fungal resistance is not known.

The pleiotropic syndrome exhibited in the cuticular mutants such as altered cuticle structure and deposition, altered chemical composition in cuticular lipids, organ fusions, changes in and cell and organ shape or resistance to pathogens suggest that plants adapt to the cuticular defects by compensatory mechanisms. To investigate such an adaptive compensatory mechanism a meta-analyses tool (MASTA; microarray overlap search tool and analysis) was developed and used for an *in-silico* analysis of gene expression profiles in hundreds of datasets (Voisin et al., 2009). This led to the identification of the *SERRATE* (*SE*) gene, which encodes a nuclear protein of RNA-processing multiprotein complexes, making it likely that small-RNA signaling is involved in the cuticular defect syndrome. The importance of the *SE* gene was confirmed with double mutants such as *lcr-se* and *bdg-se* that suppress the abnormal cuticle syndrome and resistance to *Botrytis cinerea.* These results support the hypothesis that various cuticular defects might induce a common signaling pathway that depends on the *SE* gene (Voisin et al., 2009). It will now be interesting to see if this type of analysis can be further used to identify aspects more specific to the fungal resistance response.

The evidence provided by the effect of ectopic treatments with cutin monomers, overexpression of cutinase, ectopic treatments with cutinase and various cuticular mutants with increased permeability lead several scenarios that might explain the resistance of plants in relation to defective cuticles (Chassot et al., 2008). A permeable cuticle could involve a faster perception of putative products of the cuticle released upon the action of the cutinase. In addition, cuticle monomers might be over-produced in cuticular mutants from an incomplete cuticle polymer synthesis. The perception of such monomers would generate intracellular signals and trigger multifactorial defenses. The induced defenses might involve the production/release of ROS, antimicrobial proteins and of antifungal metabolites. A permeable cuticle might also allow a faster passage of potential elicitors from *Botrytis cinerea* or its inoculation medium through the epidermis wall into the cells where they might trigger a faster and more intensive defense reaction. The surprising potential for defense against *Botrytis cinerea* unveiled in CUTE plants and in the various cuticle mutants warrants further research to understand the molecular basis of this phenomenon (**Figure 1**).

A puzzling question concerns the full susceptibility of *Arabidopsis thaliana* to *Botrytis cinerea.* This is intriguing, since *Botrytis cinerea* releases cutinase and lipase during the penetration of leaves (Comménil et al., 1998) yet no resistance is visible. In contrast, our own experiments showed that when cutinase or lipase is applied on the surface of *Arabidopsis thaliana* leaves resistance and ROS are induced (Chassot et al., 2007; L'Haridon et al., 2011). Possibly, the timing or the quantity of enzymes produced by the fungus *in planta* is sufficient for penetration but not for inducing resistance. Alternatively, *Botrytis cinerea,* like other pathogens, might suppress induced defense responses in the plant. One possible suppressor could be oxalic acid, a know pathogenicity factor of *Botrytis cinerea* (Germeier et al., 1994; Pezet et al., 2004) and suppressor of ROS (Cessna et al., 2000). Several experimental lines support this hypothesis. For instance, biocontrol bacteria selected for their ability to metabolize oxalate can protect *Arabidopsis thaliana* against *Botrytis cinerea* (Schoonbeek et al., 2007). Also, transgenic plants overexpressing a fungal oxalate decarboxylase show an earlier and increased accumulation of ROS and an enhanced tolerance after inoculation with *Botrytis cinerea* (L'Haridon et al., 2011) or *Sclerotinia sclerotiorum* (Walz et al., 2008). This might explain why *Arabidopsis thaliana* is susceptible to *Botrytis cinerea,* despite the release of cutinase and lipase.

FUTURE DIRECTIONS

How plants perceive changes in the level of cutin monomers is still not known and is a question that needs to be addressed. The experimental evidence accumulated so far makes it reasonable to

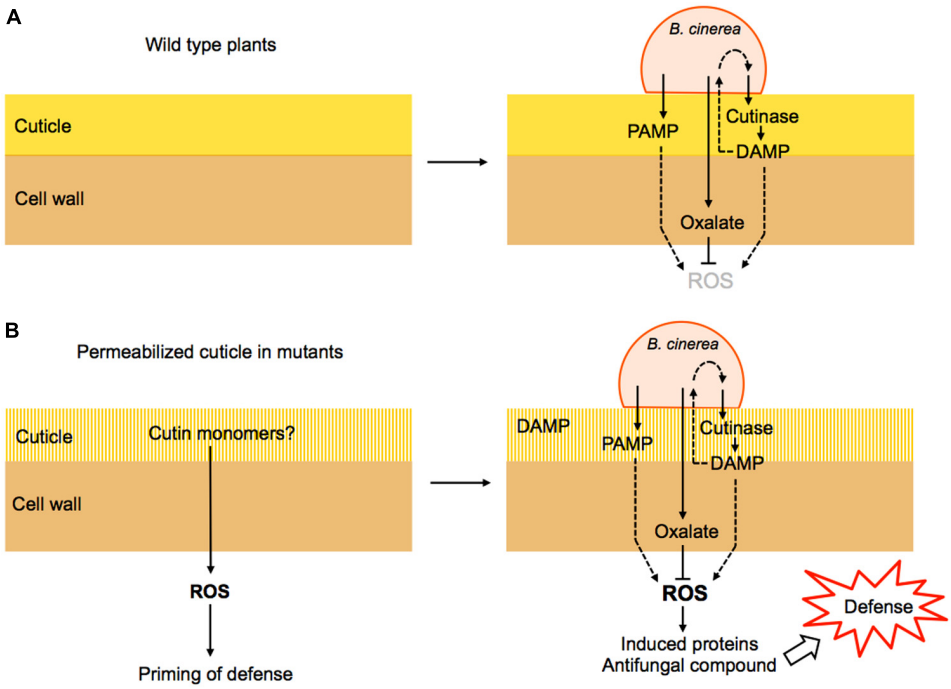


FIGURE 1 | Hypothetical model explaining cuticle-derived resistance to *B. cinerea*. **(A)** During the infection of a wild type plant, *B. cinerea* releases cutinase and PAMPs that lead to its recognition and potentially to ROS formation and defense activation. However, the production of oxalate by *B. cinerea* interferes with ROS production and prevents efficient defenses thus allowing colonization. **(B)** In various mutants affected in the cuticle and its permeability (see **Table 1**), ROS are produced constitutively leading to the priming of defenses. Upon infection, fungal oxalate is insufficient to scavenge ROS and the plant defense is successful. The products of the activity of fungal cutinase are referred to as DAMPs that can also be perceived by the fungus and activate more cutinase expression.

assume that plants are equipped to perceive cutin monomers or other related products possibly by receptors. A genetic screening would be an approach of choice to identify such receptors. In fact, we are currently screening *Arabidopsis thaliana* mutants or ecotypes that lack an increase in resistance to *Botrytis cinerea* after treatment with fungal cutinase. A series of mutants and ecotypes could be identified, all displaying an increased in susceptibility to *Botrytis cinerea*. These results are now being followed up; one predicts that such mutants could be blocked in either a putative receptor for cutinase-generated monomers or alternatively in any step downstream of it.

Using the available genome-wide gene expression microarray data, one can identify common genetic elements during the resistance syndrome in cuticle deficient mutants. Using the MASTA (Reina-Pinto et al., 2009b), differentially expressed gene lists can be generated and classified according to the gene ontology (GO). Using this strategy a list of 25 upregulated genes statistically significant under the GO category "response to fungus" can be identified. These genes point toward common functions that might relate to the resistance syndrome in cuticle deficient mutants and they deserve further attention.

Another intriguing question is the chemical nature of the fungitoxicity in the diffusates of cuticular mutants. It is not clear whether the same chemical causes the observed activity for each mutant; a bioassay-assisted chemical identification is under way to clarify this point.

ACKNOWLEDGMENTS

The support of the Swiss National Science Foundation to Jean-Pierre Métraux is gratefully acknowledged. Thanks are also extended to Antony Buchala for critical reviewing of the manuscript.

REFERENCES

Ahmed, A., Crawford, T., Gould, S., Ha, Y. S., Hollrah, M., Noor-E-Ain, F., et al. (2003). Synthesis of (R)- and (S)-10,16-dihydroxyhexadecanoic acid: cutin stereochemistry and fungal activation. *Phytochemistry* 63, 47–52. doi: 10.1016/S0031-9422(03)00003-7

Beisson, F., Li-Beisson, Y., and Pollard, M. (2012). Solving the puzzles of cutin and suberin polymer biosynthesis. *Curr. Opin. Plant Biol.* 15, 329–337. doi: 10.1016/j.pbi.2012.03.003

Beneloujaephajri, E., Costa, A., L'Haridon, F., Metraux, J.-P., and Binda, M. (2013). Production of reactive oxygen species and wound-induced resistance in *Arabidopsis thaliana* against *Botrytis cinerea* are preceded and depend on a burst of calcium. *BMC Plant Biol.* 13:160. doi: 10.1186/1471-2229-13-160

Benikhlef, L., L'Haridon, F., Abou-Mansour, E., Serrano, M., Binda, M., Costa, A., et al. (2013). Perception of soft mechanical stress in *Arabidopsis* leaves activates disease resistance. *BMC Plant Biol.* 13:133. doi: 10.1186/1471-2229-13-133

Bernard, A., and Joubès, J. (2013). *Arabidopsis* cuticular waxes: advances in synthesis, export and regulation. *Prog. Lipid Res.* 52, 110–129. doi: 10.1016/j.plipres.2012.10.002

Bessire, M., Borel, S., Fabre, G., Carraça, L., Efremova, N., Yephremov, A., et al. (2011). A member of the PLEIOTROPIC DRUG RESISTANCE family of ATP binding cassette transporters is required for the formation of a functional cuticle in *Arabidopsis*. *Plant Cell* 23, 1958–1970. doi: 10.1105/tpc.111.083121

Bessire, M., Chassot, C., Jacquat, A. C., Humphry, M., Borel, S., Petetot, J. M. C., et al. (2007). A permeable cuticle in *Arabidopsis* leads to a strong resistance to *Botrytis cinerea*. *EMBO J.* 26, 2158–2168. doi: 10.1038/sj.emboj.7601658

Bittner, F., Oreb, M., and Mendel, R. R. (2001). ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in *Arabidopsis thaliana*. *J. Biol. Chem.* 276, 40381–40384. doi: 10.1074/jbc.C100472200

Bourdenx, B., Bernard, A., Domergue, F., Pascal, S., Léger, A., Roby, D., et al. (2011). Overexpression of *Arabidopsis* ECERIFERUM1 promotes wax very-long-chain alkane biosynthesis and influences plant response to biotic and abiotic stresses. *Plant Physiol.* 156, 29–45. doi: 10.1104/pp.111.172320

Buxdorf, K., Rubinsky, G., Barda, O., Burdman, S., Aharoni, A., and Levy, M. (2014). The transcription factor SlSHINE3 modulates defense responses in tomato plants. *Plant Mol. Biol.* 84, 37–47. doi: 10.1007/s11103-013-0117-1

Cessna, S. G., Sears, V. E., Dickman, M. B., and Low, P. S. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *Plant Cell* 12, 2191–2200. doi: 10.1105/tpc.12.11.2191

Chassot, C., Nawrath, C., and Metraux, J. (2007). Cuticular defects lead to full immunity to a major plant pathogen. *Plant J.* 49, 972–980. doi: 10.1111/j.1365-313X.2006.03017.x

Chassot, C., Nawrath, C., and Métraux, J. P. (2008). The cuticle: not only a barrier for defence. *Plant Signal. Behav.* 3, 142–144. doi: 10.4161/psb.3.2.5071

Cheng, W.-H., Endo, A., Zhou, L., Penney, J., Chen, H.-C., Arroyo, A., et al. (2002). A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell* 14, 2723–2743. doi: 10.1105/tpc.006494

Comménil, P., Belingheri, L., and Dehorter, B. (1998). Antilipase antibodies prevent infection of tomato leaves by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol.* 52, 1–14. doi: 10.1006/pmpp.1997.0130

Curvers, K., Seifi, H., Mouille, G., De Rycke, R., Asselbergh, B., Van Hecke, A., et al. (2010). ABA-deficiency causes changes in cuticle permeability and pectin composition that influence tomato resistance to *Botrytis cinerea*. *Plant Physiol.* 154, 847–860. doi: 10.1104/pp.110.158972

De Tullio, M. C. (2010). Antioxidants and redox regulation: changing notions in a changing world. *Plant Physiol. Biochem.* 48, 289–291. doi: 10.1016/j.plaphy.2010.02.011

Deising, H., Nicholson, R. L., Haug, M., Howard, R. J., and Mendgen, K. (1992). Adhesion pad formation and the involvement of cutinase and esterases in the attachment of uredospores to the host cuticle. *Plant Cell* 4, 1101–1111. doi: 10.1105/tpc.4.9.1101

Dickman, M. B., Ha, Y. S., Yang, Z., Adams, B., and Huang, C. (2003). A protein kinase from *Colletotrichum trifolii* is induced by plant cutin and is required for appressorium formation. *Mol. Plant Microbe Interact.* 16, 411–421. doi: 10.1094/MPMI.2003.16.5.411

Domínguez, E., Cuartero, J., and Heredia, A. (2011a). An overview on plant cuticle biomechanics. *Plant Sci.* 181, 77–84. doi: 10.1016/j.plantsci.2011.04.016

Domínguez, E., Heredia-Guerrero, J. A., and Heredia, A. (2011b). The biophysical design of plant cuticles: an overview. *New Phytol.* 189, 938–949. doi: 10.1111/j.1469-8137.2010.03553.x

Fauth, M., Schweizer, P., Buchala, A., Markstadter, C., Riederer, M., Kato, T., et al. (1998). Cutin monomers and surface wax constituents elicit H_2O_2 in conditioned cucumber hypocotyl segments and enhance the activity of other H_2O_2 elicitors. *Plant Physiol.* 117, 1373–1380. doi: 10.1104/pp.117.4.1373

Francis, S. A., Dewey, F. M., and Gurr, S. J. (1996). The role of cutinase in germling development and infection by *Erysiphe graminis* f sp hordei. *Physiol. Mol. Plant Pathol.* 49, 201–211. doi: 10.1006/pmpp.1996.0049

Germeier, C., Hedke, K., and Von Tiedemann, A. (1994). The use of pH indicators in diagnostic media for acid-producing plant-pathogens. *Z. Pflanzenkrankh. Pflanzensch.*101, 498–507.

Gilbert, R. D., Johnson, A. M., and Dean, R. A. (1996). Chemical signals responsible for appressorium formation in the rice blast fungus *Magnaporthe grisea*. *Physiol. Mol. Plant Pathol.* 48, 335–346. doi: 10.1006/pmpp.1996.0027

Goodwin, S. M., and Jenks, M. A. (2005). "Plant cuticle function as a barrier to water loss," in *Plant Abiotic Stress*, eds M. A. Jenks and P. M. Hasegawa (Oxford: Blackwell Publishing Inc.), 14–36.

Hansjakob, A., Bischof, S., Bringmann, G., Riederer, M., and Hildebrandt, U. (2010). Very-long-chain aldehydes promote in vitro prepenetration processes of *Blumeria graminis* in a dose- and chain length-dependent manner. *New Phytol.* 188, 1039–1054. doi: 10.1111/j.1469-8137.2010.03419.x

Hansjakob, A., Riederer, M., and Hildebrandt, U. (2011). Wax matters: absence of very-long-chain aldehydes from the leaf cuticular wax of the glossy11 mutant of maize compromises the prepenetration processes of *Blumeria graminis*. *Plant Pathol.* 60, 1151–1161. doi: 10.1111/j.1365-3059.2011.02467.x

Hansjakob, A., Riederer, M., and Hildebrandt, U. (2012). Appressorium morphogenesis and cell cycle progression are linked in the grass powdery mildew fungus *Blumeria graminis*. *Fungal Biol.* 116, 890–901. doi: 10.1016/j.funbio.2012.05.006

Hegde, Y., and Kolattukudy, P. E. (1997). Cuticular waxes relieve self-inhibition of germination and appressorium formation by the conidia of *Magnaporthe grisea*. *Physiol. Mol. Plant Pathol.* 51, 75–84. doi: 10.1006/pmpp.1997.0105

Heredia, A. (2003). Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer. *Biochim. Biophys. Acta* 1620, 1–7. doi: 10.1016/S0304-4165(02)00510-X

Javelle, M., Vernoud, V., Depège-Fargeix, N., Arnould, C., Oursel, D., Domergue, F., et al. (2010). Overexpression of the epidermis-specific homeodomain-leucine zipper iv transcription factor OUTER CELL LAYER1 in maize identifies target genes involved in lipid metabolism and cuticle biosynthesis. *Plant Physiol.* 154, 273–286. doi: 10.1104/pp.109.150540

Kamper, J. T., Kamper, U., Rogers, L. M., and Kolattukudy, P. E. (1994). Identification of regulatory elements in the cutinase promoter from *Fusarium solani* f.sp. pisi (Nectria haematococca). *J. Biol. Chem.* 269, 9195–9204.

Kolattukudy, P. E. (1985). Enzymatic penetration of the plant cuticle by fungal pathogens. *Annu. Rev. Phytopathol.* 23, 223–250. doi: 10.1146/annurev.py.23.090185.001255

Kolattukudy, P. E., Rogers, L. M., Li, D. X., Hwang, C. S., and Flaishman, M. A. (1995). Surface signaling in pathogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4080–4087. doi: 10.1073/pnas.92.10.4080

Kunst, L., and Samuels, L. (2009). Plant cuticles shine: advances in wax biosynthesis and export. *Curr. Opin. Plant Biol.* 12, 721–727. doi: 10.1016/j.pbi.2009.09.009

Kurdyukov, S., Faust, A., Trenkamp, S., Bär, S., Franke, R., Efremova, N., et al. (2006a). Genetic and biochemical evidence for involvement of HOTHEAD in the biosynthesis of long-chain α-,ω-dicarboxylic fatty acids and formation of extracellular matrix. *Planta* 224, 315–329. doi: 10.1007/s00425-005-0215-7

Kurdyukov, S., Faust, A., Nawrath, C., Bar, S., Voisin, D., Efremova, N., et al. (2006b). The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. *Plant Cell* 18, 321–339. doi: 10.1105/tpc.105.036079

Lee, S. B., and Suh, M. C. (2013). recent advances in cuticular wax biosynthesis and its regulation in *Arabidopsis*. *Mol. Plant* 6, 246–249. doi: 10.1093/mp/sss159

L'Haridon, F., Besson-Bard, A., Binda, M., Serrano, M., Abou-Mansour, E., Balet, F., et al. (2011). A permeable cuticle is associated with the release of reactive oxygen species and induction of innate immunity. *PLoS Pathog.* 7:e1002148. doi: 10.1371/journal.ppat.1002148

Lin, T. S., and Kolattukudy, P. E. (1978). Induction of a bio-polyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani*-f.sp. pisi. *J. Bacteriol.* 133, 942–951.

Lolle, S. J., Hsu, W., and Pruitt, R. E. (1998). Genetic analysis of organ fusion in *Arabidopsis thaliana*. *Genetics* 149, 607–619.

Mang, H. G., Laluk, K. A., Parsons, E. P., Kosma, D. K., Cooper, B. R., Park, H. C., et al. (2009). The *Arabidopsis* RESURRECTION1 gene regulates a novel antagonistic interaction in plant defense to biotrophs and necrotrophs. *Plant Physiol.* 151, 290–305. doi: 10.1104/pp.109.142158

Martin, J. T. (1964). Role of cuticle in the defense against plant disease. *Annu. Rev. Phytopathol.* 2, 81–100. doi: 10.1146/annurev.py.02.090164.000501

Ménard, R., Verdier, G., Ors, M., Erhardt, M., Beisson, F., and Shen, W.-H. (2014). Histone H2B monoubiquitination is involved in the regulation of cutin and wax composition in *Arabidopsis thaliana*. *Plant Cell Physiol.* 55, 455–466. doi: 10.1093/pcp/pct182

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., et al. (2011). ROS signaling: the new wave? *Trends Plant Sci.* 16, 300–309. doi: 10.1016/j.tplants.2011.03.007

Muller, C., and Riederer, M. (2005). Plant surface properties in chemical ecology. *J. Chem. Ecol.* 31, 2621–2651. doi: 10.1007/s10886-005-7617-7

Nadakuduti, S. S., Pollard, M., Kosma, D. K., Allen, C., Ohlrogge, J. B., and Barry, C. S. (2012). Pleiotropic phenotypes of the sticky peel mutant provide new insight

into the role of CUTIN DEFICIENT2 in epidermal cell function in tomato. *Plant Physiol.* 159, 945–960. doi: 10.1104/pp.112.198374

Nawrath, C., Schreiber, L., Franke, R. B., Geldner, N., Reina-Pinto, J. J., and Kunst, L. (2013). Apoplastic diffusion barriers in *Arabidopsis. Arabidopsis Book* 11:e0167. doi: 10.1199/tab.0167

Nielsen, K. A., Nicholson, R. L., Carver, T. L. W., Kunoh, H., and Oliver, R. P. (2000). First touch: an immediate response to surface recognition in conidia of *Blumeria graminis. Physiol. Mol. Plant Pathol.* 56, 63–70. doi: 10.1006/pmpp.1999.0241

Parker, D. M., and Koller, W. (1998). Cutinase and other lipolytic esterases protect bean leaves from infection by *Rhizoctonia solani. Mol. Plant Microbe Interact.* 11, 514–522. doi: 10.1094/MPMI.1998.11.6.514

Pezet, R., Viret, O., and Gindro, K. (2004). "Plant-microbe interaction: the *Botrytis* grey mould of grapes. Biology, biochemistry, epidemiology and control management," in *Advances in Plant Physiology*, Vol. 7, ed. A. Hemantaranjan (Jodhpur: Scientific Publishers), 179–193.

Podila, G. K., Rogers, L. M., and Kolattukudy, P. E. (1993). Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides. Plant Physiol.* 103, 267–272.

Pollard, M., Beisson, F., Li, Y., and Ohlrogge, J. B. (2008). Building lipid barriers: biosynthesis of cutin and suberin. *Trends Plant Sci.* 13, 236–246. doi: 10.1016/j.tplants.2008.03.003

Pruitt, R. E., Vielle-Calzada, J.-P., Ploense, S. E., Grossniklaus, U., and Lolle, S. J. (2000). FIDDLEHEAD, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1311–1316. doi: 10.1073/pnas.97.3.1311

Reina-Pinto, J. J., and Yephremov, A. (2009a). Surface lipids and plant defenses. *Plant Physiol. Biochem.* 47, 540–549. doi: 10.1016/j.plaphy.2009.01.004

Reina-Pinto, J. J., Voisin, D., Teodor, R., and Yephremov, A. (2009b). Probing differentially expressed genes against a microarray database for in silico suppressor/enhancer and inhibitor/activator screens. *Plant J.* 61, 166–175. doi: 10.1111/j.1365-313X.2009.04043.x

Reisberg, E. E., Hildebrandt, U., Riederer, M., and Hentschel, U. (2013). Distinct phyllosphere bacterial communities on *Arabidopsis* wax mutant leaves. *PLoS ONE* 8:e78613. doi: 10.1371/journal.pone.0078613

Reisige, K., Gorzelanny, C., Daniels, U., and Moerschbacher, B. M. (2006). The C28 aldehyde octacosanal is a morphogenetically active component involved in host plant recognition and infection structure differentiation in the wheat stem rust fungus. *Physiol. Mol. Plant Pathol.* 68, 33–40. doi: 10.1016/j.pmpp.2006.05.006

Ringelmann, A., Riedel, M., Riederer, M., and Hildebrandt, U. (2009). Two sides of a leaf blade: *Blumeria graminis* needs chemical cues in cuticular waxes of Lolium perenne for germination and differentiation. *Planta* 230, 95–105. doi: 10.1007/s00425-009-0924-4

Samuels, L., Kunst, L., and Jetter, R. (2008). Sealing plant surfaces: cuticular wax formation by epidermal cells. *Annu. Rev. Plant Biol.* 59, 683–707. doi: 10.1146/annurev.arplant.59.103006.093219

Schnurr, J., Shockey, J., and Browse, J. (2004). The acyl-CoA synthetase encoded by LACS2 is essential for normal cuticle development in *Arabidopsis. Plant Cell* 16, 629–642. doi: 10.1105/tpc.017608

Schoonbeek, H.-J., Jacquat-Bovet, A.-C., Mascher, F., and Metraux, J.-P. (2007). Oxalate-degrading bacteria can protect *Arabidopsis thaliana* and crop plants against *Botrytis cinerea. Mol. Plant Microbe Interact.* 20, 1535–1544. doi: 10.1094/MPMI-20-12-1535

Schreiber, L. (2010). Transport barriers made of cutin, suberin and associated waxes. *Trends Plant Sci.* 15, 546–553. doi: 10.1016/j.tplants.2010.06.004

Schweizer, P., Felix, G., Buchala, A., Muller, C., and Métraux, J. P. (1996a). Perception of free cutin monomers by plant cells. *Plant J.* 10, 331–341. doi: 10.1046/j.1365-313X.1996.10020331.x

Schweizer, P., Jeanguenat, A., Whitacre, D., Métraux, J. P., and Mosinger, E. (1996b). Induction of resistance in barley against *Erysiphe graminis* f sp hordei by free cutin monomers. *Physiol. Mol. Plant Pathol.* 49, 103–120. doi: 10.1006/pmpp.1996.0043

Schweizer, P., Jeanguénat, A., Métraux, J. P., and Mösinger, E. (1994). "Plant protection by free cutin monomers in two cereal pathosystems," in *Advances Molecular Genetics Plant-Microbe Interactions*, eds M. J. Daniels, J. A. Downie, and A. E. Osbourn (Dordrecht: Kluwer), 371–374. doi: 10.1007/978-94-011-0177-6_55

Seo, P. J., Lee, S. B., Suh, M. C., Park, M.-J., Go, Y. S., and Park, C.-M. (2011). The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in *Arabidopsis. Plant Cell* 23, 1138–1152. doi: 10.1105/tpc.111.083485

Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Metraux, J. P., et al. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. *Plant Cell* 12, 721–737. doi: 10.1105/tpc.12.5.721

Tang, D., Simonich, M. T., and Innes, R. W. (2007). Mutations in LACS2, a long-chain acyl-coenzyme a synthetase, enhance susceptibility to avirulent *Pseudomonas syringae* but confer resistance to *Botrytis cinerea* in *Arabidopsis. Plant Physiol.* 144, 1093–1103. doi: 10.1104/pp.106.094318

Torres, M. A. (2010). ROS in biotic interactions. *Physiol. Plant.* 138, 414–429. doi: 10.1111/j.1399-3054.2009.01326.x

Voisin, D., Nawrath, C., Kurdyukov, S., Franke, R. B., Reina-Pinto, J. J., Efremova, N., et al. (2009). Dissection of the complex phenotype in cuticular mutants of *Arabidopsis* reveals a role of SERRATE as a mediator. *PLoS Genet.* 5:e1000703. doi: 10.1371/journal.pgen.1000703

Walz, A., Zingen-Sell, I., Loeffler, M., and Sauer, M. (2008). Expression of an oxalate oxidase gene in tomato and severity of disease caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum. Plant Pathol.* 57, 453–458. doi: 10.1111/j.1365-3059.2007.01815.x

Wellesen, K., Durst, F., Pinot, F., Benveniste, I., Nettesheim, K., Wisman, E., et al. (2001). Functional analysis of the LACERATA gene of *Arabidopsis* provides evidence for different robes of fatty acid omega- hydroxylation in development. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9694–9699. doi: 10.1073/pnas.171285998

Woloshuk, C. P., and Kolattukudy, P. E. (1986). Mechanism by which contact with plant cuticle triggers cutinase gene-expression in the spores of *Fusarium solani* f.s*p. pisi. Proc. Natl. Acad. Sci. U.S.A.* 83, 1704–1708. doi: 10.1073/pnas.83.6.1704

Xia, Y., Gao, Q.-M., Yu, K., Lapchyk, L., Navarre, D., Hildebrand, D., et al. (2009). An intact cuticle in distal tissues is essential for the induction of systemic acquired resistance in plants. *Cell Host Microbe* 5, 151–165. doi: 10.1016/j.chom.2009.01.001

Xia, Y., Yu, K., Navarre, D., Seebold, K., Kachroo, A., and Kachroo, P. (2010). The glabra1 mutation affects cuticle formation and plant responses to microbes. *Plant Physiol.* 154, 833–846. doi: 10.1104/pp.110.161646

Xiong, L., Ishitani, M., Lee, H., and Zhu, J.-K. (2001). The *Arabidopsis* LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. *Plant Cell* 13, 2063–2083. doi: 10.1105/tpc.13.9.2063

Yeats, T. H., and Rose, J. K. C. (2013). The formation and function of plant cuticles. *Plant Physiol.* 163, 5–20. doi: 10.1104/pp.113.222737

Yephremov, A., Wisman, E., Huijser, P., Huijser, C., Wellesen, K., and Saedler, H. (1999). Characterization of the FIDDLEHEAD gene of *Arabidopsis* reveals a link between adhesion response and cell differentiation in the epidermis. *Plant Cell* 11, 2187–2201. doi: 10.1105/tpc.11.11.2187

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 April 2014; paper pending published: 15 May 2014; accepted: 26 May 2014; published online: 13 June 2014.

*Citation: Serrano M, Coluccia F, Torres M, L'Haridon F and Métraux J-P (2014) The cuticle and plant defense to pathogens. Front. Plant Sci. **5**:274. doi: 10.3389/fpls.2014.00274*

This article was submitted to Plant–Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 03 September 2014
doi: 10.3389/fpls.2014.00435

Genome-wide transcriptional profiling of *Botrytis cinerea* genes targeting plant cell walls during infections of different hosts

Barbara Blanco-Ulate[1,2], Abraham Morales-Cruz[1], Katherine C. H. Amrine[1], John M. Labavitch[2], Ann L. T. Powell[2] and Dario Cantu[1]*

[1] Department of Viticulture and Enology, University of California, Davis, Davis, CA, USA
[2] Department of Plant Sciences, University of California, Davis, Davis, CA, USA

Edited by:
Vincenzo Lionetti, Sapienza Università di Roma, Italy

Reviewed by:
Barbara De Coninck, University of Leuven (KU Leuven), Belgium
Carmen González Bosch, Universidad de Valencia, Spain

***Correspondence:**
Dario Cantu, Department of Viticulture and Enology, University of California, Davis, One Shields Ave., Davis, CA 95616, USA
e-mail: dacantu@ucdavis.edu

Cell walls are barriers that impair colonization of host tissues, but also are important reservoirs of energy-rich sugars. Growing hyphae of necrotrophic fungal pathogens, such as *Botrytis cinerea* (*Botrytis*, henceforth), secrete enzymes that disassemble cell wall polysaccharides. In this work we describe the annotation of 275 putative secreted Carbohydrate-Active enZymes (CAZymes) identified in the *Botrytis* B05.10 genome. Using RNAseq we determined which *Botrytis* CAZymes were expressed during infections of lettuce leaves, ripe tomato fruit, and grape berries. On the three hosts, *Botrytis* expressed a common group of 229 potentially secreted CAZymes, including 28 pectin backbone-modifying enzymes, 21 hemicellulose-modifying proteins, 18 enzymes that might target pectin and hemicellulose side-branches, and 16 enzymes predicted to degrade cellulose. The diversity of the *Botrytis* CAZymes may be partly responsible for its wide host range. Thirty-six candidate CAZymes with secretion signals were found exclusively when *Botrytis* interacted with ripe tomato fruit and grape berries. Pectin polysaccharides are notably abundant in grape and tomato cell walls, but lettuce leaf walls have less pectin and are richer in hemicelluloses and cellulose. The results of this study not only suggest that *Botrytis* targets similar wall polysaccharide networks on fruit and leaves, but also that it may selectively attack host wall polysaccharide substrates depending on the host tissue.

Keywords: *Botrytis*, noble rot, plant pathogenic fungi, CAZymes, RNAseq, tomato, grape, lettuce

INTRODUCTION

The cell wall matrix is one of the first and largest plant structures that pathogens encounter when interacting with potential hosts. The composition and architecture of cell walls vary between plant species, organs and developmental stages. Two co-extensive networks of polysaccharides comprise up to 80% of the mass of most plant cell walls. A network of cellulose microfibrils is cross-linked via hydrogen-bonded hemicelluloses and is embedded within a second network, a matrix of simple and branched pectin polysaccharides (Carpita and Gibeaut, 1993). The integrity of the hemicellulose-cellulose microfibril network provides much of the strength and rigidity of the cell wall (Harris and Stone, 2008; Scheller and Ulvskov, 2010). The pectin network influences the wall's porosity and provides structural coherence (Ishii et al., 2001; Vincken et al., 2003). Homogalacturonan (HG) and rhamnogalacturonans (RG-I and RG-II) are the major pectins of the primary walls of dicots and non-graminaceous monocots (Voragen et al., 2009). Pectins are important for cell-to-cell adhesion and they are particularly abundant in the middle lamella and corners between adjacent cells (Mohnen, 2008). Structural glycoproteins, soluble proteins, ions and metabolites are also located within the polysaccharide networks of most cell walls (Cassab, 1998; Keegstra, 2010).

Plant cell walls that are recalcitrant to decomposition by microorganisms and walls that favor the timely activation and correct allocation of host defenses are more likely to resist pathogen infections (Cantu et al., 2008a; Underwood, 2012). For example, plant cell wall-associated kinases and receptors are crucial to sense invading pathogens and to promptly induce immune responses, including structural reinforcements of the wall and production of anti-pathogen compounds (Cantu et al., 2008a; Hematy et al., 2009). Furthermore, pre-formed defense proteins (e.g., extracellular pathogenesis-related proteins) and their locations within the plant cell wall matrix contribute to processes that prime host tissues for resistance (Powell et al., 2000; Cantu et al., 2008b).

Necrotrophic pathogens, such as *Botrytis*, have evolved complex strategies to overcome the plant immune system (Weiberg et al., 2013) and to destroy the pectin-rich middle lamellae and primary cell walls of the host, inducing cell death and compromising the integrity of host tissues (Tiedemann, 1997; Van Baarlen et al., 2004; Cantu et al., 2008a; Curvers et al., 2010). *Botrytis* is considered a generalist pathogen because it is capable of

infecting a wide variety of plant hosts and organs. During infections, *Botrytis* secretes diverse proteins and enzymes that modify the host cell walls (Van Kan, 2006; Zhang and van Kan, 2013a). Some of these proteins, such as the polygalacturonase BcPG1, have been demonstrated to be important virulence factors in multiple host tissues (Ten Have et al., 1998; Valette-Collet et al., 2003; Espino et al., 2005; Kars et al., 2005a; Brito et al., 2006; Nafisi et al., 2014). Characterizing the cell wall-degrading enzymes deployed by *Botrytis* on different hosts and tissues may help identify virulence functions that *Botrytis* uses on all hosts and those that are important on specific hosts, organs, or developmental stages.

The Carbohydrate-Active enZymes (CAZymes) are proteins with predicted catalytic and carbohydrate-binding modules that degrade, modify, or create glycosidic bonds. Therefore, some CAZymes are candidates for proteins that participate in the modification and breakdown of cell wall polysaccharides (Cantarel et al., 2009). The assignment of a gene to a particular CAZyme family can predict the catalytic properties of the protein it encodes and its possible substrates (Cantarel et al., 2009; Park et al., 2010). Sequence homology to known CAZyme genes in combination with computational prediction of protein secretion (SignalP; Petersen et al., 2011) has been used extensively for *in silico* identification and classification of the repertoire of cell wall degrading enzymes of pathogenic fungi with sequenced genomes (Floudas et al., 2012; Suzuki et al., 2012; Blanco-Ulate et al., 2013a,b,c,d).

Genome-wide transcriptional profiling approaches have been applied successfully to study the regulation of pathogen virulence factors in plant hosts (Noël et al., 2001; Ithal et al., 2007; Jeon et al., 2007; O'Connell et al., 2012; Schmidtke et al., 2012; Cantu et al., 2013; Wiemann et al., 2013; Zhang et al., 2013). In this study we (i) identified in the current release of the publically available *Botrytis* genome (strain B05.10 v.1; Amselem et al., 2011) genes encoding putatively secreted CAZymes, (ii) analyzed the phylogenetic relationships of these genes, and (iii) profiled their expression when *Botrytis* interacts with three plant hosts. The plant hosts chosen for this study, ripe tomato fruit, ripe grape berries and lettuce leaves, represent to important postharvest commodities, which are highly susceptible to infections by *Botrytis*. Our results suggest that *Botrytis* not only expresses a rich repertoire of activities that target the many diverse structures of the plant cell walls, but also that some of these functions are differentially regulated depending on the host.

MATERIALS AND METHODS

ANNOTATION OF *BOTRYTIS* CAZymes

Transcriptome sequences of *Botrytis cinerea* (strain B05.10 v.1; Amselem et al., 2011) were obtained from http://www.broadinstitute.org/annotation/genome/botrytis_cinerea. The transcriptome was annotated for sequences encoding Carbohydrate-Active enZymes (CAZymes; http://www.cazy.org) with the CAZymes Analysis Toolkit (http://mothra.ornl.gov/cgi-bin/cat/cat.cgi; Park et al., 2010) with an e value < 1e-2, a bit score threshold of 55 and a rule level of support of 40. Functional annotation of the CAZymes genes was carried out with Blast2GO v.2.7.1 (http://www.blast2go.com/start-blast2go; Götz et al., 2008), which performed a BLASTx search against the non-redundant (nr) protein database of NCBI; default parameters were used. The predicted CAZymes from *Botrytis* were then clustered in protein tribes based on sequence similarities using BLASTp alignments (*e*-value <1e−6) and Tribe-MCL (Enright et al., 2002) following methods described in Haas et al. (2009). The presence of secretion signal peptides was evaluated for all genes in the transcriptome using SignalP v.4.0 (http://www.cbs.dtu.dk/services/SignalP-4.0/; Petersen et al., 2011) with the following parameters: 0.50 D-cutoff values for SignalP-TM and 0.45 for SignalP-noTM.

One of the limitations of *in silico* analyses of secretion peptides is the occurrence of false positives and false negatives (Petersen et al., 2011; Melhem et al., 2013). SignalP v.4.0 was reported to have a higher false negative rate (8.80%) than false positive rate (3.30%) when predicting secretion signals in plant proteins (Melhem et al., 2013). A literature search of previously validated secreted *Botrytis* CAZymes was performed to identify possible false negatives resulting from the SignalP prediction. Two CAZyme-encoding genes, *BcPME1* (*BC1G_06840*) and *BcXyn11A* (*BC1G_02167*), had SignalP prediction scores below the 0.50/0.45 D-cutoff values, but both genes have been experimentally proven to encode secreted proteins (Valette-Collet et al., 2003; Kars et al., 2005b; Brito et al., 2006; Shah et al., 2009a,b; Fernández-Acero et al., 2010; Li et al., 2012); hence, they were included in the dataset of secreted *Botrytis* CAZymes. The existence of other false negatives still needs to be experimentally evaluated for each CAZyme that did not pass the SignalP thresholds.

PHYLOGENETIC ANALYSES

The protein sequences of 7 CAZyme subfamilies including genes with putative roles in degrading plant cell walls, based on manual curation of CAZymes and functional annotations, were analyzed. Multiple global sequence alignments were conducted with MUSCLE (Edgar, 2004) for all protein sequences in a particular tribe using default parameters. Phylogenetic analyses were conducted in MEGA v.5.2.2 (Tamura et al., 2011) using the Neighbor-Joining method with 1000 bootstrap replicates (Felsenstein, 1985; Saitou and Nei, 1987). All positions containing gaps and missing data were eliminated.

BIOLOGICAL MATERIAL

Tomato (*Solanum lycopersicum*) cv. Ailsa Craig was provided by the Tomato Genetics Research Center (UC Davis). Tomato plants were grown in the field in Davis, California. Fruit were tagged at 3 days post-anthesis (dpa) and harvested at the red ripe stage (42 dpa). The ripening stages of the fruit were confirmed by color, size and texture measurements.

The *Botrytis* strain B05.10 used to inoculate the tomato fruit was provided by Dr. J. A. L. van Kan (Department of Phytopathology, Wageningen University). Conidia were collected from sporulating cultures grown on 1% potato dextrose agar. Tomato fruit were disinfected and inoculated as in Cantu et al. (2008a). At the time of inoculation, fruit were wounded at seven sites to a depth of 2 mm and a diameter of 1 mm. Wounded sites were inoculated with 10 μL of a water suspension containing 5000 conidia. All fruit samples were incubated at 20°C in high humidity for 3 days. When material was collected for analysis, the tomato fruit were deseeded, frozen, and ground to fine powder in liquid nitrogen. Three biological replicates were produced;

each replicate was an independent pool of 8–10 *Botrytis*-infected tomato fruit.

Ripe (23 brix) grape berries (*Vitis vinifera* cv. Sémillon) showing the initial symptoms of *Botrytis* infections were collected from the Dolce Winery Vineyards (Napa Valley, California). Fruit were field inoculated by spraying conidia of the *Botrytis* strain BcDW1 (Blanco-Ulate et al., 2013a). Transcript polymorphisms detected in the RNAseq data suggest that other strains also infected the berries in the vineyard (results not shown). Determination of the initial stage of *Botrytis* infection (S1) was based on the time at which individual berries showed a partial color change from green to pink, but the berries still maintained their turgor and tissue integrity. The S1 stage of botrytized-grape berries was confirmed by the amount of fungal biomass present in the berries (described below). On the same day of harvest, individual infected grape berries were deseeded, frozen, and ground to fine powder in liquid nitrogen. Four biological replicates were generated from independent pools of 10–15 *Botrytis*-infected grape berries.

BOTRYTIS BIOMASS DETERMINATION

Fungal biomass was quantified using the QuickStix Kit for *Botrytis* (EnviroLogix), which utilizes the monoclonal antibody BC12.CA4 (Meyer and Dewey, 2000) as described by Cantu et al. (2008b). One gram of ground tissue (pericarp and epidermis) from each biological replication was suspended in the kit buffer, 1:40 m/v for tomato fruit and 1:20 m/v for grape berries. The amount of material that cross-reacted with the antibody was measured in 500 µl of the tissue suspension. The intensity of the test line was determined with the QuickStix reader (Envirologix) and converted into fungal biomass (µg/gFW of fruit) based on standard curves using known amounts of dry mycelium diluted into extracts of healthy tomato fruit tissue (Cantu et al., 2009a).

RNA SEQUENCING AND DATA PROCESSING

Total RNA was extracted from two grams of infected tissues (pericarp and epidermis) from each biological replicate as described in Blanco-Ulate et al. (2013e). RNA concentration and purity were measured using the NanoDrop 2000c Spectrophotometer (Thermo Scientific). RNA integrity was checked by agarose gel electrophoresis. The Illumina TruSeq RNA Sample preparation Kit v.2 was used to prepare cDNA libraries from 4 µg of total RNA. Libraries were barcoded individually and analyzed with the High Sensitivity DNA Analysis Kit using an Agilent 2100 Bioanalyzer (Agilent Technologies). Sequencing was carried out on an Illumina HiSeq machine at the DNA Technologies Service Core at UC Davis. The Illumina raw reads were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) and are accessible through GEO (GSE57588 accession; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57588). Quality trimming of raw reads was done with Sickle v.1.21 (https://github.com/ucdavis-bioinformatics/sickle) with a threshold of 20 (Q >20) and adapter trimming was done with Scythe v.0.991 (https://github.com/ucdavis-bioinformatics/scythe) with a prior of 0.4.

The sequences of the *Botrytis* (strain B05.10 v.1) and grape (v. 12X, http://www.genoscope.cns.fr/externe/Download/Projets/Projet_ML/data/12X/annotation/) transcriptomes were combined and used as a reference for mapping *Botrytis*-infected grape reads. Likewise transcriptomes of *Botrytis* and tomato (ITAG2.3, ftp://ftp.solgenomics.net/tomato_genome/annotation/ITAG2.3_release/) were merged and used as reference for *Botrytis*-infected tomato reads. Bowtie2 v.2.1.0 (Langmead and Salzberg, 2012) was used to align the processed reads to the combined references with the parameters—end-to-end—sensitive. Read counts were extracted from the bowtie2 alignments using the script sam2counts.py v.0.91 (https://github.com/vsbuffalo/sam2counts).

Raw counts of *Botrytis* genes expressed at 2 days post-infection of lettuce leaves were obtained from De Cremer et al. (2013). In this study, lettuce leaves (*Lactuca sativa* cv. Salinas) of 5 week-old plants were inoculated in the growth chamber with a spore suspension of *Botrytis*, strain B05.10 (De Cremer et al., 2013).

DIFFERENTIAL EXPRESSION ANALYSES

The Bioconductor package DESeq v. 1.14.0 (Anders and Huber, 2010) was used to normalize the raw transcript counts of *Botrytis* genes encoding potentially secreted CAZymes from infected lettuce leaves, ripe tomato fruit and grape berries. The DESeq pipeline was used to (i) compare the expression profiles of potentially secreted CAZymes during *Botrytis* infections of lettuce leaves, ripe tomato fruit and grape berries, and (ii) identify differentially expressed (DE) genes (P-adjusted value ≤ 0.05).

QUANTITATIVE REVERSE TRANSCRIPTION PCR (qRT-PCR)

cDNA was prepared from the isolated RNA using the M-MLV Reverse Transcriptase (Promega). qRT-PCR was performed on a StepOnePlus PCR System using Fast SYBR Green Master Mix (Applied Biosystems). All qRT-PCR reactions were performed as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The *BOTRYTIS RIBOSOMAL PROTEIN-LIKE5* (*BcRPL5*, *BC1G_13576*) was used as the reference gene and processed in parallel with the genes of interest. The primer sequences to amplify the *BcRPL5*, *BcPG1,* and *BcPG2* genes were obtained from Zhang and van Kan (2013b). Transcript levels for all genes in this study were linearized using the formula $2^{(BcRPL5\ \mathrm{CT} - TARGET\ \mathrm{CT})}$ as described in Chen and Dubcovsky (2012). Data presented are means of 3–4 biological replicates of infected tomato and grape berries.

RESULTS

PREDICTED CAZyme *BOTRYTIS* GENES

The genome of *Botrytis cinerea* (strain B05.10; Amselem et al., 2011) is predicted to encode 1155 CAZymes based on a similarity search against the entire non-redundant sequences of the CAZy database using the CAZYmes Analysis Toolkit (Park et al., 2010; Supplemental Table S1). Putative secretion signals were found in 275 CAZyme *Botrytis* genes (SignalP v4.0; Petersen et al., 2011). Glycoside hydrolases (GHs) were the most abundant class of putative secreted CAZymes (48.72%); among the GHs, the GH28 subfamily was the largest group (14.18% of all GHs). Twenty-three percent of the CAZyme genes encoded carbohydrate-binding proteins (CBMs), 16.48% coded for carbohydrate esterases (CEs) and 8.06 and 3.30% encoded glycosyltransferases (GTs) and

polysaccharide lyases (PLs), respectively. Eighty-eight of these CAZy proteins were previously detected in the secretomes of *Botrytis*-infected tomato fruit and *Botrytis* grown in host-free culture systems (Supplemental Table S2; Shah et al., 2009a,b; Espino et al., 2010; Fernández-Acero et al., 2010; Li et al., 2012; Shah et al., 2012.)

Of the 275 potentially secreted CAZymes, 121 were grouped into 39 protein tribes (containing at least two proteins; Supplemental Table S2). Protein tribes are protein families defined by their sequence similarity using the Tribe-MCL algorithm (Enright et al., 2002). Members within a protein tribe are predicted to have similar molecular structures and, thus, may perform similar biochemical functions. In most cases, proteins of the same tribe share a common evolutionary history (Enright et al., 2003). The largest tribes had 6–7 proteins and belonged to the CAZyme subfamilies CE10 (tribes 0 and 2), GH28 (tribe 1), CBM1 (tribe 3), GH3 (tribe 4), and GH16 (tribe 5). Tribe 1 contained the well-characterized endo-polygalacturonases (endo-PGs, BcPG1-6), which cleave the homogalacturonan (HG pectin backbones; Jayani et al., 2005). Tribes 4 and 6 included, respectively, all putative β-glucosidases and xyloglucan (XyG) transglucosylase/hydrolases (XTHs) present in the *Botrytis* genome. Both the β-glucosidases and XTHs classes target the backbones of XyGs; β-glucosidases may be also involved in cellulose degradation (Eklöf and Brumer, 2010).

RNA SEQUENCING (RNAseq) OF *BOTRYTIS*-INFECTED PLANT TISSUES

RNAseq was performed to characterize the expression profiles of *Botrytis* genes encoding CAZymes during infections of ripe tomato fruit and grape berries. mRNA was isolated from *Botrytis*-infected tomato fruit (at 3 days post-inoculation, dpi) and grape berries (stage S1; see Methods) and analyzed by high-throughput sequencing. RNAseq data from lettuce leaves 2 days after *Botrytis* infection (De Cremer et al., 2013) were analyzed in parallel to the fruit data, in order to detect commonalities and differences of CAZyme expression between fungal infections of different host organs and species. A summary of parsed reads from each biological replication of the three *Botrytis*-infected plant hosts and the number of reads mapped to the *Botrytis* (strain B05.10) and plant transcriptomes is provided in **Table 1**.

The reads that uniquely mapped to the *Botrytis* transcriptome corresponded to more than 75% of the total predicted genes in the *Botrytis* genome. 12,766 (77.79%), 12,998 (79.21%) and 13,898 (84.69%) *Botrytis* genes were detected in lettuce leaves, tomato fruit, and grape berries, respectively. In addition, the percentage of reads uniquely mapped to the *Botrytis* transcriptome relative to the total number of mapped reads suggested that the amounts of *Botrytis* were comparable between the infected hosts (**Figure 1**). *Botrytis* infections of ripe tomato fruit at 3 dpi, ripe grapes berries in stage 1 of the noble rot and lettuce leaves at 2 dpi showed similar disease symptoms, which include water-soaked lesions with no or limited fungal sporulation, and without extensive tissue maceration, suggesting that *Botrytis* was at similar stages in its life cycle on the three hosts.

The amounts of *Botrytis* biomass in the infected tomato and grape tissue samples were measured with a monoclonal antibody-based assay (Meyer and Dewey, 2000) and strongly correlated with the number of *Botrytis* transcript reads in the corresponding

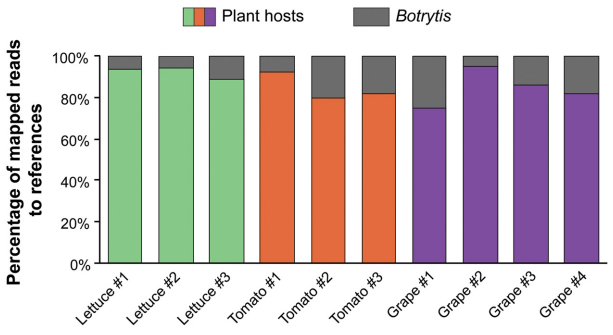


FIGURE 1 | *Botrytis* transcript abundance in infected plant tissues. Relative composition of RNAseq transcript reads between *Botrytis* and the three plant hosts (i.e., lettuce leaves, tomato fruit and grape berries).

Table 1 | Summary of trimmed and mapped reads of mRNA from *Botrytis*-infected tissues.

Samples	Quality-trimmed reads (Q >20)	Total reads mapped		Number of reads uniquely mapped	
		Number	Percentage (%)	*Botrytis*	Plant host
Lettuce #1	20,726,205	15,476,051	74.67	981,495	14,494,556
Lettuce #2	8,742,262	6,098,894	69.76	324,147	5,774,747
Lettuce #3	7,024,911	5,197,645	73.99	566,381	4,631,264
Tomato #1	15,300,253	12,693,894	82.97	971,765	11,722,129
Tomato #2	15,315,439	12,085,072	78.91	2,428,994	9,656,078
Tomato #3	17,334,445	14,020,196	80.88	2,480,527	11,539,669
Grape #1	21,529,329	16,579,455	77.01	4,083,310	12,496,145
Grape #2	25,706,831	21,673,021	84.31	983,846	20,689,175
Grape #3	22,110,864	18,024,359	81.52	2,490,373	15,533,986
Grape #4	17,372,190	13,796,319	79.42	2,448,717	11,347,602

Transcript reads from infected lettuce leaves were from De Cremer et al. (2013).

samples (Supplemental Figure S1). This confirmed that the number of *Botrytis* transcripts from infected plant tissues determined by RNAseq can be used as an indicator of the amount of *Botrytis* biomass present in the infected tissues.

CAZyme *BOTRYTIS* GENES EXPRESSED DURING HOST PLANT INFECTIONS

Most (88.40%) of the CAZyme genes predicted in the *Botrytis* genome were detected in the three infected hosts (**Figure 2A**; Supplemental Table S1). The genes commonly expressed in the three hosts include 83.23% of the CAZyme genes with predicted secretion signals (**Figure 2A**; Supplemental Table S2). The largest number of genes encoding putative secreted CAZymes belonged to the GH28 and CE10 subfamilies, each with 17 genes (Supplemental Table S2). Although CAZymes secreted by *Botrytis* are expected to target plant cell wall substrates, they could also be involved in remodeling the fungal cell wall as the pathogen grows and develops (Cantu et al., 2009b). Alternatively, they may degrade host cellular contents including starch and glycosylated proteins and secondary metabolites with sugar groups (Faure, 2002; Shah et al., 2009b; Klis et al., 2010). Some CAZymes can act on more than one polysaccharide substrate (Eklöf and Brumer, 2010). **Table 2** provides an overview of *Botrytis* CAZymes that might be relevant for the degradation of plant cell walls, as determined by manual curation of their functional annotations.

Remarkably, 98.90% of the CAZyme *Botrytis* genes with secretion signals were detected when the data of the transcriptomes from all the hosts were combined (Supplemental Table S2). Only three genes, *BC1G_09963* (CBM50|CBM18|GH18 subfamily), *BC1G_13488* (putative XTH, GH16 subfamily; tribe 5) and *BC1G_13714* (CE10 subfamily; tribe 0) were not found in any of the *Botrytis*-infected tissues.

CAZyme *Botrytis* genes that were detected on some but not all hosts (Supplemental Table S2) were identified. Thirteen percent of the genes encoding putative secreted CAZymes were expressed in infected ripe tomato fruit and grape berries but were not detected in infected lettuce leaves (**Figure 2A**; Supplemental Table S2). For example, three members of the GH16 subfamily (including two putative XTHs from tribe 5) were detected only on tomato fruit and grape berries. The well-described PG-coding gene *BcPG2* (Kars et al., 2005a) was detected only in infected lettuce leaves and tomato fruit. Two genes (*BC1G_05377* from the CBM18 subfamily and *BC1G_15017* from the AA9 subfamily) were identified only in the transcriptomes of infected lettuce leaves and grape berries.

Besides the *Botrytis* genes that appeared to be preferentially expressed in infected fruit but not in infected lettuce leaves, a few other genes seemed to be specifically expressed in a particular plant species (Supplemental Table S2). Three genes were uniquely detected in infected ripe grape berries: a putative XTH (*BC1G_09829*, GH16 subfamily and tribe 5), a candidate

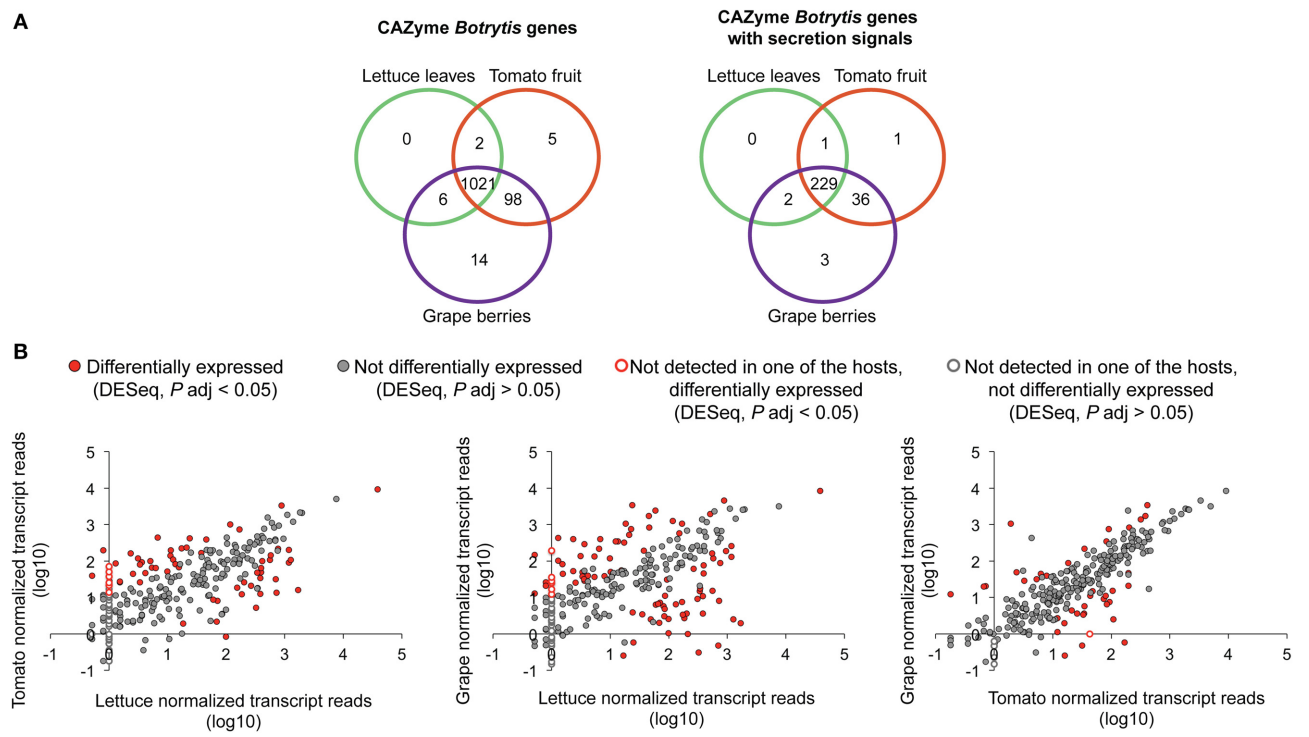


FIGURE 2 | Comparisons of CAZyme gene expression during infections of lettuce leaves, tomato fruit and grape berries. (A) Venn diagrams showing the overlapping and unique sets of CAZyme *Botrytis* genes (left) and CAZyme *Botrytis* genes with potential signal peptides (right) detected in the transcriptomes of the three infected host tissues. **(B)** Scatterplots showing all possible pair-wise correlations between DESeq-normalized counts of CAZyme *Botrytis* genes in lettuce, tomato, and grape.

Table 2 | Annotated secreted *Botrytis* enzymes involved in plant cell wall disassembly.

Plant cell wall target	CAZyme subfamily	Functional annotation	Proteins potentially secreted	Genes expressed in all three hosts
HG backbone	GH28	Polygalacturonases	11	*BcPG1, BcPG3, BcPG4, BcPG5, BcPG6, BC1G_00240, BC1G_01617, BC1G_11909, BC1G_15118*
	PL1	Pectin lyases	4	*BC1G_07527, BC1G_11690, BC1G_12017, BC1G_12517*
	PL1, PL3	Pectate lyases	4	*BC1G_00912, BC1G_09000, BC1G_07052, BC1G_10229*
	CE8	Pectin methylesterases	3	*BcPME1, BcPME2, BC1G_11144*
RG-I backbone	GH28, GH105|GH28	RG hydrolases	6	*BC1G_01234, BC1G_01923, BC1G_03619, BC1G_03464, BC1G_05961, BC1G_13970*
	GH78	α-L-Rhamnosidases	2	*BC1G_06328*
	CE12	RG acetylesterases	1	*BC1G_14009*
XyG backbone	GH3	β-Glucosidases	6	*BC1G_03179, BC1G_07110, BC1G_07622, BC1G_10231, BC1G_11439, BC1G_14169*
	GH12	XyG-specific β-glucanases	1	*BC1G_00594*
	GH16, GH16|CBM18	Glucanases and XyG transglucosy-lase/hydrolases	11	*BC1G_00409, BC1G_02932, BC1G_04948, BC1G_07945, BC1G_08924, BC1G_09991*
Xylan backbone	GH10, GH11, GH10|CBM1, GH11|CBM1	β-Xylanases	5	*BcXyn11A, BC1G_00576, BC1G_01778, BC1G_03590*
	GH43	β-Xylosidases	3	*BC1G_02487, BC1G_10797*
Mannans	GH5|CBM1	β-Mannosidases	1	*BC1G_02036*
	GH26, CBM3|GH26|CBM35|GH44	β-Mannanases	2	*BC1G_10341*
Cellulose	GH5, GH5|CBM1, GH45	1,4-β-Glucanases	10	*BC1G_02740, BC1G_03038, BC1G_07822, BC1G_08990, BC1G_09210, BC1G_13855, BC1G_13862, BC1G_16238, BcCEL5A,*
	GH6|CBM1, GH6|CBM2, GH7, GH7|CBM1	Cellulose 1,4-β-cellobiosidases	5	*BC1G_06035, BC1G_08989, BC1G_10880, BC1G_13445, BC1G_14702*
Side-chains/adducts	GH2, GH35	β-Galactosidases	3	*BC1G_02410, BC1G_03567, BC1G_12184*
	GH31	α-Xylosidases	2	*BC1G_12859, BC1G_11115*
	GH43, GH93	α-L-1,5-Arabinanases	2	*BcAra1, BC1G_13938*
	GH47, GH92	α-Mannosidases	4	*BC1G_00245, BC1G_00455, BC1G_09742, BC1G_12174*
	GH51, GH54|CBM42, GH62|CBM13	α-Arabinofuranosidases	4	*BC1G_04994, BC1G_08372, BC1G_10789, BC1G_12138*
	GH53	AG β-galactosidases	1	*BC1G_16209*
	GH95	α-L-Fucosidases	1	*BC1G_08975*
	GH115	Xylan α-1,2-glucuronosidases	1	*BC1G_13153*

The secreted CAZyme-coding genes that were commonly expressed during Botrytis infections of lettuce leaves, tomato fruit and grape berries are presented.

copper-dependent lytic polysaccharide monooxygenase (LPMO, *BC1G_00922*, AA9 subfamily) and a predicted FAD-binding oxidoreductase (*BC1G_06334*, GT22 subfamily). One gene, a putative endo-β-1,4-xylanase (*BC1G_13645*, GH11 subfamily), was detected only in tomato fruit. No CAZyme *Botrytis* genes were detected exclusively in lettuce leaves.

The lowest number of CAZyme genes was detected in *Botrytis*-infected lettuce leaves (**Figure 2A**, Supplemental Tables S1 and S2). This observation could indicate that *Botrytis* expressed a smaller set of genes in lettuce leaves, or that differences in the experimental design (e.g., inoculations in laboratory conditions vs. field infections) or sequence coverage affected the detection

levels. Some (23 genes) of the *Botrytis* genes that were not detected in infected lettuce leaves were genes with low levels (<0.01%) of mapped reads in the other plant hosts, suggesting that these genes may not have been detected in lettuce leaves perhaps because of low coverage (**Figure 2B**). However, other *Botrytis* genes missing from the infected lettuce transcriptome (4 genes) had moderate to high levels of expression (0.05–0.30%) in fruit tissues, indicating that they may not be relevant during lettuce infections (**Figure 2B**).

RELATIVE GENE EXPRESSION OF *BOTRYTIS* CAZymes WITH SIGNAL PEPTIDES IN DIFFERENT HOST TISSUES

Figure 3 describes the repertoire of potentially secreted CAZymes encoded in the *Botrytis* genome and their relative levels of expression (i.e., percentage of DESeq-normalized reads) when compared to the normalized expression of all CAZyme *Botrytis* genes with signal peptides in a given infected host tissue. Among the most highly expressed *Botrytis* genes coding for characterized or candidate plant cell wall modifying enzymes in each of the plant hosts, *BcPG1* gene had the maximum level of expression during *Botrytis* infections on all hosts (46% of reads in infected lettuce, 18.77% of reads in infected tomato fruit and 13.16% in botrytized-grape berries; Supplemental Table S2). Five other *Botrytis* genes also were highly expressed in the three hosts: a putative cellobiohydrolase gene (*BC1G_14702*, GH7|CBM1 subfamily), a candidate α-xylosidase gene (*BC1G_12859*, GH31 subfamily), *BcPME2* coding for a pectin methylesterase (PME, CE8 subfamily; Kars et al., 2005b), *BcCel5A* encoding an endo-β-glucanase (GH5 subfamily, Espino et al., 2005), and a putative endo-glucanase gene (*BC1G_13862*, GH45 subfamily).

Elevated expression (>0.50% of reads) of six genes encoding CAZymes with defined or putative roles in cell wall degradation was detected during infections of ripe fruit (tomato and grape), but not during infections of lettuce leaves (Supplemental Table S2). These putative fruit-specific genes include two genes encoding putative copper-dependent LPMOs (*BC1G_07653* and *BC1G_07658*, AA9 subfamily, *P*-adjusted value <0.005).

Eight genes were highly expressed in lettuce leaves, but were expressed at lower levels in ripe fruit (**Figure 3**, Supplemental Table S2). These included a candidate α-L-arabinofuranosidase (*BC1G_04994*, GH54|CBM42 subfamily, *P*-adjusted value <0.001), a predicted exo-PG (*BC1G_01617*, GH28 subfamily, *P*-adjusted value <0.001), and a possible β-glucosidase (*BC1G_07110*, GH3 subfamily, *P*-adjusted value <0.001).

ASSOCIATION BETWEEN EVOLUTIONARY HISTORY AND EXPRESSION LEVELS OF *BOTRYTIS* PROTEINS INVOLVED IN PLANT CELL DISASSEMBLY

The evolutionary relationships among members of seven CAZyme subfamilies corresponding to characterized and putative cell wall modifying enzymes were inferred using the Neighbor-Joining method (Saitou and Nei, 1987; **Figures 4**, **6**, **7**; see Methods for details). The CAZyme subfamilies were chosen based on their functional annotations with the additional requirement of including protein tribes with more than three members.

The phylogenetic analyses of the GH28 subfamily, which included *Botrytis* proteins involved in the hydrolysis of pectins; i.e., HG and RG-I backbones, identified two main clades (86% bootstrap value; **Figure 4A**). The first clade included the endo-PGs (tribe 1). The second clade was composed of two well-supported groups (79% bootstrap value); one group included the rhamnogalacturonan hydrolases (RGases) of the tribe 16, and the other group was comprised of exo-PGs and RGases from tribes 10 and 26, respectively. The consensus phylogenetic tree of the pectin lyases (PLs) and pectate lyases (PELs) from in the PL1 and PL3 subfamilies (**Figure 4B**) did not identify distinct clades, but confirmed the classification between protein tribes. For example, the PLs belonging to the tribe 8 group and the PELs, some of which corresponded to tribe 24, were separated in the tree. The PMEs from the CE8 subfamily (**Figure 4C**) showed a well-supported cluster (100% bootstrap value) composed of the characterized BcPME1 and BcPME2 proteins; however, the tree also supported the grouping of the tribe 11, which also included BcPME3.

Botrytis endo-PG (tribe 1) and PME (tribe 11) genes had the highest levels of expression (**Figures 4A,C**, Supplemental Table S2). However, not all the members of these tribes were expressed equally on the three plant hosts. Among the members of tribe 1, only the *BcPG1* gene was highly expressed in all hosts (**Figure 4A**). *BcPG3* had elevated expression in botrytized grape berries (1.28% of reads) but was expressed at lower levels in other infected host tissues (*P*-adjusted value <0.05); whereas the *BcPG6* gene was expressed more in infected lettuce leaves than on tomato fruit or grape berries (*P*-adjusted value <0.001), and *BcPG2* was not expressed during *Botrytis* infections of grape berries (*P*-adjusted value <0.001). The expression of the *BcPG1* and *BcPG2* genes were validated by quantitative reverse transcription PCR (qRT-PCR; **Figure 5**). A sequence alignment of the *BcPG2* gene of the B05.10 strain and the gene homolog of BcDW1 strain (primary inoculum used to induce noble rot in the grape berries; Blanco-Ulate et al., 2013a) indicated that they share 99.02% identity at the DNA level with no gaps. The mapping parameters used in this study were chosen to allow reads to map on a reference with a higher level of sequence diversity. To determine if sequence polymorphisms were responsible for the apparent lack of *BcPG2* expression, the RNAseq transcript reads from infected grapes were mapped to the predicted BcDW1 transcriptome and very similar mapping counts were obtained ($r > 0.99$; *P*- value = 2.2e−16; Supplemental Figure S2). The mapping coverage of *BcPG2* using BcDW1 as transcriptomics reference confirmed the absence of expression of this gene during *Botrytis* infections of ripe grape berries.

Although transcripts of all *BcPMEs* were abundant in all plant tissues, *Botrytis* selectively expressed specific PME genes in each host: *BcPME2* in lettuce leaves, *BcPME1* in tomato fruit and *BcPME3* in grape berries (**Figure 4C**, Supplemental Table S2). Hydrolases of the RG-I backbone (RGases; tribes 16 and 26) showed low levels of expression in all hosts, with the exception of *BC1G_05961* that was expressed at higher level in ripe fruit when compared to lettuce leaves (**Figure 4A**, *P*-adjusted value <0.001). On the other hand, *BC1G_01617* was the only

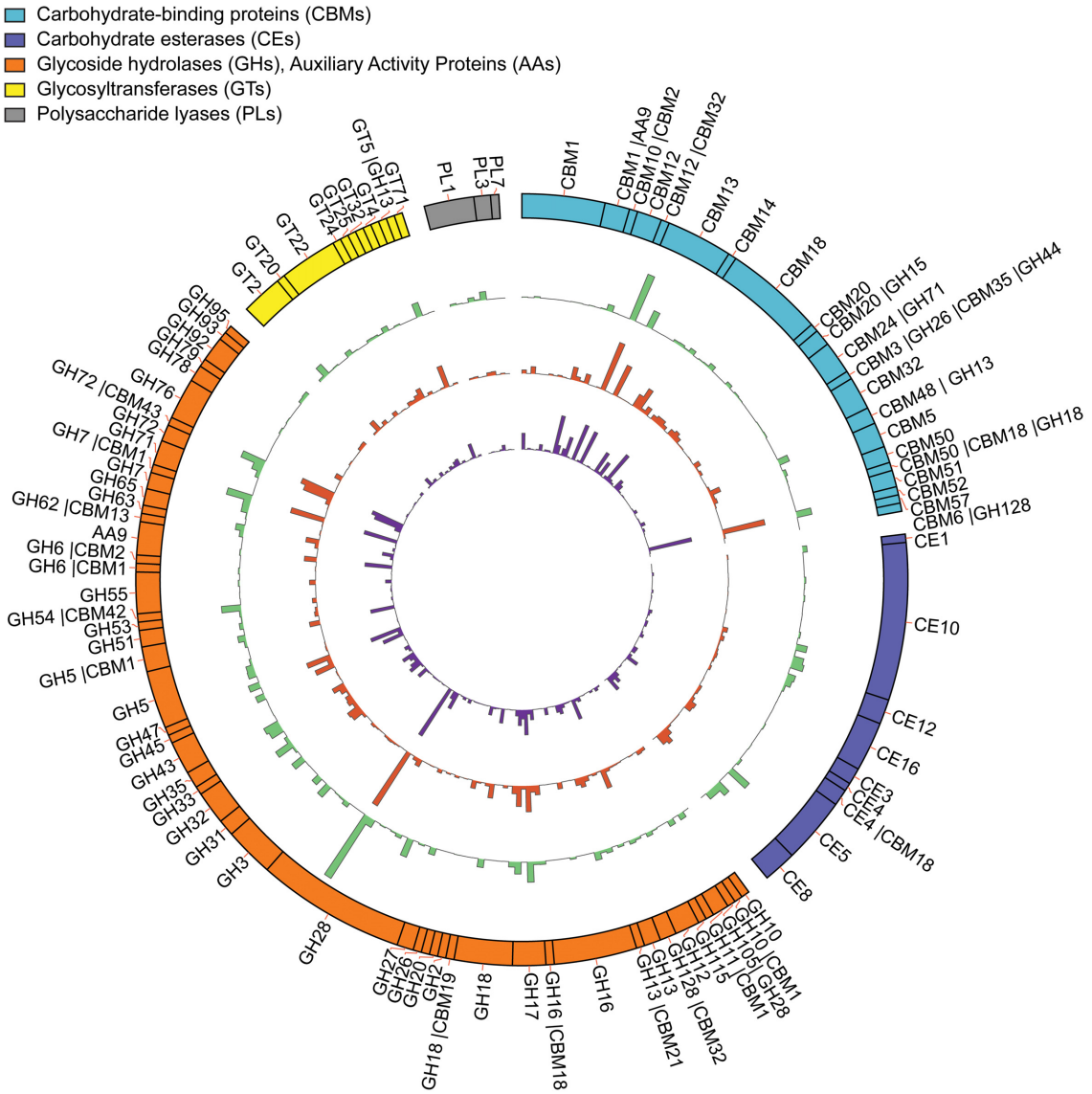


FIGURE 3 | *Botrytis* genes encoding predicted secreted CAZymes and their relative expression levels during infections of three plant hosts. The outermost ring represents all of the secreted CAZymes predicted in the *Botrytis cinerea* genome (strain B05.10). The inner four rings represent the relative expression of each CAZyme gene during *Botrytis* infections of lettuce leaves (green, second ring from outside), ripe tomato fruit (red, third ring from outside) and ripe grape berries (purple, the innermost ring). The relative expression of each gene is the log2-transformed percentage of normalized reads among the total normalized reads from all CAZyme *Botrytis* genes that possess a secretion signal peptide.

exo-PG (tribe 10) with considerable levels of expression in all hosts; the highest level of expression of this gene was in lettuce leaves (*P*-adjusted value <0.001). Members of the PL1 and PL3 subfamilies, predicted to encode PLs and PELs, had low levels of expression (<0.3%) in all plant hosts. The exception was the *BcPL-like1* gene (tribe 8), which was more highly expressed in infected lettuce leaves than ripe tomato fruit (**Figure 4B**; *P*-adjusted value <0.05).

Figure 6 depicts the phylogenetic relationships among the CAZymes that might have roles in the breakdown of XyGs and cellulose. These include putative XTHs and glucanases (GH16 subfamily), and β-glucosidases (GH3 subfamily). The consensus phylogenetic tree of the GH16 subfamily separated the putative XTHs (tribe 5) from glucanases. Most candidate XTHs (tribe 5) showed low levels of expression in the three host tissues; e.g., the *BcXTH-like1* gene had an intermediate level of expression in ripe fruit tissues (>0.35% reads in both tomato and grape hosts). Three possible glucanases (*BC1G_00409*, *BC1G_04948*, and *BC1G_02932*) from diverse tribes have high expression in the three host tissues (Supplemental Table S2).

The *Botrytis* proteins present in the AA9 subfamily include copper-dependent LPMOs proteins and other hypothetical proteins. Copper-dependent LPMOs are an auxiliary class of cell

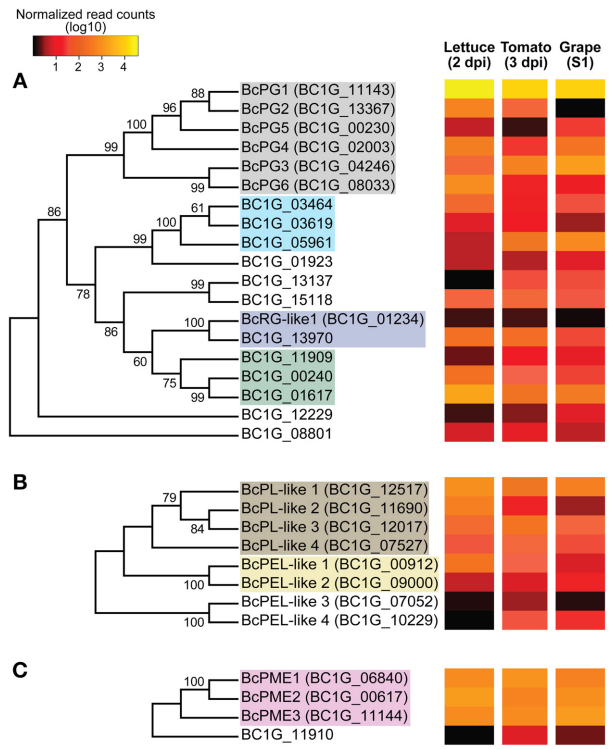


FIGURE 4 | Phylogenetic tree and expression levels of characterized and candidate enzymes that act on pectin backbones. Bootstrap consensus trees inferred from 1000 replicates using the Neighbor-Joining method for the *Botrytis* CAZymes with predicted signal peptides in the GH28 **(A)**, PL1 and PL3 **(B)** and CE8 **(C)** subfamilies. The percentages (>50%) of replicated trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Colored-boxes in the phylogenetic trees indicate protein tribes (>2 members) determined by BLASTp alignments (*e*-value < 1e−6) and Tribe-MCL (Enright et al., 2002). Boxes in **(A)**, gray = tribe 1, light blue = tribe 16, purple = tribe 26 and green = tribe 10. Boxes in **(B)** brown = tribe 8, yellow = tribe 24. In **(C)**, pink box = tribe 11 (Supplemental Table S2). The colors in the heat maps represent the numbers of DESeq-normalized transcript counts (log10) of the *Botrytis* genes in infected lettuce leaves, ripe tomato fruit and grape berries.

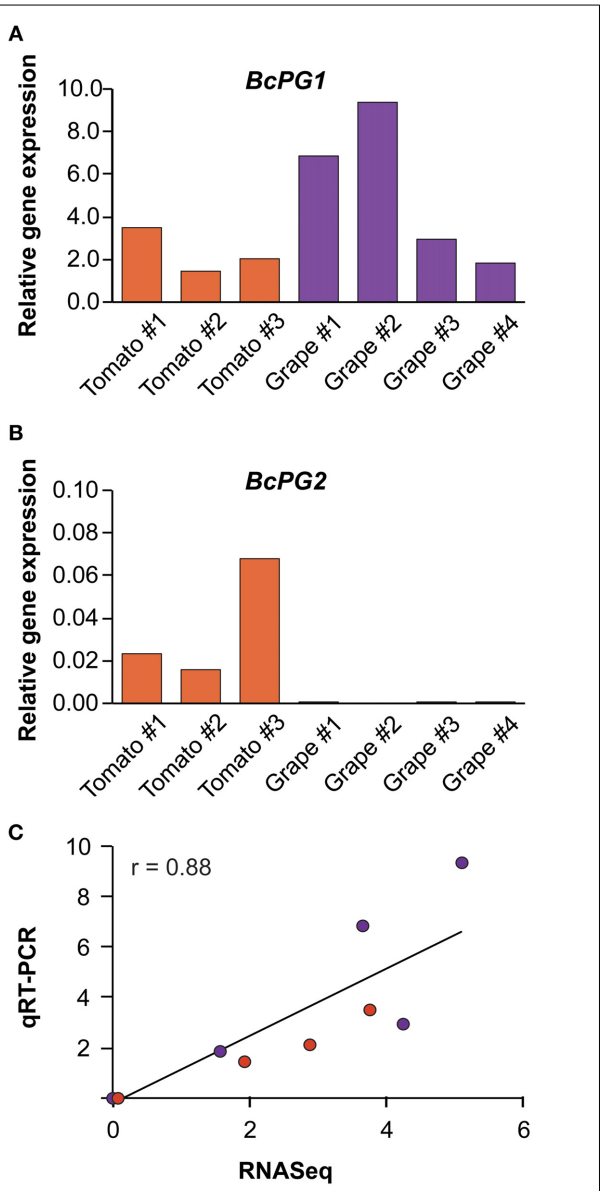


FIGURE 5 | qRT-PCR validation of the RNAseq expression results for *BcPG1* and BcPG2 genes in ripe tomato fruit and grape berries. Relative expression levels of *BcPG1* **(A)** and *BcPG2* **(B)** measured in the biological replications of *Botrytis*-infected tomato fruit and botrytized-grape berries. **(C)** The scatterplot depicts the correlation between the qRT-PCR relative expression of *BcPG1* and *BcPG2* genes and their corresponding raw reads normalized against the same reference gene (*BcRPL5*) used in the qRT-PCR analyses. The data points indicate that the *BcPG2* expression in grape berries were close to zero (average of 4.49e−5) and in tomato fruit the expression was 3.57e−2 on average; thus they overlapped in the graph. The Pearson correlation coefficient (r) is presented (*P*-value < 0.001). A linear trend is shown.

wall modifying proteins that may act on cellulose microfibrils. The phylogenetic analysis of the AA9 subfamily identified two potential clades, in one of which the LPMOs (tribe 13) grouped together. Two of these putative LPMOs (*BC1G_07653* and *BC1G_07658*) showed elevated expression in *Botrytis*-infected fruit, particularly in grape berries (**Figure 7A**; mentioned before).

Phylogenetic and gene expression analyses were done with proteins from the CE5 subfamily, some of which are involved in plant cuticle degradation (**Figure 7B**). There were two monophyletic groups in the consensus phylogenetic tree of the CE5 subfamily. Tribe 15, which included the cutinases, *BcCutA* (Van Kan et al., 1997) and *BcCutB* (Leroch et al., 2013), formed a separate group from the proteins of tribe 31, which included CAZymes without clear functional annotations. On the other hand, proteins from tribe 31 clustered together with putative acetylxylan esterases, which catalyze the deacetylation of xylan backbones, thus assisting, their subsequent degradation by hydrolytic enzymes. *BcCutA* gene was highly expressed (>0.45% of reads) by *Botrytis* in fruit tissues (*P*-adjusted value <0.001), while a putative cutinase gene (*BcCUT-like1*) was more highly expressed (2.03% of reads) during *Botrytis* infections of lettuce leaves (**Figure 7B**; *P*-adjusted value <0.001).

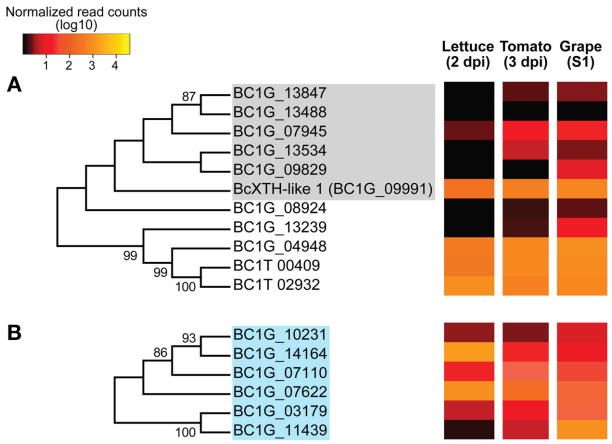


FIGURE 6 | Phylogenetic tree and expression levels of predicted xyloglucan (XyG) backbone-degrading enzymes. Bootstrap consensus trees inferred from 1000 replicates using the Neighbor-Joining method for the *Botrytis* CAZymes with predicted signal peptides in the GH16 **(A)** and GH3 **(B)** subfamilies. The percentages (>50%) of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Colored-boxes in the phylogenetic trees indicate protein tribes (> 2 members) determined by BLASTp alignments (*e*-value < 1e−6) and Tribe-MCL (Enright et al., 2002). Gray box in **(A)** correspond to tribe 5, while the light blue box in **(B)** refers to tribe 4 (Supplemental Table S2). The colors in the heat maps represent the number of DESeq-normalized transcript counts (log10) of the *Botrytis* genes in infected lettuce leaves, ripe tomato fruit and grape berries.

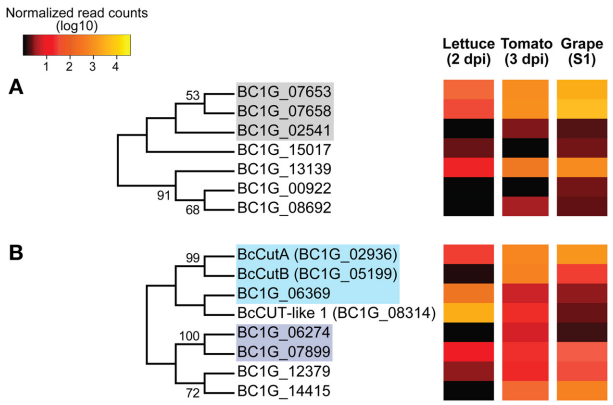


FIGURE 7 | Phylogenetic tree and expression levels of putative copper-dependent lytic polysaccharide monooxygenases (LPMOs) and cutinases. Bootstrap consensus trees inferred from 1000 replicates using the Neighbor-Joining method for the *Botrytis* CAZymes with predicted signal peptides in the AA9 **(A)** and CE5 **(B)** subfamilies. The percentages (>50%) of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Colored-boxes in the phylogenetic trees indicate protein tribes (>2 members) determined by BLASTp alignments (*e*-value < 1e−6) and Tribe-MCL (Enright et al., 2002). In **(A)**, gray box = tribe 13. Boxes in **(B)** light blue = tribe 15 and purple = tribe 31 (Supplemental Table S2). The colors in the heat maps represent the number of DESeq-normalized transcript counts (log10) of the *Botrytis* genes in infected lettuce leaves, ripe tomato fruit and grape berries.

DISCUSSION

BOTRYTIS EXPRESSES A LARGE AND DIVERSE SET OF ENZYMES TO DEGRADE PLANT CELL WALLS

The presence and expression of specific CAZymes or gene families in plant pathogenic fungi have revealed the preferences of pathogens for particular host cell wall polysaccharides and infection strategies that are adapted to the pathogen lifestyle (King et al., 2011; Zhao et al., 2014). Our results indicate that *Botrytis*, a necrotroph, expressed most (>99%) of its 1155 CAZyme genes during infections of lettuce leaves, ripe tomato fruit and grape berries, indicating that expression of a large and diverse array of cell wall-targeting proteins is an important hallmark of the necrotrophic lifestyle.

Botrytis, as a generalist pathogen, infects a wide variety of host tissues with different cell wall compositions and architectures. Therefore, identifying enzymes that *Botrytis* produces on different hosts, can help to define parts of the host cell wall polysaccharide matrix that are important targets for *Botrytis* growth or metabolism. A common set of 229 CAZyme genes with secretion signals was expressed by *Botrytis* when infecting the three different hosts. Eighty-two of these proteins were also detected in proteomic studies of the *Botrytis* secretome (Shah et al., 2009a,b; Espino et al., 2010; Fernández-Acero et al., 2010; Li et al., 2012; Shah et al., 2012).

These common CAZymes probably constitute the core enzymatic machinery utilized by *Botrytis* as it penetrates and invades various plant tissues. Pectin-degrading enzymes (e.g., characterized endo-PGs, putative RGases and candidate exo-PGs) were the most abundant plant cell wall modifying proteins among the CAZymes expressed in all hosts. This result confirms that pectin degradation is a key process during *Botrytis* infections of plant tissues, including those with higher proportions of cellulose and hemicellulose polysaccharides, such as lettuce leaves (Nunan et al., 1998; Wagstaff et al., 2010; Lunn et al., 2013).

Botrytis adapts its infection strategy to the diverse conditions present in the host cell walls. The expression of 43 *Botrytis* genes encoding predicted secreted CAZymes was detected in some but not all plant hosts. The majority (83.72%) of these genes were commonly expressed in tomato and grape tissues, but not in lettuce leaves. Among these fruit-specific genes, those encoding putative xyloglucan (XyG) transglucosylase/hydrolases and glucanases were the most abundant. A candidate exo-PG (*BC1G_13137*) gene that is only detected when in *Botrytis*-infected fruit had been detected when this pathogen was grown in minimal medium supplemented with pectins as the sole carbon source, which suggests that this enzyme is important in the degradation of host cell walls that are rich in pectins (Shah et al., 2009a). The molecules or signals lead to the expression of host-specific enzymes are not known, but some could result from the degradation of host cell walls by core CAZymes (e.g., pectin derived oligosaccharides; Körner et al., 1998; An et al., 2005).

Botrytis may promote susceptibility in the host tissues by inducing or suppressing the expression of plant cell wall degrading enzymes (AbuQamar et al., 2006; Flors et al., 2007; Cantu et al., 2008b, 2009a). Fungal and plant enzymes may cooperate to effectively digest relatively complex polysaccharides. In addition, the activity of plant enzymes can result in the disassembly of the

host cell walls beyond the site of fungal infection, which would facilitate the subsequent growth of *Botrytis* into the host tissues.

PECTIN MODIFYING ENZYMES

Botrytis produces a large collection of enzymes to degrade the backbones and side-branches of pectin polysaccharides. Breakdown of pectins during infections increases the plant cell wall's porosity, and may facilitate the degradation of other wall polysaccharides and the growth of *Botrytis*. Enzymes that target pectin backbones include PGs and RGases (GH28 subfamily), and PLs/PELs (PL1 and PL3 subfamilies). PMEs (CE8 subfamily) and RG esterases (CE12 subfamily) might cooperate in the effective degradation of pectin backbones (Van Kan, 2006; Zhang and van Kan, 2013a).

PGs hydrolyze the backbones of HGs. The *Botrytis* genome encodes 11 potentially secreted PGs, of which at least five are likely exo-PGs (i.e., predicted to remove sugar monomers from the non-reducing ends of HG or oligomeric products generated by endo-PG action on HG) and six are endo-PGs (i.e., hydrolyze the HG polymer at internal sites). When infecting lettuce leaves and ripe fruit, *Botrytis* expressed four exo-PG and five endo-PG genes (**Table 2**). *BcPG2* was not detected in botrytized grape berries. The expression of *BcPG2* was up-regulated in the presence of pectate as carbon source in a host-free transcriptomics analysis (Zhang et al., 2013). The BcPG2 protein was detected at high levels when *Botrytis* was grown in media enriched with partially esterified pectin, glucose and extracts of tomato fruit, while only small quantities of BcPG2 protein were observed in *Botrytis* cultures supplemented with highly esterified pectin substrates or kiwi fruit extracts (Shah et al., 2009a; Espino et al., 2010). Taken together, these results indicate that the expression of *BcPG2* is differentially regulated depending on the conditions present in the plant host's cell wall matrixes (e.g., esterification status of the pectin polysaccharides; presence of inhibitors or activators of *BcPG2* gene expression, availability of particular cell wall substrates, efficiency of other BcPGs in a particular tissue).

In agreement with previous reports, *BcPG1* was the most highly expressed CAZyme gene in lettuce leaves, ripe tomato fruit and grape fruit (Reignault et al., 2000; Wubben et al., 2000; Ten Have et al., 2001). Although BcPG1 is not indispensable for virulence, the Δ*bcpg1* knockout mutant is significantly less virulent in diverse hosts (Ten Have et al., 1998; Zhang and van Kan, 2013b). The expression data in this study support the conclusion that PGs, especially BcPG1, have major roles for *Botrytis* infections in a broad-range of plant tissues (Ten Have et al., 1998, 2001; Powell et al., 2000; Kars et al., 2005a; Rowe and Kliebenstein, 2007; Zhang et al., 2014).

Plants produce PG inhibiting proteins, PGIPs, to reduce the extensive pectin degradation caused by fungal, bacterial or insect attack (De Lorenzo et al., 2001). PGIPs inhibit most of the *Botrytis*'s PGs (Sharrock and Labavitch, 1994; Joubert et al., 2007). Over-expression of PGIPs has been proven to increase the abundance of this inhibitor in the cell wall matrix and to reduce *Botrytis* growth on vegetative tissues and ripe tomato fruit (Powell et al., 2000; Ferrari et al., 2003, 2006).

In addition to the production of its own endo-PGs, *Botrytis* can trigger the expression of plant genes encoding endo-PGs. Because plant endo-PGs are not inhibited by PGIPs (Cervone et al., 1990), they may assist in the breakdown of host cell wall pectins even when inhibitors of fungal enzymes are present. The tomato endo-PG, *SlPG2A*, is precociously up-regulated by *Botrytis* infections of unripe tomato fruit (Cantu et al., 2009a; Shah et al., 2012). The *SlPG2A* is considered a key cell wall degrading enzyme during tomato fruit ripening and softening (Bennett and Labavitch, 2008; Cantu et al., 2008b), and thus, its premature induction may benefit *Botrytis* infections.

PLs and PELs degrade HGs by a β-elimination rather than by hydrolysis. PLs generally act on heavily methylesterified HG backbones, and PELs are more efficient on lightly methylesterified HGs. Four PLs and four genes encoding PELs were annotated in the *Botrytis* genome, and the expression of all was detected in the three hosts (**Table 2**). However, the expression of these genes was lower than the expression of the *BcPG* genes, suggesting that *Botrytis* PLs/PELs assist PGs and are not the primary enzymes attacking HGs.

The extent of methylation and acetylation of the HG backbones can impact the activity of *Botrytis* endo-PGs and PELs (Kars et al., 2005a). PMEs catalyze the specific demethylesterification of HGs. Three putative secreted PMEs are present in the *Botrytis* genome (*BcPME1-3*; Kars et al., 2005b). Although these three PMEs were expressed in *Botrytis*-infected lettuce leaves, tomato fruit, and grape berries, *Botrytis* PME activity seems not to be essential for virulence on certain hosts. Knockout mutations in *BcPME1* and *BcPME2* did not affect *Botrytis*'s virulence on leaves of tomato and grapevine and on pear fruit (Kars et al., 2005b). However, BcPME1 was necessary for successful infections of apple fruit (Valette-Collet et al., 2003). It is possible that *Botrytis* relies on plant PMEs for demethylesterification of the HG backbones in certain host tissues (Raiola et al., 2011), or that the activity of BcPME3 can compensate for the absence of BcPME1 and BcPME2 (Kars et al., 2005b).

Plant PMEs may act as susceptibility factors by cooperating with *Botrytis* PMEs for the demethylesterification of HG backbones (Lionetti et al., 2012). For example, infection of Arabidopsis leaves by *Botrytis* alters the expression of host PME genes (AbuQamar et al., 2006), and the enhanced gene expression and activity of AtPME3 increases susceptibility to *Botrytis* (Raiola et al., 2011). In an effort to counteract the increased PME activity that results from encounters with pathogens, plants produce PME inhibitors (PMEIs; An et al., 2008; Volpi et al., 2011; Lionetti et al., 2012, 2014). In vegetative tissues, the over-expression of plant PMEIs had been effective for limiting *Botrytis* infections Lionetti et al., 2007, 2012.

The *Botrytis* genome includes six possible secreted RGases, two α-L-rhamnosidases and an RG acetylesterase (**Table 2**), which could cleave or modify RG-I backbones (Schols et al., 1994; Mutter et al., 1998; Mølgaard et al., 2000). Although expression of most of these genes was detected in the *Botrytis*-infected tissues, the low level of their expression may reflect the paucity of RG-I compared to HG pectins. RG-I is a major part of the hairy region of pectins in plant cell walls, but they are not as abundant as pectins with HG-backbones (Voragen et al., 2009).

Botrytis expressed diverse enzymes that are predicted to degrade pectin side-branches. Among these genes were four

α-arabinofuranosidases, three β-galactosidases, and two α-L-1,5-arabinanases (including *BcAra1*; **Table 2**). BcAra1 has been shown to degrade 1,5-arabinan *in vitro*. On Arabidopsis leaves but not tobacco or tomato leaves, the Δ*bcara1* knockout mutant has reduced virulence (Nafisi et al., 2014).

HEMICELLULOSE MODIFYING ENZYMES

A variety of hemicellulose-modifying enzymes is encoded in the *Botrytis*'s genome. XyG backbones are hydrolyzed by endo-acting β-1,4-glucanases or β-glucosidases, which also act on cellulose (Gilbert, 2010). All of the predicted *Botrytis* β-glucosidase genes were expressed in infected lettuce leaves, tomato fruit and grape berries; however, expression levels of some were higher on lettuce leaves (Supplemental Table S2). The β-glucosidases were among the most numerous glycosyl hydrolases in fungal genomes (Zhao et al., 2014). XTHs can act on XyG backbones. They have two possible catalytic activities: (1) XyG endo-transglucosylase (XET) activity, which results in the non-hydrolytic cleavage and ligation of XyG polymers, and (2) XyG endo-hydrolase (XEH) activity that leads to the irreversible shortening of the XyG backbone (Eklöf and Brumer, 2010). The *Botrytis* genome has six candidate XTHs (GH16 subfamily), and two of this genes (*BcXTH-like1* and *BC1G_07945*) were commonly expressed at low levels in all of the host tissues studied.

Xylans and mannans are present in the primary and secondary walls of many of *Botrytis's* hosts, but they are less abundant than XyGs. Digestion of these hemicelluloses may be important for *Botrytis*'s energy acquisition and tissue exploration efforts. Expression of four β-xylanases (including *BcXyn11A*; **Table 2**) and two β-xylosidases (*BC1G_02487* and *BC1G_10797*), which target xylan backbones, is detected when *Botrytis* infects lettuce leaves, ripe tomato fruit and post-véraison grape berries. Deletion of the *BcXyn11A* gene delayed disease symptoms and reduced the lesion size on tomato leaves and table grape berries (Brito et al., 2006). However, the contribution of BcXyn11A to overall virulence does not depend on its xylanase activity; rather, it is related to the necrosis in the host caused by the xylanase protein itself (Noda et al., 2010).

Some of the side-branches along the XyG and xylan backbones contribute to the overall strength of the hemicellulose-cellulose microfibril network (Pauly et al., 2013). Therefore, removal of these groups might affect the hemicellulose cross-linking properties and, at least locally, disrupt the wall's hemicellulose-cellulose network. In all hosts analyzed, *Botrytis* expressed two α-xylosidases (*BC1G_12859* and *BC1G_11115*) and one α-L-fucosidase (*BC1G_08975*). These enzymes could digest XyG side groups, exposing the hemicellulose's glucan backbone to further digestion. Additional enzymes that remove side-branches in hemicelluloses may be the same as or functionally equivalent to CAZymes that trim the side groups of pectins; e.g., α-arabinofuranosidases (previously described).

CELLULOSE MODIFYING ENZYMES

Botrytis expresses genes encoding predicted cellulose-degrading enzymes; these include nine endo-β-1,4-glucanases (including *BcCel5A*), five cellobiohydrolases, and the previously discussed β-glucosidases. Espino et al. (2005) demonstrated that a mutant with a deletion in *BcCel5A* (*BC1G_00642*), an endo-β -1,4-glucanase encoding gene, can infect tomato leaves and gerbera petals. Because the expression of *BcCel5A* appears to be relevant during spore germination and penetration of waxy surfaces (Leroch et al., 2013), evaluating the virulence of Δ*bccel5a* mutants in these hosts may provide information about the importance of this enzyme during infections.

As consequence of *Botrytis* infection, expression of plant endo-β-1,4-glucanases is reduced (Flors et al., 2007; Finiti et al., 2013). Transgenic suppression of endo-β-1,4-glucanases limited *Botrytis* growth and promoted the activation of defense responses in tomato fruit and Arabidopsis and tomato leaf tissues. These responses included enhanced callose deposition and expression of defense genes, e.g., *PR1* and *LoxD* (Flors et al., 2007; Finiti et al., 2013).

Copper-dependent lytic polysaccharide monooxygenases (LPMOs) cooperate with canonical cellulose-degrading enzymes and other electron transfer proteins to accelerate the degradation of cellulose microfibrils (Hemsworth et al., 2013). A number of LPMOs have been identified mainly in fungal genomes, especially in wood decay-causing fungi (Levasseur et al., 2013). *Botrytis* expressed two putative LPMOs (*BC1G_07653* and *BC1G_08692*) in lettuce leaves and fruit hosts. However, a larger number of LPMOs was expressed during infections of ripe fruit (especially grape tissues) and their expression was higher than in the other plant host tissues (Supplemental Table S2).

CUTINASES

In the absence of cracks or wounds in the plant surface, the initial interactions between a host and *Botrytis* occur at the plant cuticle. In those situations, *Botrytis* secretes an assortment of cutinases and lipases to breach the cuticle and penetrate the host (Van Kan et al., 1997; Reis et al., 2005; Van Kan, 2006; Leroch et al., 2013). Cutinases cleave ester bonds between cutin monomers (Pio and Macedo, 2009).

When infecting the three hosts analyzed, *Botrytis* expressed *BcCutA* and three other putative cutinases (*BcCutB, BcCUT-like1* and *BC1G_06369*). The expression levels of these enzymes depended on the host tissues; for example, *BcCutA* was highly expressed in fruit, but not in lettuce leaves. The expression of *BcCutA* was up-regulated during early germination of *Botrytis* spores on apple wax; this may indicate that *BcCutA* may be preferentially expressed at the surfaces of fleshy fruit, where wax accumulation is common (Leroch et al., 2013). Some *Botrytis* cutinases showed high homology to acetylxylan esterases, which are associated with the degradation of xylans. The *Botrytis* genome encodes two putative acetylxylan esterases (*BC1G_12379* and *BC1G_07899*), and one was expressed in all infected plant tissues analyzed. Results in Skamnioti et al. (2008) suggested that the functional diversification between cutinases and acetylxylan esterases may have occurred before the speciation of *Botrytis cinerea* and two other ascomycete pathogens, *Fusarium graminearum* and *Neurospora crassa*.

CONCLUDING REMARKS

The diversity of CAZyme-encoding genes in the *Botrytis* genome and their extensive expression when this necrotroph interacts

with its hosts suggests that the pathogen's ability to degrade a wide-range of cell wall polysaccharides is tightly associated with its success in infecting a broad range of plant hosts. While several proteins were previously identified by proteomic analyses, this study expands the catalog of the complex array of enzymes that *Botrytis* may secrete to digest host tissues. Pectins, particularly pectin backbones, appear to be the main target of degradation by *Botrytis* in leaf and fruit tissues. However, *Botrytis* also expresses particular CAZyme genes only when infecting certain hosts. What promotes this host-specific expression of CAZyme genes is not known. Information about the structural details of the associations between the constituents of diverse host plant cell walls is needed to fully understand how *Botrytis* benefits from the digestion of plant cell wall polysaccharides during successful infections. In addition, understanding plant responses to *Botrytis* infections, which may include altered expression of endogenous CAZyme genes (Flors et al., 2007; Cantu et al., 2008b, 2009a) or inhibitors of cell wall degrading enzymes (De Lorenzo et al., 1994; Powell et al., 2000; De Lorenzo and Ferrari, 2002; Lionetti et al., 2007, 2014) and plant cell wall fortifications (Van Baarlen et al., 2007; Finiti et al., 2013), may shed some light on the co-evolution of plant and pathogen strategies and their impact on resistance or susceptibility to fungal infections.

Measurements of enzymatic activity *in vitro* as well as *in planta* on cell wall polysaccharides may confirm the predicted enzymatic activities of some of the genes described in this study. This information may refine our understanding of important virulence functions needed for successful *Botrytis* infections. Another strategy to demonstrate that cell wall modifications have occurred during *Botrytis* infection would be to identify the accumulation of characteristic breakdown products; e.g., pectin derived oligosaccharides that result from the activity of PLs and PGs (Melotto et al., 1994; Körner et al., 1998; An et al., 2005). Validation of the role and function of the *Botrytis* cell wall modifying enzymes may also be achieved by targeted mutagenesis, with the caveat that it is expected that most of these enzymes have paralogs with redundant activities and/or their functions may depend on other proteins, including some produced by the plant host.

ACKNOWLEDGMENTS

This work was partially supported by funding to Dario Cantu from the College of Agricultural and Environmental Sciences and the Department of Viticulture and Enology (UC Davis) and to Ann L. T. Powell from NSF (IOS 0957264). Support to Barbara Blanco-Ulate was provided by the Consejo Nacional de Ciencia y Tecnología (Ministerio de Ciencia y Tecnología, Costa Rica) and by the Department of Plant Sciences (UC Davis).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00435/abstract

REFERENCES

AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., et al. (2006). Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to *Botrytis* infection. *Plant J.* 48, 28–44. doi: 10.1111/j.1365-313X.2006.02849.x

Amselem, J., Cuomo, C. A., van Kan, J. A. L., Viaud, M., Benito, E. P., Couloux, A., et al. (2011). Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS Genet.* 7:e1002230. doi: 10.1371/journal.pgen.1002230

An, H. J., Lurie, S., Greve, L. C., Rosenquist, D., Kirmiz, C., Labavitch, J. M., et al. (2005). Determination of pathogen-related enzyme action by mass spectrometry analysis of pectin breakdown products of plant cell walls. *Anal. Biochem.* 338, 71–82. doi: 10.1016/j.ab.2004.11.004

An, S. H., Sohn, K. H., Choi, H. W., Hwang, I. S., Lee, S. C., and Hwang, B. K. (2008). Pepper pectin methylesterase inhibitor protein CaPMEI1 is required for antifungal activity, basal disease resistance and abiotic stress tolerance. *Planta* 228, 61–78. doi: 10.1007/s00425-008-0719-z

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol.* 11:R106. doi: 10.1186/gb-2010-11-10-r106

Bennett, A. B., and Labavitch, J. M. (2008). Ethylene and ripening-regulated expression and function of fruit cell wall modifying proteins. *Plant Sci.* 175, 130–136. doi: 10.1016/j.plantsci.2008.03.004

Blanco-Ulate, B., Rolshausen, P. E., and Cantu, D. (2013a). Draft genome sequence of *Botrytis cinerea* BcDW1, inoculum for noble rot of grape berries. *Genome Announc.* 1:e00252-13. doi: 10.1128/genomeA.00252-13

Blanco-Ulate, B., Rolshausen, P. E., and Cantu, D. (2013b). Draft genome sequence of *Neofusicoccum parvum* isolate UCR-NP2, a fungal vascular pathogen associated with grapevine cankers. *Genome Announc.* 1:e00339-13. doi: 10.1128/genomeA.00339-13

Blanco-Ulate, B., Rolshausen, P. E., and Cantu, D. (2013c). Draft genome sequence of the ascomycete *Phaeoacremonium aleophilum* strain UCR-PA7, a causal agent of the Esca disease complex in grapevines. *Genome Announc.* 1:e00390-13. doi: 10.1128/genomeA.00390-13

Blanco-Ulate, B., Rolshausen, P. E., and Cantu, D. (2013d). Draft genome sequence of the grapevine dieback fungus *Eutypa lata* UCR-EL1. *Genome Announc.* 1:e00228-13. doi: 10.1128/genomeA.00228-13

Blanco-Ulate, B., Vincenti, E., Powell, A. L. T., and Cantu, D. (2013e). Tomato transcriptome and mutant analyses suggest a role for plant stress hormones in the interaction between fruit and *Botrytis cinerea*. *Front. Plant Sci.* 4:142. doi: 10.3389/fpls.2013.00142

Brito, N., Espino, J., and González, C. (2006). The endo-β-1,4-xylanase Xyn11A is required for virulence in *Botrytis cinerea*. *Mol. Plant Microbe Interact.* 19, 25–32. doi: 10.1094/MPMI-19-0025

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, 233–238. doi: 10.1093/nar/gkn663

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J. M., Bennett, A. B., and Powell, A. L. T. (2009a). Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires NOR but not RIN or ethylene. *Plant Physiol.* 150, 1434–1449. doi: 10.1104/pp.109.138701

Cantu, D., Greve, L. C., Labavitch, J. M., and Powell, A. L. T. (2009b). Characterization of the cell wall of the ubiquitous plant pathogen *Botrytis cinerea*. *Mycol. Res.* 113, 1396–1403. doi: 10.1016/j.mycres.2009.09.006

Cantu, D., Segovia, V., MacLean, D., Bayles, R., Chen, X., Kamoun, S., et al. (2013). Genome analyses of the wheat yellow (stripe) rust pathogen *Puccinia striiformis* f. sp. tritici reveal polymorphic and haustorial expressed secreted proteins as candidate effectors. *BMC Genomics* 14:270. doi: 10.1186/1471-2164-14-270

Cantu, D., Vicente, A., Greve, L., Dewey, F., Bennett, A., Labavitch, J., et al. (2008b). The intersection between cell wall disassembly, ripening and fruit susceptibility to *Botrytis cinerea*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 859–864. doi: 10.1073/pnas.0709813105

Cantu, D., Vicente, A. R., Labavitch, J. M., Bennett, A. B., and Powell, A. L. T. (2008a). Strangers in the matrix: plant cell walls and pathogen susceptibility. *Trends Plant Sci.* 13, 610–617. doi: 10.1016/j.tplants.2008.09.002.

Carpita, N. C., and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* 3, 1–30. doi: 10.1111/j.1365-313X.1993.tb00007.x

Cassab, G. I. (1998). Plant cell wall proteins. *Annu. Rev. Plant Physiol. Plant Mol.Biol.* 49, 281–309. doi: 10.1146/annurev.arplant.49.1.281

Cervone, F., De Lorenzo, G., Pressey, R., Darvill, A. G., and Albersheim, P. (1990). Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants? *Phytochem*. 29, 447–449. doi: 10.1016/0031-9422(90)85094-V

Chen, A., and Dubcovsky, J. (2012). Wheat TILLING mutants show that the vernalization gene VRN1 down-regulates the flowering repressor VRN2 in leaves but is not essential for flowering. *PLoS Genetics* 8:e1003134. doi: 10.1371/journal.pgen.1003134

Curvers, K., Seifi, H., Mouille, G. D., Rycke, R., Asselbergh, B., Van Hecke, A., et al. (2010). ABA-deficiency causes changes in cuticle permeability and pectin composition that influence tomato resistance to *Botrytis cinerea*. *Plant Physiol.* 154, 847–860. doi: 10.1104/pp.110.158972

De Cremer, K., Mathys, J., Vos, C., Froenicke, L., Michelmore, R. W., Cammue, B. P. A., et al. (2013). RNAseq-based transcriptome analysis of *Lactuca sativa* infected by the fungal necrotroph *Botrytis cinerea*. *Plant Cell Environ.* 36, 1992–2007. doi: 10.1111/pce.12106

De Lorenzo, G., Cervone, F., Bellincampi, D., Caprari, C., Clark, A. J., Desiderie, A., et al. (1994). Polygalacturonase, PGIP and oligogalacturonides in cell-cell communication. *Biochem. Soc. Trans.* 22, 394–397.

De Lorenzo, G., D'Ovidio, R., and Cervone, F. (2001). The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu. Rev. Phytopathol.* 39, 313–335. doi: 10.1146/annurev.phyto.39.1.313

De Lorenzo, G., and Ferrari, S. (2002). Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. *Curr. Opin. Plant Biol.* 5, 295–299. doi: 10.1016/S1369-5266(02)00271-6

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Eklöf, J. M., and Brumer, H. (2010). The XTH gene family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling. *Plant Physiol.* 153, 456–466. doi: 10.1104/pp.110.156844

Enright, A. J., Kunin, V., and Ouzounis, C. A. (2003). Protein families and TRIBES in genome sequence space. *Nucleic Acids Res.* 31, 4632–4638. doi: 10.1093/nar/gkg495

Enright, A. J., Van Dongen, S., and Ouzounis, C. A. (2002). An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Res.* 30, 1575–1584. doi: 10.1093/nar/30.7.1575

Espino, J., Brito, N., Noda, J., and Gonzalez, C. (2005). *Botrytis cinerea* endo-ß-1, 4-glucanase Cel5A is expressed during infection but is not required for pathogenesis. *Physiol. Mol. Plant Pathol.* 66, 213–221. doi: 10.1016/j.pmpp.2005.06.005

Espino, J., Gutiérrez-Sánchez, G., Brito, N., Shah, P., Orlando, R., and González, C. (2010). The *Botrytis cinerea* early secretome. *Proteomics* 10, 3020–3034. doi: 10.1002/pmic.201000037

Faure, D. (2002). The family-3 glycoside hydrolases: from housekeeping functions to host-microbe interactions. *Appl. Environ. Microbiol.* 68, 1485–1490. doi: 10.1128/AEM.68.4.1485-1490.2002

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.2307/2408678

Fernández-Acero, F. J., Colby, T., Harzen, A., Carbú, M., Wieneke, U., Cantoral, J. M., et al. (2010). 2-DE proteomic approach to the *Botrytis cinerea* secretome induced with different carbon sources and plant-based elicitors. *Proteomics* 10, 2270–2280. doi: 10.1002/pmic.200900408

Ferrari, S., Galletti, R., Vairo, D., Cervone, F., and De Lorenzo, G. (2006). Antisense expression of the *Arabidopsis thaliana AtPGIP1* gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to *Botrytis cinerea*. *Mol. Plant Microbe Interact.* 19, 931–936. doi: 10.1094/MPMI-19-0931

Ferrari, S., Vairo, D., Ausubel, F. M., Cervone, F., and De Lorenzo, G. (2003). Tandemly duplicated Arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. *Plant Cell* 15, 93–106. doi: 10.1105/tpc.005165

Finiti, I., Leyva, M., López-Cruz, J., Calderan Rodrigues, B., Vicedo, B., Angulo, C., et al. (2013). Functional analysis of endo-1, 4-β-glucanases in response to *Botrytis cinerea* and *Pseudomonas syringae* reveals their involvement in plant–pathogen interactions. *Plant Biol.* 15, 819–831. doi: 10.1111/j.1438-8677.2012.00701.x

Flors, V., Leyva, M., Vicedo, B., Finiti, I., Real, M. D., Garcia-Agustin, P., et al. (2007). Absence of the endo-β-1, 4-glucanses Cel1 and Cel2 reduces susceptibility to *Botrytis cinerea* in tomato. *Plant J.* 52, 1027–1040. doi: 10.1111/j.1365-313X.2007.03299.x

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R. A., Henrissat, B., et al. (2012). The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science* 336, 1715–1719. doi: 10.1126/science.1221748

Gilbert, H. J. (2010). The biochemistry and structural biology of plant cell wall deconstruction. *Plant Physiol.* 153, 444–455. doi: 10.1104/pp.110.156646

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., et al. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* 36, 3420–3435. doi: 10.1093/nar/gkn176

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., et al. (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* 461, 393–398. doi: 10.1038/nature08358

Harris, P. J., and Stone, B. A. (2008). "Chemistry and molecular organization of plant cell walls," in *Biomass Recalcitrance: Deconstructing the Plant Cell Wall for Bioenergy*, ed M. E. Himmel (London, UK: Blackwell Publishing), 61–93.

Hematy, K., Cherk, C., and Somerville, S. (2009). Host–pathogen warfare at the plant cell wall. *Curr. Opin. Plant Biol.* 12, 406–413. doi: 10.1016/j.pbi.2009.06.007

Hemsworth, G. R., Davies, G. J., and Walton, P. H. (2013). Recent insights into copper-containing lytic polysaccharide mono-oxygenases. *Curr. Opin. Struc. Biol.* 23, 660–668. doi: 10.1016/j.sbi.2013.05.006

Ishii, T., Matsunaga, T., and Hayashi, N. (2001). Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness of pumpkin tissue. *Plant Physiol.* 126, 1698–1705. doi: 10.1104/pp.126.4.1698

Ithal, N., Recknor, J., Nettleton, D., Hearne, L., Maier, T., Baum, T. J., et al. (2007). Parallel genome-wide expression profiling of host and pathogen during soybean cyst nematode infection of soybean. *Mol. Plant Microbe Interact.* 20, 293–305. doi: 10.1094/MPMI-20-3-0293

Jayani, R. S., Saxena, S., and Gupta, R. (2005). Microbial pectinolytic enzymes: a review. *Process Biochem.* 40, 2931–2944. doi: 10.1016/j.procbio.2005.03.026

Jeon, J., Park, S. Y., Chi, M. H., Choi, J., Park, J., Rho, H. S., et al. (2007). Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. *Nat. Genet*. 39, 561–565. doi: 10.1038/ng2002

Joubert, D. A., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M. A., et al. (2007). A polygalacturonase-inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* leaves without any evidence for *in vitro* interaction. *Mol. Plant Microbe Interact.* 20, 392–402. doi: 10.1094/MPMI-20-4-0392

Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. A. E., and van Kan, J. A. L. (2005a). Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *Plant J.* 43, 213–225. doi: 10.1111/j.1365-313X.2005.02436.x

Kars, I., McCalman, M., Wagemakers, L., and van Kan, J. A. L. (2005b). Functional analysis of *Botrytis cinerea* pectin methlesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of strain B05.10. *Mol. Plant Pathol.* 6, 641–652. doi: 10.1111/j.1364-3703.2005.00312.x

Keegstra, K. (2010). Plant cell walls. *Plant Physiol.* 154, 483–486. doi: 10.1104/pp.110.161240

King, B. C., Waxman, K. D., Nenni, N. V., Walker, L. P., Bergstrom, G. C., and Gibson, D. M. (2011). Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. *Biotechnol. Biofuels* 4:4. doi: 10.1186/1754-6834-4-4

Klis, F. M., Brul, S., and De Groot, P. W. J. (2010). Covalently linked wall proteins in ascomycetous fungi. *Yeast* 27, 489–493. doi: 10.1002/yea.1747

Körner, R., Limberg, G., Mikkelsen, J. D., and Roepstorff, P. (1998). Characterization of enzymatic pectin digests by matrix−assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* 33, 836–842.

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Leroch, M., Kleber, A., Silva, E., Coenen, T., Koppenhofer, D., Shmaryahu, A., et al. (2013). Transcriptome profiling of *Botrytis cinerea* conidial germination reveals upregulation of infection-related genes during the prepenetration stage. *Eukaryot. Cell* 12, 614–626. doi: 10.1128/EC.00295-12

Levasseur, A., Drula, E., Lombard, V., Coutinho, P. M., and Henrissat, B. (2013). Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol. Biofuels* 6:41. doi: 10.1186/1754-6834-6-41

Li, B., Wang, W., Zong, Y., Qin, G., and Tian, S. (2012). Exploring pathogenic mechanisms of *Botrytis cinerea* secretome under different ambient pH based

on comparative proteomic analysis. *J. Proteome Res.* 11, 4249–4260. doi: 10.1021/pr300365f

Lionetti, V., Cervone, F., and Bellincampi, D. (2012). Methyl esterification of pectin plays a role during plant–pathogen interactions and affects plant resistance to diseases. *J. Plant Physiol.* 169, 1623–1630. doi: 10.1016/j.jplph.2012.05.006

Lionetti, V., Raiola, A., Cervone, F., and Bellincampi, D. (2014). Transgenic expression of pectin methylesterase inhibitors limits tobamovirus spread in tobacco and Arabidopsis. *Mol. Plant Pathol.* 15, 265–274. doi: 10.1111/mpp.12090

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., et al. (2007). Overexpression of pectin methylesterase inhibitors in Arabidopsis restricts fungal infection by *Botrytis cinerea*. *Plant Physiol.* 143, 1871–1880. doi: 10.1104/pp.106.090803

Lunn, D., Phan, T. D., Tucker, G. A., and Lycett, G. W. (2013). Cell wall composition of tomato fruit changes during development and inhibition of vesicle trafficking is associated with reduced pectin levels and reduced softening. *Plant Physiol. Biochem.* 66, 91–97. doi: 10.1016/j.plaphy.2013.02.005

Melhem, H., Min, X. J., and Butler, G. (2013). "The impact of SignalP 4.0 on the prediction of secreted proteins," in *Computational Intelligence in Bioinformatics and Computational Biology (CIBCB), 2013 IEEE Symposium* (Singapore), 16–22.

Melotto, E., Greve, L. C., and Labavitch, J. M. (1994). Cell wall metabolism in ripening fruit. VII. Biologically active pectin oligomers in ripening tomato (Lycopersicon esculentum Mill.) Fruits. *Plant Physiol.* 106, 575–581.

Meyer, U. M., and Dewey, F. M. (2000). Efficacy of different immunogens for raising monoclonal antibodies *to Botrytis cinerea*. *Mycol. Res.* 104, 979–987. doi: 10.1017/S0953756200002501

Mohnen, D. (2008). Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.* 11, 266–277. doi: 10.1016/j.pbi.2008.03.006

Mølgaard, A., Kauppinen, S., and Larsen, S. (2000). Rhamnogalacturonan acetylesterase elucidates the structure and function of a new family of hydrolases. *Structure* 8, 373–383. doi: 10.1016/S0969-2126(00)00118-0

Mutter, M., Renard, C. M., Beldman, G., Schols, H. A., and Voragen, A. G. (1998). Mode of action of RG-hydrolase and RG-lyase toward rhamnogalacturonan oligomers. Characterization of degradation products using RG-rhamnohydrolase and RG-galacturonohydrolase. *Carbohyd. Res.* 311, 155–164. doi: 10.1016/S0008-6215(98)00188-8

Nafisi, M., Stranne, M., Zhang, L., van Kan, J., and Sakuragi, Y. (2014). The endo-arabinanase BcAra1 is a novel host-specific virulence factor of the necrotic fungal phytopathogen *Botrytis cinerea*. *Mol. Plant Microbe Interact.* 27, 781–792. doi: 10.1094/MPMI-02-14-0036-R

Noda, J., Brito, N., and Gonzalez, C. (2010). The *Botrytis cinerea* xylanase Xyn11A contributes to virulence with its necrotizing activity, not with its catalytic activity. *BMC Plant Biol.* 10:38. doi: 10.1186/1471-2229-10-38

Noël, L., Thieme, F., Nennstiel, D., and Bonas, U. (2001). cDNA−AFLP analysis unravels a genome−wide hrpG−regulon in the plant pathogen *Xanthomonas campestris* pv. vesicatoria. *Mol. Microbiol.* 41, 1271–1281. doi: 10.1046/j.1365-2958.2001.02567.x

Nunan, K. J., Sims, I. M., Bacic, A., Robinson, S. P., and Fincher, G. B. (1998). Changes in cell wall composition during ripening of grape berries. *Plant Physiol.* 118, 783–792. doi: 10.1104/pp.118.3.783

O'Connell, R. J., Thon, M. R., Hacquard, S., Amyotte, S. G., Kleemann, J., Torres, M. F., et al. (2012). Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. *Nat. Genet.* 44, 1060–1065. doi: 10.1038/ng.2372

Park, B. H., Karpinets, T. V., Syed, M. H., Leuze, M. R., and Uberbacher, E. C. (2010). CAZymes Analysis Toolkit (CAT): web service for searching and analyzing carbohydrate-active enzymes in a newly sequenced organism using CAZy database. *Glycobiology* 20, 1574–1584. doi: 10.1093/glycob/cwq106

Pauly, M., Gille, S., Liu, L., Mansoori, N., Souza, A., Schultink, A., et al. (2013). Hemicellulose biosynthesis. *Planta* 238, 627–642. doi: 10.1007/s00425-013-1921-1

Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. doi: 10.1038/nmeth.1701

Pio, T. F., and Macedo, G. A. (2009). Cutinases: properties and industrial applications. *Adv. Appl. Microbiol.* 66, 77–95. doi: 10.1016/S0065-2164(08)00804-6

Powell, A. L. T., van Kan, J. A. L., ten Have, A., Visser, J., Greve, L. C., Bennett, A. B., et al. (2000). Transgenic expression of pear *PGIP* in tomato limits fungal colonization. *Mol. Plant Microbe Interact.* 13, 942–950. doi: 10.1094/MPMI.2000.13.9.942

Raiola, A., Lionetti, V., Elmaghraby, I., Immerzeel, P., Mellerowicz, E. J., Salvi, G., et al. (2011). Pectin methylesterase is induced in Arabidopsis upon infection and is necessary for a successful colonization by necrotrophic pathogens. *Mol. Plant Microbe Interact.* 24, 432–440. doi: 10.1094/MPMI-07-10-0157

Reignault, P., Kunz, C., Delage, N., Moreau, E., Vedel, R., Hamada, W., et al. (2000). Host-and symptom-specific pectinase isozymes produced by *Botrytis cinerea*. *Mycol. Res.* 104, 421–428. doi: 10.1017/S0953756299001501

Reis, H., Pfiffi, S., and Hahn, M. (2005). Molecular and functional characterization of a secreted lipase from *Botrytis cinerea*. *Mol. Plant Pathol.* 6, 257–267. doi: 10.1111/j.1364-3703.2005.00280.x

Rowe, H. C., and Kliebenstein, D. J. (2007). Elevated genetic variation within virulence-associated Botrytis cinerea polygalacturonase loci. *Mol. Plant Microbe Interact.* 20, 1126–1137. doi: 10.1094/MPMI-20-9-1126

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Scheller, H. V., and Ulvskov, P. (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289. doi: 10.1146/annurev-arplant-042809-112315

Schmidtke, C., Findeiss, S., Sharma, C. M., Kuhfuss, J., Hoffmann, S., Vogel, J., et al. (2012). Genome-wide transcriptome analysis of the plant pathogen *Xanthomonas* identifies sRNAs with putative virulence functions. *Nucleic Acids Res.* 40, 2020–2031. doi: 10.1093/nar/gkr904

Schols, H. A., Voragen, A. G., and Colquhoun, I. J. (1994). Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. *Carbohyd. Res.* 256, 97–111. doi: 10.1016/0008-6215(94)84230-2

Shah, P., Atwood, J. A., Orlando, R., El Mubarek, H., Podila, G. K., and Davis, M. R. (2009b). Comparative proteomic analysis of *Botrytis cinerea* secretome. *J. Proteome Res.* 8, 1123–1130. doi: 10.1021/pr8003002

Shah, P., Gutierrez-Sanchez, G., Orlando, R., and Bergmann, C. (2009a). A proteomic study of pectin-degrading enzymes secreted by *Botrytis cinerea* grown in liquid culture. *Proteomics* 9, 3126–3135. doi: 10.1002/pmic.200800933

Shah, P., Powell, A. L., Orlando, R., Bergmann, C., and Gutierrez-Sanchez, G. (2012). Proteomic analysis of ripening tomato fruit infected by *Botrytis cinerea*. *J. Proteome Res.* 11, 2178–2192. doi: 10.1021/pr200965c

Sharrock, K. R., and Labavitch, J. M. (1994). Polygalacturonase inhibitors of Bartlett pear fruits: differential effects on *Botrytis cinerea* polygalacturonase isozymes, and influence on products of fungal hydrolysis of pear cell walls and on ethylene induction in cell culture. *Physiol. Mol. Plant Pathol.* 45, 305–319. doi: 10.1016/S0885-5765(05)80061-X

Skamnioti, P., Furlong, R. F., and Gurr, S. J. (2008). The fate of gene duplicates in the genomes of fungal pathogens. *Commun. Integr. Biol.* 1, 196–198. doi: 10.4161/cib.1.2.7144

Suzuki, H., MacDonald, J., Syed, K., Salamov, A., Hori, C., Aerts, A., et al. (2012). Comparative genomics of the white-rot fungi, *Phanerochaete carnosa* and *P. chrysosporium*, to elucidate the genetic basis of the distinct wood types they colonize. *BMC Genomics* 13:444. doi: 10.1186/1471-2164-13-444

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Ten Have, A., Breuil, W. O., Wubben, J. P., Visser, J., and van Kan, J. A. L. (2001). *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. *Fungal Genet. Biol.* 33, 97–105. doi: 10.1006/fgbi.2001.1269

Ten Have, A., Mulder, W., Visser, J., and van Kan, J. A. L. (1998). The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. *Mol. Plant Microbe Interact.* 11, 1009–1016.

Tiedemann, A. V. (1997). Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. *Physiol. Mol. Plant P* 50, 151–166. doi: 10.1006/pmpp.1996.0076

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Front. Plant Sci.* 3:85. doi: 10.3389/fpls.2012.00085

Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C., and Boccara, M. (2003). Disruption of *Botrytis cinerea* pectin methylesterase gene *Bcpme1* reduces virulence on several host plants. *Mol. Plant Microbe Interact.* 16, 360–367. doi: 10.1094/MPMI.2003.16.4.360

Van Baarlen, P., Legendre, L., and Kan, J. A.,L (2007). "Plant defence compounds against botrytis infection," in *Botrytis: Biology, Pathology and Control*, eds Y. Elad, B. Williamson, P. Tudzynski, and N. Delen (Netherlands: Springer), 143–161.

Van Baarlen, P., Staats, M., and Van Kan, J. A. (2004). Induction of programmed cell death in lily by the fungal pathogen *Botrytis elliptica*. *Mol. Plant Pathol.* 5, 559–574. doi: 10.1111/j.1364-3703.2004.00253.x

Van Kan, J. A. L. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci.* 11, 247–253. doi: 10.1016/j.tplants.2006.03.005

Van Kan, J. A. L., van't Klooster, J. W., Wagemakers, C. A. M., Dees, D. C. T., and van der Vlugt-Bergmans, C. J. B. (1997). Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato. *Mol. Plant Microbe Interact.* 10, 30–38.

Vincken, J. P., Schols, H. A., Oomen, R. J., McCann, M. C., Ulvskov, P., Voragen, A. G., et al. (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiol.* 132, 1781–1789. doi: 10.1104/pp.103.022350

Volpi, C., Janni, M., Lionetti, V., Bellincampi, D., Favaron, F., and D'Ovidio, R. (2011). The ectopic expression of a pectin methyl esterase inhibitor increases pectin methyl esterification and limits fungal diseases in wheat. *Mol. Plant Microbe Interact.* 24, 1012–1019. doi: 10.1094/MPMI-01-11-0021

Voragen, A. J., Coenen, G. J., Verhoef, R., and Schols, H. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Struct. Chem.* 20, 263–275. doi: 10.1007/s11224-009-9442-z

Wagstaff, C., Clarkson, G. J., Zhang, F., Rothwell, S. D., Fry, S. C., Taylor, G., et al. (2010). Modification of cell wall properties in lettuce improves shelf life. *J. Exp. Bot.* 61, 1239–1248. doi: 10.1093/jxb/erq038

Weiberg, A., Wang, M., Lin, F.-M., Zhao, H., Zhang, Z., Kaloshian, I., et al. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* 342, 118–123. doi: 10.1126/science.1239705

Wiemann, P., Sieber, C. M., von Bargen, K. W., Studt, L., Niehaus, E. M., Espino, J. J., et al. (2013). Deciphering the cryptic genome: genome-wide analyses of the rice pathogen *Fusarium fujikuroi* reveal complex regulation of secondary metabolism and novel metabolites. *PLoS Pathog.* 9:e1003475. doi: 10.1371/journal.ppat.1003475

Wubben, J. P., ten Have, A., van Kan, J. M. L., and Visser, J. (2000). Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacutonic acid, ambient pH and carbon catabolite, repression. *Curr. Gen.* 37, 152–157. doi: 10.1007/s002940050022

Zhang, L., Hua, C., Stassen, J. H., Chatterjee, S., Cornelissen, M., and van Kan, J. A. L. (2013). Genome-wide analysis of pectate-induced gene expression in *Botrytis cinerea*: identification and functional analysis of putative d-galacturonate transporters. *Fungal Genet. Biol.* doi: 10.1016/j.fgb.2013.10.002. [Epub ahead of print].

Zhang, L., Kars, I., Essenstam, B., Liebrand, T. W., Wagemakers, L., Elberse, J., et al. (2014). Fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the arabidopsis receptor-like protein RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1. *Plant Physiol.* 164, 352–364. doi: 10.1104/pp.113.230698

Zhang, L., and van Kan, J. A. L. (2013a). "14 Pectin as a barrier and nutrient source for fungal plant pathogens," in *Agricultural Applications: The Mycota,* ed F. Kempken (Berlin, Germany: Springer Berlin Heidelberg), 361–375.

Zhang, L., and van Kan, J. A. L. (2013b). *Botrytis cinerea* mutants deficient in d-galacturonic acid catabolism have a perturbed virulence on *Nicotiana benthamiana* and Arabidopsis, but not on tomato. *Mol. Plant Pathol.* 14, 19–29. doi: 10.1111/j.1364-3703.2012.00825.x

Zhao, Z., Liu, H., Wang, C., and Xu, J. R. (2014). Correction: comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics* 15:6. doi: 10.1186/1471-2164-15-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 May 2014; accepted: 15 August 2014; published online: 03 September 2014.

Citation: Blanco-Ulate B, Morales-Cruz A, Amrine KCH, Labavitch JM, Powell ALT and Cantu D (2014) Genome-wide transcriptional profiling of Botrytis cinerea genes targeting plant cell walls during infections of different hosts. Front. Plant Sci. ***5****:435. doi: 10.3389/fpls.2014.00435*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 28 May 2014
doi: 10.3389/fpls.2014.00228

Plant cell wall dynamics and wall-related susceptibility in plant–pathogen interactions

Daniela Bellincampi, Felice Cervone and Vincenzo Lionetti*

Dipartimento di Biologia e Biotecnologie "Charles Darwin," Sapienza Università di Roma, Rome, Italy

Edited by:
Jean-Pierre Metraux, Université de Fribourg, Switzerland

Reviewed by:
Jurriaan Ton, University of Sheffield, UK
Simone Ferrari, Sapienza Università di Roma, Italy

***Correspondence:**
Vincenzo Lionetti, Dipartimento di Biologia e Biotecnologie "Charles Darwin," Sapienza Università di Roma, Rome 00185, Italy
e-mail: vincenzo.lionetti@uniroma1.it

The cell wall is a dynamic structure that often determines the outcome of the interactions between plants and pathogens. It is a barrier that pathogens need to breach to colonize the plant tissue. While fungal necrotrophs extensively destroy the integrity of the cell wall through the combined action of degrading enzymes, biotrophic fungi require a more localized and controlled degradation of the cell wall in order to keep the host cells alive and utilize their feeding structures. Also bacteria and nematodes need to degrade the plant cell wall at a certain stage of their infection process, to obtain nutrients for their growth. Plants have developed a system for sensing pathogens and monitoring the cell wall integrity, upon which they activate defense responses that lead to a dynamic cell wall remodeling required to prevent the disease. Pathogens, on the other hand, may exploit the host cell wall metabolism to support the infection. We review here the strategies utilized by both plants and pathogens to prevail in the cell wall battleground.

Keywords: cell wall, cell wall integrity, host cell wall metabolism reprogramming, plant defense, susceptibility factors

INTRODUCTION

Phytopathogenic fungi, bacteria, and nematodes infect, grow and reproduce themselves on the plant tissues and, at least at the early stages of infection, require breaking the integrity of the host cell wall. Beyond the cuticle layer, the interaction with the plant cell wall and the extent of the wall degradation are determined by the lifestyle of the pathogen. Plants perceive a diverse set of microbial molecules referred to as microbial/pathogen associated molecular patterns (MAMPs/PAMPs; Boller and He, 2009) through high-affinity cell surface pattern recognition receptors (PRRs) leading to intracellular signaling, transcriptional reprogramming, and biosynthesis of defense metabolites that limit the microbial infection (Dangl et al., 2013). Emerging evidences indicate that plant cells also exploit sophisticated mechanisms of sensing the alteration of cell wall integrity (CWI) during biotic stress (Hamann, 2012; Pogorelko et al., 2013a). For instance, they perceive endogenous molecules produced in damaged tissues (the so-called damage-associated molecular patterns, or DAMPs) through membrane receptors (Ferrari et al., 2013). The loss of CWI induced by pathogens activates a variety of defense responses including a cell wall remodeling required to prevent the disease. To escape recognition, pathogens produce effector proteins that counteract the plant defenses (Giraldo and Valent, 2013) and, sometimes, exploit the host cell wall metabolism to favor the infection process (Cantu et al., 2008b).

CELL WALL DYNAMICS DURING INFECTION BY MICROBIAL PATHOGENS

Infection by fungal necrotrophs is a complex process that includes conidial attachment, germination, host penetration, lesion formation and expansion, and tissue maceration followed by sporulation (Prins et al., 2000). Penetration may be achieved by degrading the external cuticle through the action of cutinases and lipases (Laluk and Mengiste, 2010). The role of the cuticle in plant defense is discussed elsewhere (Chassot and Metraux, 2005; Chassot et al., 2007). Once penetrated the cuticle, necrotrophs have a spatial and temporal strategy of attacking the plant cell wall by producing several cell wall degrading enzymes (CWDEs) belonging to multiple families (**Figure 1A**). The diversity of these enzymes mirrors the structural complexity and the dynamics of the cell wall as well as the lifestyle and host adaptation of the pathogen (King et al., 2011). The extensive degradation of cell wall polysaccharides by necrotrophs is sensed by plants. The leucine-rich repeat receptor-like kinase (LRR-RLK) ERECTA (ER) and the heterotrimeric G-protein are involved in cell wall remodeling during *Arabidopsis* defense response against *Plectosphaerella cucumerina* and probably control CWI (Llorente et al., 2005; Sanchez-Rodriguez et al., 2009). The impairment of cellulose synthases involved in secondary cell wall deposition is also a mechanism of sensing CWI and enhances disease resistance of *Arabidopsis* to *P. cucumerina* and *Ralstonia solanacearum* (Hernandez-Blanco et al., 2007).

One of the strategies used by plants to limit the degradation of the cell wall polysaccharides by microbial CWDEs is the production of proteinaceous inhibitors (**Figures 1A,B**). Polygalacturonases (PGs) are pathogenicity factors produced at the earlier stages of a microbial infection that depolymerize the homogalacturonan (HG), i.e., the main component of pectin in dicots but also present in monocots (Caprari et al., 1993; D'Ovidio et al., 2004). Against microbial and insect PGs, plants produce cell wall-associated polygalacturonase-inhibiting proteins (PGIPs; Spadoni et al., 2006). The over expression of PGIPs improves the resistance to fungal and bacterial necrotrophs in different plants (Aguero et al., 2005; Ferrari et al., 2012). The PG-PGIP interaction results in the accumulation of elicitor-active oligogalacturonides

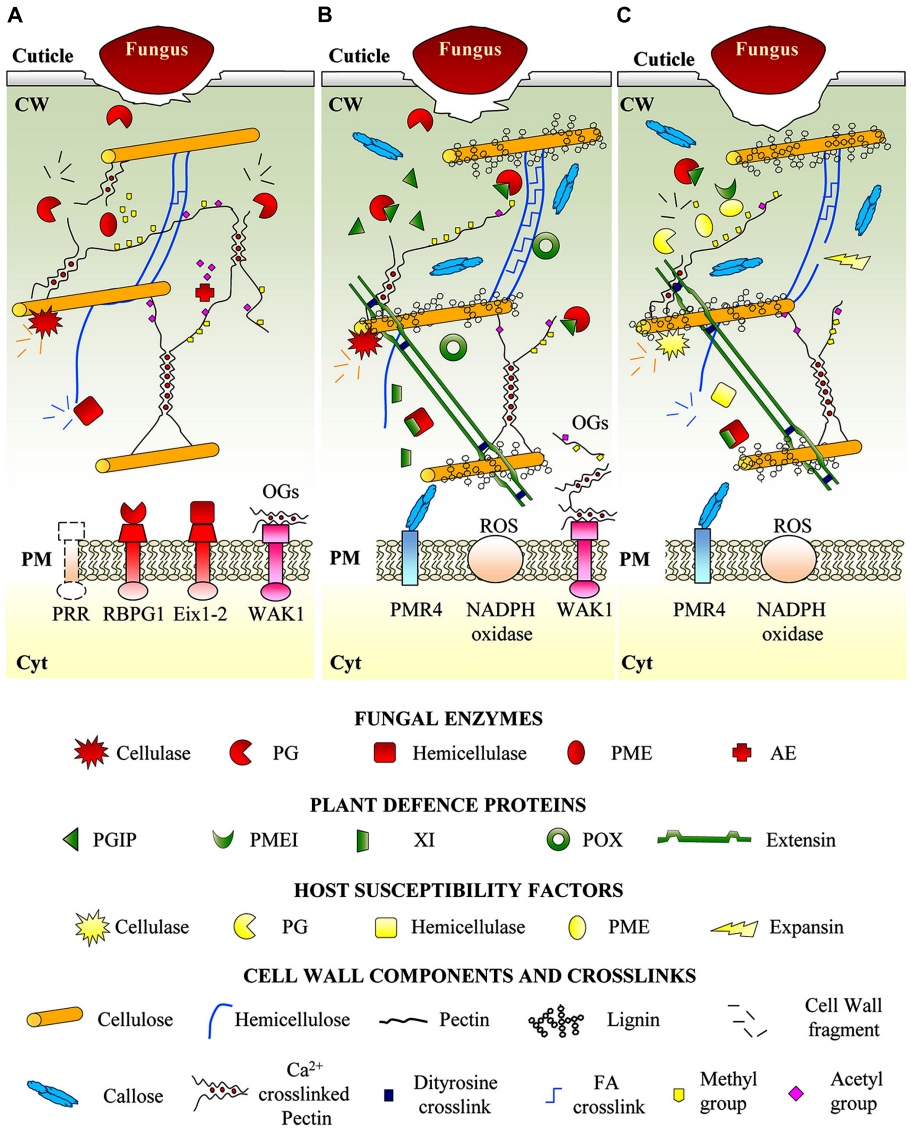


FIGURE 1 | Cell wall dynamics during necrotrophs invasion. **(A)** Necrotrophic fungi secrete a large arsenal of cell wall degrading enzymes (CWDEs) like PGs, hemicellulases and cellulases, assisted by PMEs and AEs in the apoplastic space to degrade cell wall polymers and facilitate the availability of nutrients. PGs and EIXs have been proposed to function as PAMPs recognized by the membrane receptors RBPG1 and Eix1 or 2, respectively. **(B)** As first line of defense, plants produce a variety of CWDE inhibitors to hinder degradation by microbial CWDEs. For instance, the inhibition of PG degrading activity by PGIPs induces the accumulation of elicitor-active pectin fragments (OGs) perceived by WAK1 receptors. It cannot be excluded the presence of other not yet identified receptors sensing damage of other cell wall components. The perception of cell wall damage triggers specific signaling pathways activating defense responces aimed to reinforce cell wall structure. The more evident defense strategies are callose and lignin deposition, induction of peroxidases/ROS-mediated crosslinks between cell wall structural proteins and polysaccharides. **(C)** Necrotrophs force plants to cooperate in disease exploiting plant cellulases, expansins, PGs and PMEs as susceptibility factors. PM, plasma membrane; CW, cell wall; Cyt, cytoplasm; OGs, oligogalacturonides; WAK1, wall associated kinase 1; AEs, acetyl esterases; PGs, polygalacturonases; EIXs, ethylen induced xylanases; PME, pectin methylesterases; PMEI, pectin methylesterase inhibitor; FA, ferulic acid; Eix1-2, receptors of ethylene induced xylanases; RBPG1, Responsiveness to Botrytis PolyGalacturonase 1; Ca^{2+}, calcium ions; XI, xylanase inhibitor; PRR, pattern recognition receptor; POX, peroxidase; ROS, reactive oxygen species.

(OGs) that are perceived in *Arabidopsis* by the receptor Wall Associated Kinase 1 (WAK1; Brutus et al., 2010) to activate the plant immune responses (Ferrari et al., 2013). Accumulation and sensing of OGs in response to pathogens is critical for monitoring the pectin integrity and, in general, a tissue injury (De Lorenzo et al., 2011). Alteration of pectin integrity caused by the expression of PGII from *Aspergillus niger* in tobacco and *Arabidopsis* causes a constitutive activation of defense genes and resistance against *Botrytis cinerea* (Ferrari et al., 2008). Recently, *B. cinerea* and *A. niger* PGs have been proposed to function themselves as PAMPs recognized by the *Arabidopsis* Receptor-Like Responsiveness to Botrytis PolyGalacturonase 1 (RBPG1) belonging to

a super clade of LRR receptor-like proteins (RLPs; Zhang et al., 2014).

Xylan is the major hemicellulose polymer in cereals. To counteract xylan degradation by microbial endoxylanases, graminaceous monocots produce the *Triticum aestivum* xylanase inhibitor (TAXI), the xylanase inhibitor protein (XIP) and the thaumatin-like xylanase inhibitor (TL-XI; Bellincampi et al., 2004; Juge, 2006). The constitutive expression of TAXI-III in wheat reduces susceptibility to *Fusarium graminearum* (Moscetti et al., 2013). On the other hand, fungal xylanases function as PAMPs by eliciting defense responses and promoting necrosis (Noda et al., 2010; Sella et al., 2013). Ethylene inducing xylanases (EIXs) produced by *Trichoderma* species are perceived in tomato, by two specific LRR-RLPs receptors, LeEix1 and LeEix2 (Ron and Avni, 2004). Both receptors bind Eixs, while only LeEix2 mediates defense responses. LeEix1 heterodimerizes with LeEix2 upon application of the Eixs and attenuates Eix-induced internalization and signaling of the LeEix2 receptor (Bar et al., 2010). Xyloglucan, i.e., the main hemicellulosic polysaccharide in the primary walls of dicots and non-graminaceous monocots, is degraded by microbial xyloglucan-specific endoglucanases (XEGs). Fungal XEGs are inhibited by xyloglucan endoglucanase inhibiting proteins (XEGIPs), which so far have been characterized in tomato, carrot and tobacco (Juge, 2006).

Reinforcement of the cell wall is initiated at the pathogen penetration sites in response to cell wall damage (**Figure 1B**). Deposition of callose by the callose synthase PMR4 occurs upon infection of *Arabidopsis* with *P. cucumerina* and *Alternaria brassicicola* (Ton and Mauch-Mani, 2004; Flors et al., 2008). Callose deposition is triggered by PAMPs and DAMPs, is affected by environmental conditions and requires the apoplastic accumulation of the hydrolysis products of glucosinolates or benzoxazinoid metabolites (Galletti et al., 2008; Ahmad et al., 2011; Luna et al., 2011).

Deposition of lignin has been associated to resistance of cotton to *Verticillium dahliae* and of *Camelina sativa* to *Sclerotinia sclerotiorum* (Xu et al., 2011; Eynck et al., 2012). Lignin makes the cell wall more resistant to CWDEs and also prevents the diffusion of pathogen-produced toxins (Sattler and Funnell-Harris, 2013). The cell wall may also be reinforced by cross-links and insolubilization of structural proteins like the hydroxyproline-rich glycoproteins (HRGPs) by peroxidase-mediated isodityrosine linkages formed in response to pathogen attach (Deepak et al., 2010). Plant peroxidases catalyze cross-links between phenolic compounds in the secondary walls and between polysaccharides and ferulic acid (FA) upon attack by necrotrophs (Passardi et al., 2004). Crosslinks between FA and polysaccharides enhance the recalcitrance of the cell wall to digestion by microbial CWDEs and the overall resistance to fungi (Bily et al., 2003). On the other hand, fungal FA esterases may shear FA from the cell wall polysaccharides (Udatha et al., 2012).

The activities of pectin methyl esterases (PMEs) from both plants and pathogens and the degree and pattern of pectin methyl esterification are critical for the outcome of plant–pathogen infections (Lionetti et al., 2012). The cell walls containing highly methylesterified pectin are somewhat protected against the action of microbial PGs and pectate lyases (PLs; Arancibia and Motsenbocker, 2006). PMEs, which remove methyl esters from pectin, are controlled by PME inhibitor proteins (PMEIs) either during growth and development (Raiola et al., 2004; Rocchi et al., 2011; Reca et al., 2012) and during plant–pathogen interactions (Lionetti et al., 2012). The biochemical and structural bases of the enzyme/inhibitor interaction have been elucidated (Mattei et al., 2002; Di Matteo et al., 2005). *Arabidopsis* over expressing PMEIs have a lower level of PME activity, a higher degree of pectin esterification and a concomitant reduced susceptibility to *B. cinerea* and *Pectobacterium carotovorum* (Lionetti et al., 2007; Raiola et al., 2011). The ectopic expression in wheat of a PME inhibitor from kiwi reduces the susceptibility to *F. graminearum* and *Bipolaris sorokiniana* (Volpi et al., 2011). The transcription factor MYB46 which affect the secondary cell wall biosynthesis (Zhong et al., 2007), regulates the expression of genes encoding several cell wall proteins including PMEI and mediates disease susceptibility of *Arabidopsis* to *B. cinerea* (Ramirez et al., 2011). Recently, jasmonic acid has been proposed to modulate the degree of methylesterification in potato to protect pectin degradation by PLs produced by *Dickeya dadantii* (Taurino et al., 2014). Acetylation of the cell wall polysaccharides is also a determinant of plant–pathogen interaction. An *Arabidopsis* mutant with reduced acetylation displays increased tolerance to *B. cinerea* (Manabe et al., 2011). *Arabidopsis* and *Brachypodium distachyon* plants expressing xylan or pectin acetylesterases from *A. nidulans* activate specific defense responses and are more resistant to *B. cinerea* and *B. sorokiniana* (Pogorelko et al., 2013b).

Biotrophic and hemi-biotrophic fungi acquire nutrients from the host cells without causing their death. They often attack the plant surface and penetrate the external barriers by developing appressoria and exploiting the mechanical pressure (Wilson and Talbot, 2009). In order to breach the host cuticle they also secrete oxidases, esterases, cutinases, and lipases (Feng et al., 2011). Small amounts of CWDEs associated with local softening and loosening of plant cell walls are produced by biotrophic microorganisms (Zhao et al., 2013) (**Figure 2A**). Plants contrast invasion of biotrophs by the apposition of "papillae," cell wall thickening early produced at the site of pathogen penetration (**Figure 2B**). Papillae contain callose, as most abundant constituent, cellulose, hemicelluloses, pectins, lignin, and structural proteins such as arabinogalactan proteins and HRGPs (Aist, 1976; Celio et al., 2004; Voigt, 2014). Transgenic *Arabidopsis* plants overexpressing the callose synthase PMR4 show an early and elevated deposition of callose at the sites of penetration which prevents the haustoria formation and further penetration by *Golovinomyces cichoracearum* and *Blumeria graminis* (Ellinger et al., 2013). Papillae are also the sites where antimicrobial peptides, toxic secondary metabolites and reactive oxygen species (ROS) accumulate and contribute to plant resistance (Bednarek et al., 2009; Daudi et al., 2012). Lignification and cross-links of proteins in the papillar cell wall may entrap the penetration peg of biotrophic fungi and render the cell wall more resistant to the mechanical pressure exerted by fungal appressoria (Bechinger et al., 1999; O'Brien et al., 2012). Lignin downregulation may also activate defense responses and increases the resistance to the hemibiotroph *Colletotrichum trifolii* in alfalfa (*Medicago sativa* L.) (Gallego-Giraldo et al., 2011).

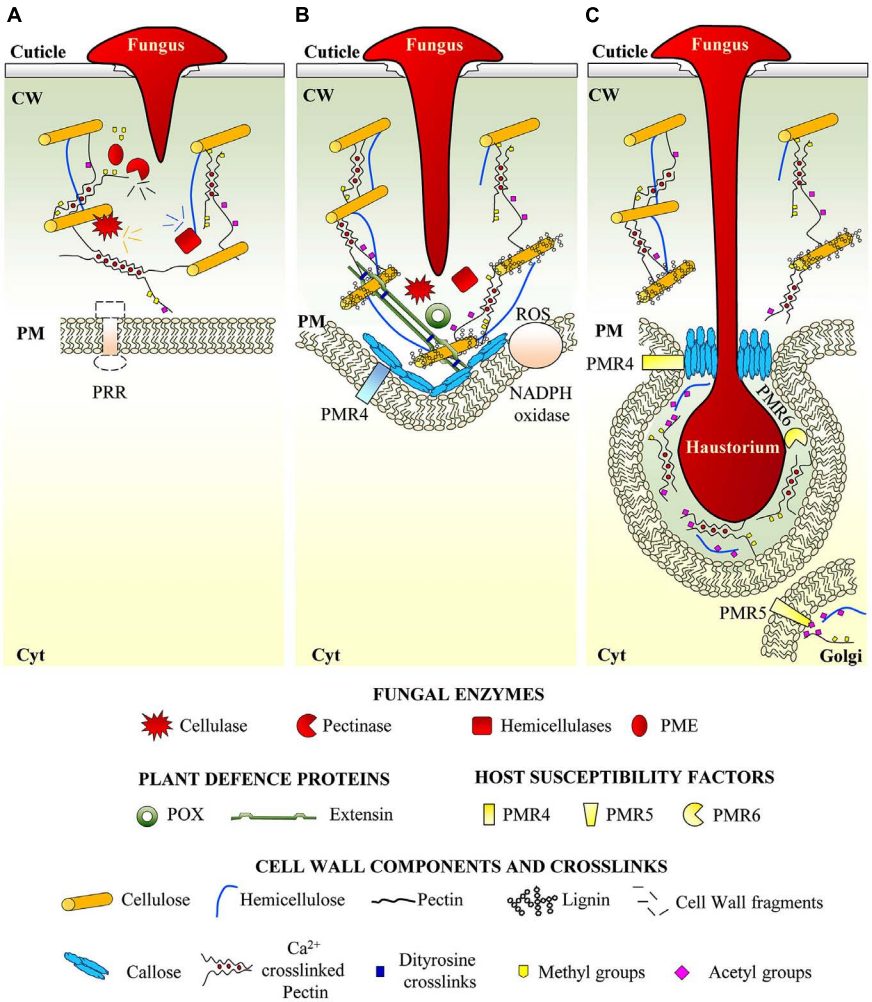


FIGURE 2 | Cell wall dynamics in plant-biotrophic fungi interaction. **(A)** Biotrophic fungi use appressorial mechanical pressure and secrete cell wall degrading enzymes to penetrate plant cell wall. **(B)** Plants perceive fungal biotrophs penetration with not yet identified receptors and respond with "papillae" apposition between cell wall and plasma membrane. Papillae, in addition to new cell wall material are also sites of accumulation of ROS possibly involved in cell wall reinforcement. **(C)** At a later stage of infection, fungus forms the haustorium feeding organ invaginated into the host membranes and plant cell wall. Biotrophs locally affect cell wall metabolism by induction of susceptibility factors (callose synthase PMR4, *O*-acetyltransferase PMR5 and pectate lyase PMR6) to modify the extrahaustorial matrix to improve the accessibility of nutrients or to ensure the mechanical stability of the haustorium. PM, plasma membrane; CW, cell wall; Cyt, cytoplasm; PG, polygalacturonase; PME, pectin methylesterase; PRR, pattern recognition receptor; POX, peroxidase; ROS, reactive oxygen species.

A reduced cellulose content by mutation of cellulose synthase *CESA3,* involved in primary cell wall formation, leads to production of lignin, and makes *Arabidopsis* more resistant to different powdery mildew pathogens (Ellis and Turner, 2001; Ellis et al., 2002; Cano-Delgado et al., 2003). RLKs belonging to the *Catharanthus roseus* (CrRLK)-like protein family are implicated in CWI mechanisms. Among these, THESEUS1 (THE1) is required for lignification in response to inhibition of cellulose biosynthesis (Hematy et al., 2007).

Alteration of pectin integrity can trigger plant immunity also against hemibiotrophs (Ferrari et al., 2008; Bethke et al., 2013). *Arabidopsis* PMEs, triggered by a Jasmonic acid dependent pathway, contribute to plant immunity against *Pseudomonas syringae*(Bethke et al., 2013).

MICROBIAL PATHOGENS EXPLOIT THE HOST CELL WALL METABOLISM TO FACILITATE PATHOGENESIS

Necrotrophs can force plants to cooperate in disease by altering the host cell wall and favoring the cell wall accessibility to CWDEs (Hok et al., 2010; **Figure 1C**). The *Arabidopsis* AtPME3 is induced upon infection with *B. cinerea* and *P. carotovorum* and functions as susceptibility factor required for the initial colonization of the host tissue (Raiola et al., 2011). A PG (LePG) and expansin (LeExp1) cooperatively contribute to cell wall loosening during tomato ripening; their expression is induced by necrotrophic pathogens to successfully infect fruits (Cantu et al., 2009). Transgenic tomato fruits with suppressed expression of LePG and LeExp1 exhibit a reduced susceptibility to *B. cinerea* (Cantu et al., 2008a). Silencing of two putative endo β-1,4-endoglucanases, involved in the

hydrolysis of cellulose or hemicellulose during ripening, cause a reduced susceptibility of tomato fruits to *B. cinerea* (Flors et al., 2007).

Biotrophic fungi, at a later stage of infection, produce a limited and localized degradation of the cell wall in the epidermal or mesophyll cells (Herbert et al., 2004). On the other hand, they form intracellular structures, like the "haustoria," i.e., feeding organs invaginated into the host membranes to acquire nutrients. Biotrophs need to avoid the host defense responses and carefully regulate the cell-wall degradation at the border of their feeding structures to allow fungal accommodation and haustorium function (**Figure 2C**). A screening for *Arabidopsis* powdery mildew-resistant mutants allowed isolating two pectin-related genes, *PMR5* and *PMR6* which are pathogen-induced and required for susceptibility to *G. cichoracearum* and *G. orontii* (Vogel et al., 2002, 2004). *PMR5* encodes a protein with unknown function that shares sequence similarity with genes encoding polysaccharide *O*-acetyltransferase (Gille and Pauly, 2012). Therefore, acetylation may be a host susceptibility mechanism that is reprogrammed by biotrophs during infection. PMR6 encodes a putative PL that is, possibly, recruited by the fungi as a susceptible factor to reduce Ca^{++}-pectate domains at the level of haustoria-plasma membrane and facilitate cell wall porosity and accessibility of host nutrients (Vogel et al., 2002). Callose deposition may also work in favor of the pathogen by contributing to the stability and function of the haustoria and acting as a barrier that renders haustoria less susceptible to toxic metabolites that are produced by the host and accumulate in the site of infection (Jacobs et al., 2003). On the other hand, callose may limit the diffusion of pathogen-derived elicitors, thus reducing the activation of defense responses (Underwood, 2012).

Many bacterial pathogens utilize a type III secretion system to inject effector proteins directly into the host cytoplasm and manipulate the host cellular activities to their own advantage (Buttner and He, 2009). The effector AvrPto of *P. syringae* suppresses a set of *Arabidopsis* genes that encode cell wall-related defense proteins such as HRGPs (Hauck et al., 2003).

CELL WALL DYNAMICS IN PLANT INTERACTIONS WITH NEMATODES AND VIRUSES

Changes in the cell wall metabolism occur during plant infection by nematodes (Barcala et al., 2010; Bohlmann and Sobczak, 2014). Like biotrophic pathogens, root-knot and cyst nematodes need to establish feeding structures inside the plant tissue to allow the uptake of nutrients (Davis et al., 2004; Williamson and Kumar, 2006). This process is assisted by the secretion of CWDEs such as pectinases and cellulases produced by the nematodes, (Vanholme et al., 2004; Davis et al., 2008) and by the local expression of host proteins like expansins and cellulases (Wieczorek et al., 2006, 2008). The sugar beet cyst nematode *Heterodera schactii* infects *Arabidopsis* roots and exploits the host-encoded AtPME3. Transgenic plants overexpressing AtPME3 exhibit an increased susceptibility to the nematode. It has been proposed that AtPME3 locally reduces the pectin esterification and improves the cell wall loosening of pre-syncytial cells during the early stages of syncytium formation (Hewezi et al., 2008).

Callose deposition at the level of plasmodesmata (PD) limits the cell-to-cell spreading of plant viruses. Due to the small diameter of the PD pore, some viruses utilize the viral movement proteins (MPs) to modify the PD size exclusion limit. Specific interactions of viral MPs with PME are often required (Chen et al., 2000). In addition to MP-PME interaction, the PME-dependent formation of methanol has also been reported to be important for viral cell-to-cell movement (Dorokhov et al., 2012; Komarova et al., 2014). The overexpression of PME inhibitor proteins in tobacco and *Arabidopsis* contrasts the cell-to-cell and systemic movement of tobamoviruses (Lionetti et al., 2013).

CONCLUSION

The cell wall is the battleground where plants and pathogens attempt to prevail by implementing contrasting wall-reinforcing and wall-weakening strategies. When pathogens start degrading the plant cell wall components, plants are capable of perceiving the loss of wall integrity and subsequently activate the defense signaling pathways. Pathogens try to escape the plant defenses and sometimes take advantage of the host cell wall metabolism to facilitate their entry into the tissue. These dynamic processes vary according to the lifestyle of the pathogen and the type of plant pathogen interaction. While necrotrophy involves a strong and diffused molecular warfare that may provoke extended lesions of the tissue, during biotrophy the battle involves a weaker cell wall degradation mainly localized at the point of penetration and at the level of the feeding apparatus. Perception of cell wall damage as well as the pathogen- and host-induced cell wall remodeling occurs in both cases. The damage of specific cell wall polysaccharides during infection may be perceived by receptors as THE1, ER and WAK1. Plants may also rely on the recognition of CWDEs by LRR-RLPs receptors, as RBPG1 and LeEIX1-2. Cell wall fragments may be released during infection and sensed as damage signals. Analysis of cell wall mutants has shed light on the relationship between cell wall remodeling and plant response to pathogens. The expression of endogenous and microbial CWDEs and their inhibitors is also a valuable approach for studying the dynamics of the cell wall during plant–pathogen interactions as well as a strategy to improve plant protection.

ACKNOWLEDGMENTS

We would like to apologize to those people whose relevant publications could not be cited because of space constraints. This research was supported by the European Research Council (ERC Advanced Grant, 233083).

REFERENCES

Aguero, C. B., Uratsu, S. L., Greve, C., Powell, A. L. T., Labavitch, J. M., Meredith, C. P., et al. (2005). Evaluation of tolerance to pierce's disease and botrytis in transgenic plants of *Vitis vinifera L. expressing the pear PGIP gene. Mol. Plant Pathol.* 6, 43–51. doi: 10.1111/j.1364-3703.2004.00262.x

Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y. H., Martin, J., Smart, L., et al. (2011). Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. *Plant Physiol.* 157, 317–327. doi: 10.1104/pp.111.180224

Aist, J. R. (1976). Papillae and related wound plugs of plant cells. *Annu. Rev. Phytopathol.* 14, 145–163. doi: 10.1146/annurev.py.14.090176.001045

Arancibia, R. A., and Motsenbocker, C. E. (2006). Pectin methylesterase activity in vivo differs from activity in vitro and enhances polygalacturonase-mediated

pectin degradation in tabasco pepper. *J. Plant Physiol.* 163, 488–496. doi: 10.1016/j.jplph.2005.06.022

Bar, M., Sharfman, M., Ron, M., and Avni, A. (2010). BAK1 is required for the attenuation of ethylene-inducing xylanase (Eix)-induced defense responses by the decoy receptor LeEix1. *Plant J.* 63, 791–800. doi: 10.1111/j.1365-313X.2010.04282.x

Barcala, M., Garcia, A., Cabrera, J., Casson, S., Lindsey, K., Favery, B., et al. (2010). Early transcriptomic events in microdissected *Arabidopsis* nematode-induced giant cells. *Plant J.* 61, 698–712. doi: 10.1111/j.1365-313X.2009.04098.x

Bechinger, C., Giebel, K. F., Schnell, M., Leiderer, P., Deising, H. B., and Bastmeyer, M. (1999). Optical measurements of invasive forces exerted by appressoria of a plant pathogenic fungus. *Science* 285, 1896–1899. doi: 10.1126/science.285.5435.1896

Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubsky, J., Mansurova, M., et al. (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323, 101–106. doi: 10.1126/science.1163732

Bellincampi, D., Camardella, L., Delcour, J. A., Desseaux, V., D'Ovidio, R., Durand, A., et al. (2004). Potential physiological role of plant glycosidase inhibitors. *Biochim. Biophys. Acta* 1696, 265–274. doi: 10.1016/j.bbapap.2003.10.011

Bethke, G., Grundman, R. E., Sreekanta, S., Truman, W., Katagiri, F., and Glazebrook, J. (2013). *Arabidopsis* pectin methylesterases contribute to immunity against pseudomonas syringae. *Plant Physiol.* 164, 1093–1107. doi: 10.1104/pp.113.227637

Bily, A. C., Reid, L. M., Taylor, J. H., Johnston, D., Malouin, C., Burt, A. J., et al. (2003). Dehydrodimers of ferulic acid in maize grain pericarp and aleurone: resistance factors to *Fusarium graminearum*. *Phytopathology* 93, 712–719. doi: 10.1094/PHYTO.2003.93.6.712

Bohlmann, H., and Sobczak, M. (2014). The plant cell wall in the feeding sites of cyst nematodes. *Front. Plant Sci.* 5:89. doi: 10.3389/fpls.2014.00089

Boller, T., and He, S. Y. (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* 324, 742–744. doi: 10.1126/science.1171647

Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9452–9457. doi: 10.1073/pnas.1000675107

Buttner, D., and He, S. Y. (2009). Type III protein secretion in plant pathogenic bacteria. *Plant Physiol.* 150, 1656–1664. doi: 10.1104/pp.109.139089

Cano-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003). Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *Plant J.* 34, 351–362. doi: 10.1046/j.1365-313X.2003.01729.x

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J. M., Bennett, A. B., and Powell, A. L. T. (2009). Ripening-regulated susceptibility of tomato fruit to *botrytis cinerea* requires nor but not rin or ethylene. *Plant Physiol.* 150, 1434–1449. doi: 10.1104/pp.109.138701

Cantu, D., Vicente, A. R., Greve, L. C., Dewey, F. M., Bennett, A. B., Labavitch, J. M., et al. (2008a). The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 859–864. doi: 10.1073/pnas.0709813105

Cantu, D., Vicente, A. R., Labavitch, J. M., Bennett, A. B., and Powell, A. L. (2008b). Strangers in the matrix: plant cell walls and pathogen susceptibility. *Trends Plant Sci.* 13, 610–617. doi: 10.1016/j.tplants.2008.09.002

Caprari, C., Bergmann, C., Migheli, Q., Salvi, G., Albersheim, P., Darvill, A., et al. (1993). *Fusarium moniliforme* secretes four endopolygalacturonases derived from a single gene product. *Physiol. Mol. Plant Pathol.* 43, 453–462. doi: 10.1006/pmpp.1993.1073

Celio, G. J., Mims, C. W., and Richardson, E. A. (2004). Ultra-structure and immuno cytochemistry of the host–pathogen interface in poinsettia leaves infected with powdery mildew. *Can. J. Bot.* 82, 421–429. doi: 10.1139/b04-019

Chassot, C., and Metraux, J. P. (2005). The cuticle as source of signals for plant defense. *Plant Biosyst.* 139, 28–31. doi: 10.1080/11263500500056344

Chassot, C., Nawrath, C., and Metraux, J. P. (2007). Cuticular defects lead to full immunity to a major plant pathogen. *Plant J.* 49, 972–980. doi: 10.1111/j.1365-313X.2006.03017.x

Chen, M. H., Sheng, J., Hind, G., Handa, A. K., and Citovsky, V. (2000). Interaction between the tobacco mosaic virus movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. *EMBO J.* 19, 913–920. doi: 10.1093/emboj/19.5.913

D'Ovidio, R., Mattei, B., Roberti, S., and Bellincampi, D. (2004). Polygalacturonases, polygalacturonase–inhibiting proteins and pectic oligomers in plant–pathogen interactions. *Biochim. Biophys. Acta* 1696, 237–244. doi: 10.1016/j.bbapap.2003.08.012

Dangl, J. L., Horvath, D. M., and Staskawicz, B. J. (2013). Pivoting the plant immune system from dissection to deployment. *Science* 341, 746–751. doi: 10.1126/science.1236011

Daudi, A., Cheng, Z. Y., O'Brien, J. A., Mammarella, N., Khan, S., Ausubel, F. M., et al. (2012). The apoplastic oxidative burst peroxidase in *arabidopsis* is a major component of pattern-triggered immunity. *Plant Cell* 24, 275–287. doi: 10.1105/tpc.111.093039

Davis, E. L., Hussey, R. S., and Baum, T. J. (2004). Getting to the roots of parasitism by nematodes. *Trends Parasitol.* 20, 134–141. doi: 10.1016/j.pt.2004.01.005

Davis, E. L., Hussey, R. S., Mitchum, M. G., and Baum, T. J. (2008). Parasitism proteins in nematode–plant interactions. *Curr. Opin. Plant Biol.* 11, 360–366. doi: 10.1016/j.pbi.2008.04.003

Deepak, S., Shailasree, S., Kini, R. K., Muck, A., Mithofer, A., and Shetty, S. H. (2010). Hydroxyproline-rich glycoproteins and plant defense. *J. Phytopathol.* 158, 585–593. doi: 10.1111/j.1439-0434.2010.01669.x

De Lorenzo, G., Brutus, A., Savatin, D. V., Sicilia, F., and Cervone, F. (2011). Engineering plant resistance by constructing chimeric receptors that recognize damage-associated molecular patterns (DAMPs). *FEBS Lett.* 585, 1521–1528. doi: 10.1016/j.febslet.2011.04.043

Di Matteo, A., Giovane, A., Raiola, A., Camardella, L., Bonivento, D., De Lorenzo, G., et al. (2005). Structural basis for the interaction between pectin methylesterase and a specific inhibitor protein. *Plant Cell* 17, 849–858. doi: 10.1105/tpc.104.028886

Dorokhov, Y. L., Komarova, T. V., Petrunia, I. V., Frolova, O. Y., Pozdyshev, D. V., and Gleba, Y. Y. (2012). Airborne signals from a wounded leaf facilitate viral spreading and induce antibacterial resistance in neighboring plants. *PLoS Pathog.* 8:e1002640. doi: 10.1371/journal.ppat.1002640

Ellinger, D., Naumann, M., Falter, C., Zwikowics, C., Jamrow, T., Manisseri, C., et al. (2013). Elevated early callose deposition results in complete penetration resistance to powdery mildew in *Arabidopsis*. *Plant Physiol.* 161, 1433–1444. doi: 10.1104/pp.112.211011

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J. G. (2002). The *Arabidopsis* mutant cev1 links cell wall signaling to jasmonate and ethylene responses. *Plant Cell* 14, 1557–1566. doi: 10.1105/tpc.002022

Ellis, C., and Turner, J. G. (2001). The *Arabidopsis* mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *Plant Cell* 13, 1025–1033. doi: 10.1105/tpc.13.5.1025

Eynck, C., Seguin-Swartz, G., Clarke, W. E., and Parkin, I. A. P. (2012). Monolignol biosynthesis is associated with resistance to Sclerotinia sclerotiorum in *Camelina sativa*. *Mol. Plant Pathol.* 13, 887–899. doi: 10.1111/j.1364-3703.2012.00798.x

Feng, J., Wang, F., Hughes, G. R., Kaminskyj, S., and Wei, Y. D. (2011). An important role for secreted esterase in disease establishment of the wheat powdery mildew fungus *Blumeria graminis* f. *sp tritici*. *Can. J. Microbiol.* 57, 211–216. doi: 10.1139/W10-120

Ferrari, S., Galletti, R., Pontiggia, D., Manfredini, C., Lionetti, V., Bellincampi, D., et al. (2008). Transgenic expression of a fungal endo-polygalacturonase increases plant resistance to pathogens and reduces auxin sensitivity. *Plant Physiol.* 146, 669–681. doi: 10.1104/pp.107.109686

Ferrari, S., Savatin, D. V., Sicilia, F., Gramegna, G., Cervone, F., and De Lorenzo, G. (2013). Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Front. Plant Sci.* 4:49. doi: 10.3389/fpls.2013.00049

Ferrari, S., Sella, L., Janni, M., Favaron, F., and D'Ovidio, R. (2012). Transgenic expression of polygalacturonase–inhibiting proteins in *Arabidopsis* and wheat increases resistance to the flower pathogen *Fusarium graminearum*. *Plant Biol. (Stuttg.)* 14, 31–38. doi: 10.1111/j.1438-8677.2011.00449.x

Flors, V., Leyva, M. D., Vicedo, B., Finiti, I., Real, M. D., Garcia-Agustin, P., et al. (2007). Absence of the endo-beta-1,4-glucanases Cel1 and Cel2 reduces susceptibility to *Botrytis cinerea* in tomato. *Plant J.* 52, 1027–1040. doi: 10.1111/j.1365-313X.2007.03299.x

Flors, V., Ton, J., van Doorn, R., Jakab, G., Garcia-Agustin, P., and Mauch-Mani, B. (2008). Interplay between JA, SA and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. *Plant J.* 54, 81–92. doi: 10.1111/j.1365-313X.2007.03397.x

Gallego-Giraldo, L., Jikumaru, Y., Kamiya, Y., Tang, Y. H., and Dixon, R. A. (2011). Selective lignin downregulation leads to constitutive defense response expression in alfalfa (*Medicago sativa* L.). *New Phytol.* 190, 627–639. doi: 10.1111/j.1469-8137.2010.03621.x

Galletti, R., Denoux, C., Gambetta, S., Dewdney, J., Ausubel, F. M., De Lorenzo, G., et al. (2008). The AtrbohD-mediated oxidative burst elicited by oligogalacturonides in *Arabidopsis* is dispensable for the activation of defense responses effective against *Botrytis cinerea*. *Plant Physiol.* 148, 1695–1706. doi: 10.1104/pp.108.127845

Gille, S., and Pauly, M. (2012). O-acetylation of plant cell wall polysaccharides. *Front. Plant Sci.* 3:12. doi: 10.3389/fpls.2012.00012

Giraldo, M. C., and Valent, B. (2013). Filamentous plant pathogen effectors in action. *Nat. Rev. Microbiol.* 11, 800–814. doi: 10.1038/nrmicro3119

Hamann, T. (2012). Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms. *Front. Plant Sci.* 3:77. doi: 10.3389/fpls.2012.00077

Hauck, P., Thilmony, R., and He, S. Y. (2003). A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis plants. Proc. Natl. Acad. Sci. U.S.A.* 100, 8577–8582. doi: 10.1073/pnas.1431173100

Hematy, K., Sado, P. E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., et al. (2007). A receptor-like kinase mediates the response of *Arabidopsis* cells to the inhibition of cellulose synthesis. *Curr. Biol.* 17, 922–931. doi: 10.1016/j.cub.2007.05.018

Herbert, C., O'Connell, R., Gaulin, E., Salesses, V., Esquerre-Tugaye, M. T., and Dumas, B. (2004). Production of a cell wall-associated endopolygalacturonase by *Colletotrichum lindemuthianum* and pectin degradation during bean infection. *Fungal Genet. Biol.* 41, 140–147. doi: 10.1016/j.fgb.2003.09.008

Hernandez-Blanco, C., Feng, D. X., Hu, J., Sanchez-Vallet, A., Deslandes, L., Llorente, F., et al. (2007). Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. *Plant Cell* 19, 890–903. doi: 10.1105/tpc.106.048058

Hewezi, T., Howe, P., Maier, T. R., Hussey, R. S., Mitchum, M. G., Davis, E. L., et al. (2008). Cellulose binding protein from the parasitic nematode *Heterodera schachtii* interacts with *Arabidopsis* pectin methylesterase: cooperative cell wall modification during parasitism. *Plant Cell* 20, 3080–3093. doi: 10.1105/tpc.108.063065

Hok, S., Attard, A., and Keller, H. (2010). Getting the most from the host: how pathogens force plants to cooperate in disease. *Mol. Plant Microbe Interact.* 23, 1253–1259. doi: 10.1094/MPMI-04-10-0103

Jacobs, A. K., Lipka, V., Burton, R. A., Panstruga, R., Strizhov, N., Schulze-Lefert, P., et al. (2003). An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* 15, 2503–2513. doi: 10.1105/tpc.016097

Juge, N. (2006). Plant protein inhibitors of cell wall degrading enzymes. *Trends Plant Sci.* 11, 359–367. doi: 10.1016/j.tplants.2006.05.006

King, B. C., Waxman, K. D., Nenni, N. V., Walker, L. P., Bergstrom, G. C., and Gibson, D. M. (2011). Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. *Biotechnol. Biofuels* 4:4. doi: 10.1186/1754-6834-4-4

Komarova, T. V., Sheshukova, E. V., and Dorokhov, Y. L. (2014). Cell wall methanol as a signal in plant immunity. *Front. Plant Sci.* 5:101. doi: 10.3389/fpls.2014.00101

Laluk, K., and Mengiste, T. (2010). Necrotroph attacks on plants: wanton destruction or covert extortion? *Arabidopsis Book* 8:e0136. doi: 10.1199/tab.0136

Lionetti, V., Cervone, F., and Bellincampi, D. (2012). Methyl esterification of pectin plays a role during plant–pathogen interactions and affects plant resistance to diseases. *J. Plant Physiol.* 169, 1623–1630. doi: 10.1016/j.jplph.2012.05.006

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., et al. (2007). Overexpression of pectin methylesterase inhibitors in *Arabidopsis* restricts fungal infection by *Botrytis cinerea*. *Plant Physiol.* 143, 1871–1880. doi: 10.1104/pp.106.090803

Lionetti, V., Raiola, A., Cervone, F., and Bellincampi, D. (2013). Transgenic expression of pectin methylesterase inhibitors limits tobamovirus spread in tobacco and *Arabidopsis. Mol. Plant Pathol.* 15, 265–274. doi: 10.1111/mpp.12090

Llorente, F., Alonso-Blanco, C., Sanchez-Rodriguez, C., Jorda, L., and Molina, A. (2005). ERECTA receptor-like kinase and heterotrimeric G protein from *Arabidopsis* are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina. Plant J.* 43, 165–180. doi: 10.1111/j.1365-313X.2005.02440.x

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. (2011). Callose deposition: a multifaceted plant defense response. *Mol. Plant Microbe Interact.* 24, 183–193. doi: 10.1094/MPMI-07-10-0149

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., et al. (2011). Loss-of-function mutation of reduced wall acetylation2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea. Plant Physiol.* 155, 1068–1078. doi: 10.1104/pp.110.168989

Mattei, B., Raiola, A., Caprari, C., Federici, L., Bellincampi, D., De Lorenzo, G., et al. (2002) "Studies on plant inhibitors of pectin modifying enzymes: polygalacturonase-inhibiting protein (PGIP) and pectin methylesterase inhibitor (PMEI)," in *Carbohydrate Bioengineering Interdisciplinary approaches,* eds T. T. Teeri, B. Svensson, H. J. Gilbert, and T. Feizi (CambridgeRoyal Society of Chemistry), 160–167.

Moscetti, I., Tundo, S., Janni, M., Sella, L., Gazzetti, K., Tauzin, A., et al. (2013). Constitutive expression of the xylanase inhibitor TAXI-III delays fusarium head blight symptoms in durum wheat transgenic plants. *Mol. Plant Microbe Interact.* 26, 1464–1472. doi: 10.1094/MPMI-04-13-0121-R

Noda, J., Brito, N., and González, C. (2010). The Botrytis cinerea xylanase Xyn11A contributes to virulence with its necrotizing activity, not with its catalytic activity. *BMC Plant Biol.* 10, 1–15 doi: 10.1186/1471-2229-10-38

O'Brien, J. A., Daudi, A., Butt, V. S., and Bolwell, G. P. (2012). Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta* 236, 765–779. doi: 10.1007/s00425-012-1696-9

Passardi, F., Penel, C., and Dunand, C. (2004). Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends Plant Sci.* 9, 534–540. doi: 10.1016/j.tplants.2004.09.002

Pogorelko, G., Lionetti, V., Bellincampi, D., and Zabotina, O. (2013a). Cell wall integrity: Targeted post–synthetic modifications to reveal its role in plant growth and defense against pathogens. *Plant Signal. Behav.* 8, 1–8. doi: 10.4161/psb.25435

Pogorelko, G., Lionetti, V., Fursova, O., Sundaram, R. M., Qi, M. S., Whitham, S. A., et al. (2013b). *Arabidopsis* and *Brachypodium distachyon* transgenic plants expressing *Aspergillus nidulans* acetylesterases have decreased degree of polysaccharide acetylation and increased resistance to pathogens. *Plant Physiol.* 162, 9–23. doi: 10.1104/pp.113.214460

Prins, T. W., Tudzynski, P., Von Tiedemann, A., Tudzynski, B., ten Have, A., Hansen, M. E., et al. (2000). "Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens," in *Fungal Pathology*, ed. J. Kronstad (Dordrecht: Kluwer Academic Publishers), 33–64. doi: 10.1007/978-94-015-9546-9_2

Raiola, A., Camardella, L., Giovane, A., Mattei, B., De Lorenzo, G., Cervone, F., et al. (2004). Two *Arabidopsis thaliana* genes encode functional pectin methylesterase inhibitors. *FEBS Lett.* 557, 199–203. doi: 10.1016/S0014-5793(03)01491-1

Raiola, A., Lionetti, V., Elmaghraby, I., Immerzeel, P., Mellerowicz, E. J., Salvi, G., et al. (2011). Pectin methylesterase is induced in *Arabidopsis* upon infection and is necessary for a successful colonization by necrotrophic pathogens. *Mol. Plant Microbe Interact.* 24, 432–440. doi: 10.1094/MPMI-07-10-0157

Ramirez, V., Agorio, A., Coego, A., Garcia-Andrade, J., Hernandez, M. J., Balaguer, B., et al. (2011). MYB46 modulates disease susceptibility to *Botrytis cinerea* in *Arabidopsis. Plant Physiol.* 155, 1920–1935. doi: 10.1104/pp.110.171843

Reca, I. B., Lionetti, V., Camardella, L., D'Avino, R., Giardina, T., Cervone, F., et al. (2012). A functional pectin methylesterase inhibitor protein (SolyPMEI) is expressed during tomato fruit ripening and interacts with PME-1. *Plant Mol. Biol.* 79, 429–442. doi: 10.1007/s11103-012-9921-2

Rocchi, V., Janni, M., Bellincampi, D., Giardina, T., and D'Ovidio, R. (2011). Intron retention regulates the expression of pectin methylesterase inhibitor (Pmei) genes during wheat growth and development. *Plant Biol.* 14, 365–373. doi: 10.1111/j.1438-8677.2011.00508.x

Ron, M., and Avni, A. (2004). The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell* 16, 1604–1615. doi: 10.1105/tpc.022475

Sanchez-Rodriguez, C., Estevez, J. M., Llorente, F., Hernandez-Blanco, C., Jorda, L., Pagan, I., et al. (2009). The ERECTA receptor-like kinase regulates cell wall-mediated resistance to pathogens in *Arabidopsis thaliana. Mol. Plant Microbe Interact.* 22, 953–963. doi: 10.1094/MPMI-22-8-0953

Sattler, S. E., and Funnell-Harris, D. L. (2013). Modifying lignin to improve bioenergy feedstocks: strengthening the barrier against pathogens? *Front. Plant Sci.* 4:70. doi: 10.3389/fpls.2013.00070

Sella, L., Gazzetti, K., Faoro, F., Odorizzi, S., D'Ovidio, R., Schafer, W., et al. (2013). A *Fusarium graminearum* xylanase expressed during wheat infection is a necrotizing factor but is not essential for virulence. *Plant Physiol. Biochem.* 64, 1–10. doi: 10.1016/j.plaphy.2012.12.008

Spadoni, S., Zabotina, O., Di Matteo, A., Mikkelsen, J. D., Cervone, F., DL, G., et al. (2006). Polygalacturonase-inhibiting protein (PGIP) interacts with pectin through a binding site formed by four clustered residues of arginine and lysine. *Plant Physiol.* 141, 557–564. doi: 10.1104/pp.106.076950

Taurino, M., Abelenda, J. A., Rio-Alvarez, I., Navarro, C., Vicedo, B., Farmaki, T., et al. (2014). Jasmonate-dependent modifications of the pectin matrix during potato development function as a defense mechanism targeted by Dickeya dadantii virulence factors. *Plant J.* 77, 418–429. doi: 10.1111/tpj.12393

Ton, J., and Mauch-Mani, B. (2004). Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *Plant J.* 38, 119–130. doi: 10.1111/j.1365-313X.2004.02028.x

Udatha, D. B. R. K., Mapelli, V., Panagiotou, G., and Olsson, L. (2012). Common and distant structural characteristics of feruloyl esterase families from *Aspergillus oryzae. PLoS ONE* 7:e39473. doi: 10.1371/journal.pone.0039473

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Front. Plant Sci.* 3:85. doi: 10.3389/fpls.2012.00085

Vanholme, B., De Meutter, J., Tytgat, T., Van Montagu, M., Coomans, A., and Gheysen, G. (2004). Secretions of plant-parasitic nematodes: a molecular update. *Gene* 332, 13–27. doi: 10.1016/j.gene.2004.02.024

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. C. (2002). PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis. Plant Cell* 14, 2095–2106. doi: 10.1105/tpc.003509

Vogel, J. P., Raab, T. K., Somerville, C. R., and Somerville, S. C. (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. *Plant J.* 40, 968–978. doi: 10.1111/j.1365-313X.2004.02264.x

Voigt, C. A. (2014). Callose-mediated resistance to pathogenic intruders in plant defense-related papillae. *Front. Plant Sci.* 5:168. doi: 10.3389/fpls.2014.00168

Volpi, C., Janni, M., Lionetti, V., Bellincampi, D., Favaron, F., and D'Ovidio, R. (2011). The ectopic expression of a pectin methyl esterase inhibitor increases pectin methyl esterification and limits fungal diseases in wheat. *Mol. Plant Microbe Interact.* 24, 1012–1019. doi: 10.1094/MPMI-01-11-0021

Wieczorek, K., Golecki, B., Gerdes, L., Heinen, P., Szakasits, D., Durachko, D. M., et al. (2006). Expansins are involved in the formation of nematode-induced syncytia in roots of *Arabidopsis thaliana. Plant J.* 48, 98–112. doi: 10.1111/j.1365-313X.2006.02856.x

Wieczorek, K., Hofmann, J., Blochl, A., Szakasits, D., Bohlmann, H., and Grundler, F. M. W. (2008). *Arabidopsis* endo-1,4-beta-glucanases are involved in the formation of root syncytia induced by *Heterodera schachtii. Plant J.* 53, 336–351. doi: 10.1111/j.1365-313X.2007.03340.x

Williamson, V. M., and Kumar, A. (2006). Nematode resistance in plants: the battle underground. *Trends Genet.* 22, 396–403. doi: 10.1016/j.tig.2006.05.003

Wilson, R. A., and Talbot, N. J. (2009). Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae. Nat. Rev. Microbiol.* 7, 185–195. doi: 10.1038/nrmicro2032

Xu, L., Zhu, L. F., Tu, L. L., Liu, L. L., Yuan, D. J., Jin, L., et al. (2011). Lignin metabolism has a central role in the resistance of cotton to the wilt fungus Verticillium dahliae as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry. *J. Exp. Bot.* 62, 5607–5621. doi: 10.1093/jxb/err245

Zhang, L., Kars, I., Essenstam, B., Liebrand, T. W., Wagemakers, L., Elberse, J., et al. (2014). Fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the *Arabidopsis* receptor-like protein responsiveness to *Botrytis polygalacturonases1. Plant Physiol.* 164, 352–364. doi: 10.1104/pp.113.230698

Zhao, Z. T., Liu, H. Q., Wang, C. F., and Xu, J. R. (2013). Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics* 14:274. doi: 10.1186/1471-2164-14-274

Zhong, R., Richardson, E. A., and Ye, Z. H. (2007). The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in *Arabidopsis. Plant Cell* 19, 2776–2792. doi: 10.1105/tpc.107.053678

Conflict of Interest Statement: The Review Editor, Dr Ferrari, declares that, despite being affiliated to the same institution as authors Dr Daniela Bellincampi and Dr Felice Cervone, the review process was handled objectively. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 March 2014; paper pending published: 10 April 2014; accepted: 06 May 2014; published online: 28 May 2014.

*Citation: Bellincampi D, Cervone F and Lionetti V (2014) Plant cell wall dynamics and wall-related susceptibility in plant–pathogen interactions. Front. Plant Sci. **5**:228. doi: 10.3389/fpls.2014.00228*

This article was submitted to Plant–Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 06 May 2014
doi: 10.3389/fpls.2014.00178

The role of the cell wall in plant immunity

Frederikke G. Malinovsky[1]*, Jonatan U. Fangel[2] and William G. T. Willats[2]

[1] DNRF Center DynaMo and Copenhagen Plant Science Center, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Copenhagen, Denmark
[2] Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

Edited by:
Vincenzo Lionetti, Sapienza Università di Roma, Italy

Reviewed by:
Thorsten Hamann, Norwegian University of Science and Technology, Norway
Kian Hématy, Institut National de la Recherche Agronomique, France

****Correspondence:***
Frederikke G. Malinovsky, DNRF Center DynaMo and Copenhagen Plant Science Center, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg Centret, Copenhagen, Denmark
e-mail: fgm@PLEN.ku.dk

The battle between plants and microbes is evolutionarily ancient, highly complex, and often co-dependent. A primary challenge for microbes is to breach the physical barrier of host cell walls whilst avoiding detection by the plant's immune receptors. While some receptors sense conserved microbial features, others monitor physical changes caused by an infection attempt. Detection of microbes leads to activation of appropriate defense responses that then challenge the attack. Plant cell walls are formidable and dynamic barriers. They are constructed primarily of complex carbohydrates joined by numerous distinct connection types, and are subject to extensive post-synthetic modification to suit prevailing local requirements. Multiple changes can be triggered in cell walls in response to microbial attack. Some of these are well described, but many remain obscure. The study of the myriad of subtle processes underlying cell wall modification poses special challenges for plant glycobiology. In this review we describe the major molecular and cellular mechanisms that underlie the roles of cell walls in plant defense against pathogen attack. In so doing, we also highlight some of the challenges inherent in studying these interactions, and briefly describe the analytical potential of molecular probes used in conjunction with carbohydrate microarray technology.

Keywords: plant cell wall, defense, PTI, PAMP, DAMP, callose, chitin, immunity

INTRODUCTION

The dynamic interplay between pathogen and plant host is the product of millions of years of co-evolution. This struggle is often described in terms of an "arms race," a fitting term considering the investment required and the significance for both sides. The goal for the plant is to keep healthy and fertile, but for pathogens the strategy varies. Biotrophic and hemibiotrophic microorganisms rely on keeping their host alive and unaware of their presence, at least until later stages of infection. In contrast, necrotrophs live and feed on dead and dying cells and can therefore use a more forceful attack strategy (Davidsson et al., 2013). The frontline of the plant defense system consists of physical and chemical barriers such as the cell wall, waxes, hairs, antimicrobial enzymes, and secondary metabolites. If these obstacles are overcome, the pathogen is still confronted by elaborate surveillance systems in which molecular sentinels operate to activate resistance responses (Jones and Dangl, 2006).

Molecular components that serve essential functions for the fitness or survival of microbes are often highly conserved. For plants, detection of such microbial fingerprints also referred to as pathogen-associated molecular patterns (PAMPs), is a warning of impending attack (Medzhitov and Janeway, 1997). Consequently, a key aspect of plant innate immunity is the ability to recognize PAMPs such as bacterial flagellin, lipopolysaccharides, peptidoglycans, and fungal chitin (Boller and Felix, 2009). In addition to sensing PAMPs, the ability to sense a compromised "self" by detecting damage-associated molecular patterns (DAMPs) such as released plant cell wall fragments is a central part of plant defense (Boller and Felix, 2009). Both PAMP- and DAMP-recognition activates PAMP-triggered immunity (PTI) which in general prevents microbial colonization. To escape detection and PTI induction, a common strategy among adapted microorganisms is to secrete a range of effector proteins that can modulate PTI components (Jones and Dangl, 2006). The stealth afforded by the microbial effectors can in turn be counteracted in the plant by an intracellular surveillance system consisting of an array of nucleotide-binding leucine-rich-repeat proteins that seek to detect the presence of such effector proteins, and enable induction of effector-triggered immunity (ETI). ETI is often associated with a localized cell death termed the hypersensitive response which functions to restrict spread of this more progressive stage of microbial attack (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Hence the important feature of PTI is the ability to sense *infectious-self* and *non-self*, whereas for ETI it is the ability to sense microbe-mediated modifications inferred on points of vulnerability in the host. By guarding these weak points or even setting up decoys to confuse invaders, ETI is an efficient safety net for more progressed infections (van der Hoorn and Jones, 2004; Jones and Dangl, 2006).

Pathogen-associated molecular pattern perception is mediated by ligand-binding surface-exposed transmembrane pattern-recognition receptors (PRRs) of either the receptor-like kinase (RLK) or receptor-like proteins (RLPs) families. Both types of modular proteins are single-pass transmembrane proteins with extracellular domains, but where RLKs have an intracellular kinase domain; RLPs lack this cytosolic signaling domain (Monaghan and Zipfel, 2012). The archetypical bacterial PRRs are elongation factor Tu (EF-Tu) receptor (EFR), a leucine-rich repeat RLK (LRR-RLK) that recognizes the abundant cytoplasmic protein EF-Tu; and the related flagellin sensing 2 (FLS2) that recognizes

flagellin, the principal component of bacterial flagella. These two PAMPs are often characterized by their minimal requirement peptide epitopes, elf18 and flg22 (Gomez-Gomez et al., 1999; Kunze et al., 2004; Boller and Felix, 2009). Sensing of the elf18 peptide appears to be restricted to the *Brassicaceae* family, while in rice recognition of EF-Tu, occurs via the EFa50 region (Furukawa et al., 2013). Interestingly, transgenic expression of *Arabidopsis* (*Arabidopsis thaliana*) EFR in *Solanaceae* species that cannot perceive EF-Tu is sufficient to confer resistance to a broad range of phytopathogenic bacteria, suggesting high conservation of the responses downstream of PAMP recognition (Lacombe et al., 2010). PTI induction leads to a series of early and late responses. The early responses occur within minutes to hours, and consist of rapid ion fluxes across the plasma membrane, an oxidative burst, activation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs), and induction of defense-related genes. Deposition of callose, inhibition of seedling growth and PAMP induced resistance are later responses that occur within days (Boller and Felix, 2009).

RECOGNITION OF CHITIN: AN EVOLUTIONARY ARMS RACE

A good example of the intricate evolutionary arms race between a pathogen and its plant host concerns the polysaccharide chitin (**Figure 1**). Chitin, a homopolymer of β-(1→4)-linked *N*-acetylglucosamine (GlcNAc) units, is the major structural component of fungal cell walls, and is also a main constituent of insect exoskeletons, crustacean shells, and the eggs and gut linings of nematodes (Bueter et al., 2013; Hadwiger, 2013). Chitin is an obvious PAMP-candidate and an ideal point of attack during plant defense responses since glucosamine polymers are not found in plants. It is therefore not surprising that an evolutionarily conserved strategy toward fungi and insects in plants is based on secreting chitinases – hydrolytic enzymes, which can break down microbial chitin polymers (**Figure 1**; Hadwiger, 2013). However, as usual, countermeasures have also evolved, the biotrophic fungal pathogen *Cladosporium fulvum* combats the action of chitinases by secreting the apoplastic effector Avr4, a chitin-binding lectin that functions to protect the integrity of the fungal cell wall against chitinases (**Figure 1**; van den Burg et al., 2006). Heterologous expression of Avr4 in *Arabidopsis* or tomato camouflages the chitin thereby increasing the virulence of several fungal pathogens (van Esse et al., 2007). That is unless the host harbors Cf-4, an extracellular membrane-anchored LRR protein that mediates Avr4 perception (**Figure 1**) and activates the hypersensitive response (Thomas et al., 1997; Takken et al., 1999). In addition to this forceful degradation strategy, chitin is also recognized as a PAMP in rice (*Oryza sativa*) by a dual recognition system consisting of the lysine motif (LysM)–RLK CHITIN ELICITOR RECEPTOR KINASE-1 OsCERK1 and the LysM-RLP CHITIN OLIGOSACCHARIDE ELICITOR-BINDING PROTEIN OsCEBiP (Shimizu et al., 2010). In *Arabidopsis* there is no contribution to signaling from the major chitin-oligosaccharide binding CEBiP, and AtCERK1 seems to act alone as the chitin PRR (**Figure 1**; Iizasa et al., 2010; Petutschnig et al., 2010; Shinya et al., 2012). The biological activity of chitin oligomers depends on their size, as the highest PAMP-activity is found for heptamers and octamers. Higher oligomeric chitin fragments like octamers can bind two or more AtCERK1 and this ligand-induced dimerization activates the receptor (Liu et al., 2012). To avoid chitin-induced PTI, *C. fulvum* also secretes the evolutionally conserved LysM-containing effector extracellular protein 6 (Ecp6) during infection. Ecp6 is a scavenger of chitin fragments released by chitinases and out-competes chitin-PRR binding to avoid fungal detection (**Figure 1**; Bolton et al., 2008; de Jonge et al., 2010; Sanchez-Vallet et al., 2013). Whether Ecp6 is recognized in plants is still

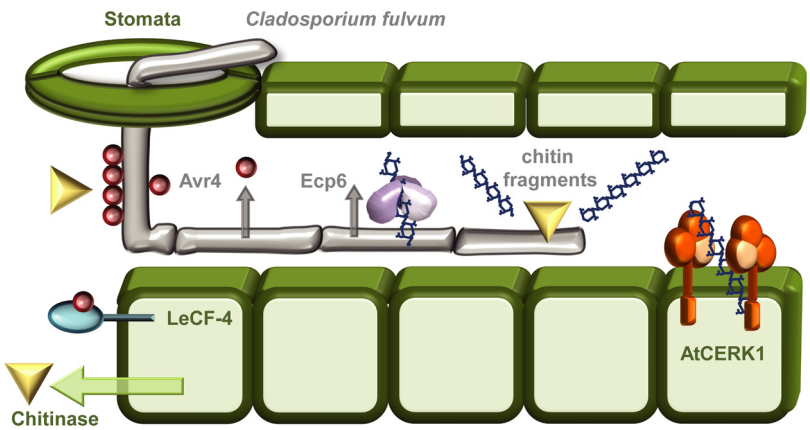


FIGURE 1 | The "arms race" between *Cladosporium fulvum* and plant hosts. As a general protective measure against fungal infections plants respond to infection attempts by secreting chitinases into the apoplastic space. The tomato leaf mould fungus *C. fulvum* can enter its host through stomatal openings, and grows as extracellular hyphae. To shield against the action of these chitin-degrading enzymes the fungus camouflages its chitin-containing cell walls by cloaking them with the chitin-binding effector Avr4 (van den Burg et al., 2006; Hadwiger, 2013). The presence of Avr4 can be recognized by the Tomato RLP Cf-4, leading to induction of the hypersensitive response (Thomas et al., 1997; Takken et al., 1999). Chitin oligomers released by chitinases are recognized as PAMPs, in *Arabidopsis* by the RLK CERK1 (Iizasa et al., 2010; Petutschnig et al., 2010), and in rice by both OsCERK1 and the RLP OsCEBiP (Shimizu et al., 2010). To escape chitin-induced PTI *C. fulvum* can secrete Ecp6 an effector that functions as a chitin-scavenger removing the chitin oligomers released by the chitinases (de Jonge et al., 2010; Kombrink and Thomma, 2013; Sanchez-Vallet et al., 2013).

unknown. In all cases, this example demonstrates the continuous battle between pathogen and host and how one party's armor is continuously being evolutionarily countered by the opponent.

PTI SIGNALING

A vital feature of plant innate immunity is the early recognition of potential pathogens via perception of PAMPs and DAMPs. This recognition is mediated by designated surface-localized PRRs and sets in train a range of early and late responses that ensure a specialized and appropriate response is transmitted by a shared downstream pathway. Some of these steps are already mapped whereas others are yet to be discovered.

After ligand recognition, several PRRs rapidly form complexes with the regulatory RLK BRI1-ASSOCIATED KINASE1 (BAK1; **Figure 2**; Monaghan and Zipfel, 2012). Indeed, complex formation between the co-receptor and the flagellin receptor FLS2 occurs within seconds of flg22 treatment (Schulze et al., 2010). BAK1 was believed to function solely as a signal enhancer rather than being involved in ligand-binding. However, it has recently been demonstrated that BAK1 acts as a co-receptor in FLS2 mediated flg22 recognition by interacting with the bound ligand (Sun et al., 2013). BAK1 belongs to the somatic embryogenesis-related kinases; a small family of five LRR-RLKs (Heese et al., 2007; Roux et al., 2011). In addition to FLS2, BAK1 has also been shown to interact with EFR and the DAMP receptors PEPR1 and PEPR2 (Krol et al., 2010; Yamaguchi et al., 2010). Apart from its association with PRRs, BAK1 is also involved in the perception of brassinosteroids through its interaction with the RLK BRASSINOSTEROID INSENSITIVE 1 (BRI1; Li et al., 2002; Nam and Li, 2002). PAMP-binding and complex formation leads to auto- and trans-phosphorylation between PRR and co-receptor, as well as the plasma membrane localized receptor-like cytoplasmic kinase BOTRYTIS-INDUCED KINASE 1 (BIK1) and related PBS1-LIKE (PBL) kinases (Lu et al., 2010; Zhang et al., 2010). BIK1 plays a central role in conveying signals from several PRRs including EFR, FLS2, CERK1, and PEPR1/PEPR2. PAMP/DAMP induced phosphorylation of BIK1 prompts dissociation from the PRR complex which may permit BIK1 to move toward and activate downstream signaling targets (Lu et al., 2010; Zhang et al., 2010; Liu et al., 2013).

CONVEYING THE SIGNAL INTO TRANSCRIPTIONAL REPROGRAMMING

Calcium ions serve as important second messengers in eukaryotes. Both abiotic and biotic stress responses lead to rapid transient fluctuations in the concentration of intracellular calcium ions (**Figure 2**). The low basal cytosolic concentration allows rapid spatial and temporal changes in the calcium flux. Such calcium signatures are sensed by stimulus-specific Ca^{2+}-binding sensors that can mediate the downstream signal transduction. Deciphering the calcium signatures enables the affected cells to respond appropriately to diverse stimuli (DeFalco et al., 2010). During PTI, CDPKs function as Ca^{2+}-sensors that are required to obtain complete transcriptional reprogramming (**Figure 2**). The four functionally redundant *Arabidopsis* CDPKs, CPK4, CPK5, CPK6, and CPK11 define a sub-clade required for flg22 induced PTI responses (Boudsocq et al., 2010). The transcriptional changes effected by the CDPKs are mostly independent of the MAPK pathway (see below), but the combination of CDPKs and MAPKs is needed to activate

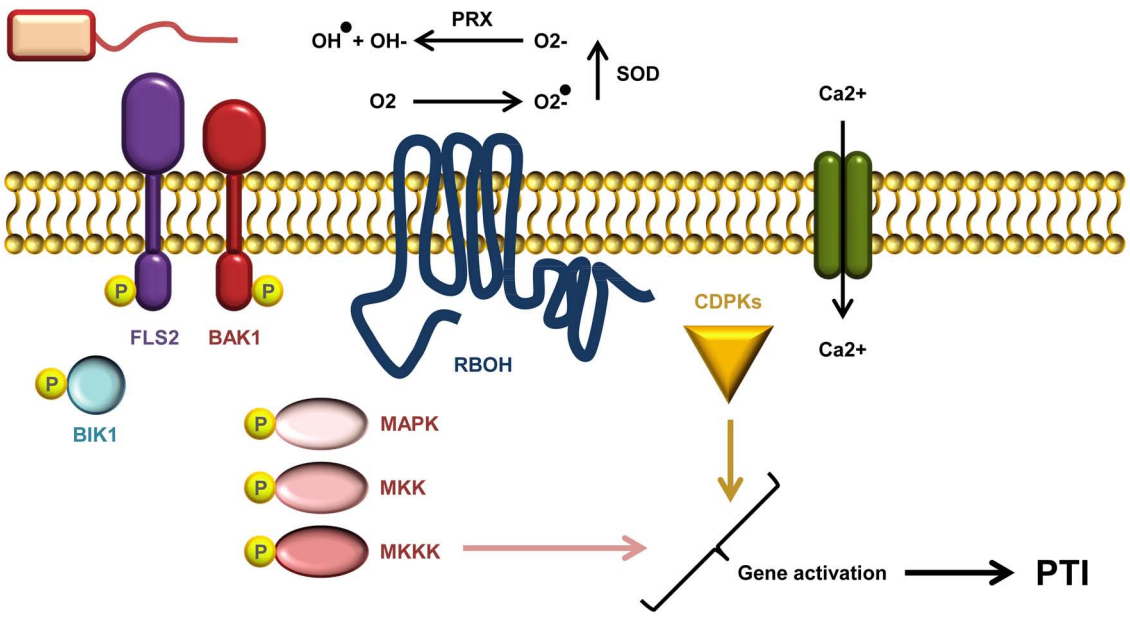


FIGURE 2 | A current model of AtFLS2 signaling. Upon flg22 binding, a complex between FLS2, BIK1, BAK1 (and other SERKs) is formed. Complex formation triggers multiple rapid phosphorylation events resulting in BIK1 release. The signal transduction downstream of ligand perception includes a Ca^{2+} burst, activation CDPKs and AtRbohD required for the ROS burst, and induction MAPK cascades. Activation of CDPKs and MAPKs is required for full induction of defense genes.

at least four early transcriptional regulatory programs induced by flg22 (Boudsocq et al., 2010; Wurzinger et al., 2011).

An oxidative burst of reactive oxygen species (ROS) such as $\cdot O_2^-$, ·OH, and H_2O_2 is one of the early measurable events in PTI (**Figure 2**). In *Arabidopsis*, the ROS burst is facilitated by the RESPIRATORY BURST OXIDASE HOMOLOGUE D (RbohD), an NADPH oxidase (Torres et al., 2002). The activity of RbohD is dependent on calcium and phosphorylation (Ogasawara et al., 2008; Kimura et al., 2012), and interestingly RbohD is phosphorylated by CDPK5 (Dubiella et al., 2013). In potato (*Solanum tuberosum*) StRbohB is phosphorylated by the plasma membrane-localized potato StCDPK5, and expression of constitutively active StCDPK5 in *Nicotiana benthamiana* leads to induction of ROS (Kobayashi et al., 2007).

Like the CDPKs, MAPK cascades also function to transmit signals within the cell via differential phosphorylation. In such cascades MAP kinase-kinase-kinases (MAPKKKs) phosphorylate MAP kinase-kinases (MAPKKs), which in turn phosphorylate MAP-kinases (**Figure 2**; Rasmussen et al., 2012). The *Arabidopsis* MAP kinases MPK3, MPK4, MPK6 as well as the recently documented MPK11 are all activated by PAMP treatment (Rasmussen et al., 2012). They designate at least two different pathways: one that triggers MPK4 activation by a module consisting of the two MAPKKs MKK1 and MKK2, and MEKK1; and another that leads to MPK3/MPK6 activation, via MKK4 and MKK5, and possibly MEKK1 (Rasmussen et al., 2012). Activation of MAPK cascades is, together with CDPK activation, vital for obtaining the transcriptional reprogramming needed to mount a full PTI response (**Figure 2**; Boudsocq et al., 2010). Even though some genes require both MAPK and CDPK activation to be induced there seems to be a branching of PTI signals on some level (Boudsocq et al., 2010; Xu et al., 2014). Loss of the flg22-induced ROS in *Arabidopsis rbohD* does not affect MPK3 and MPK6 activation, and activation of these MAP kinases is also not required for ROS burst (Xu et al., 2014).

FATE OF ACTIVATED RECEPTORS

Ubiquitination is a key signal for endosomal sorting of membrane proteins (Raiborg and Stenmark, 2009). Upon ligand perception FLS2 is being ubiquitinated by two PLANT U-BOX (PUB) E3 ligases PUB12 and PUB13 (Lu et al., 2011). This may well be part of a strategy to terminate the signal initiated by PAMP recognition. FLS2 is endocytosed and targeted for endocytic degradation approximately 30–60 min post-elicitation, and the activated FLS2 travels through early, late and multi-vesicular endosomes toward its vacuolar destination (Robatzek et al., 2006; Chinchilla et al., 2007; Beck et al., 2012b; Smith et al., 2014). The removal of ligand-bound receptors de-sensitizes the system after initial PAMP-treatment. After two hours the de-sensitized cells begin to be re-sensitized through *de novo* synthesis mediated replenishment of the receptor, preparing them for a new round of ligand perception (Smith et al., 2014). FLS2 has recently been shown to interact with two subunits of ENDOSOMAL SORTING COMPLEXES REQUIRED FOR TRANSPORT-I (ESCRT-I; Spallek et al., 2013). ESCRT complexes are responsible for identifying ubiquitinated vesicular cargoes, sorting them for degradation, and depositing them in intraluminal vesicles to prevent their recycling (Raiborg and Stenmark, 2009). Loss of the E3 ligases or ESCRT-I components affects flg22-triggered immune reactions underlining the importance of correct endosomal trafficking of FLS2 in sustaining a sufficient level of immunity (Lu et al., 2011; Spallek et al., 2013). In addition removal of activated receptors, trafficking might also play an integrated part in signaling transduction as part of the signaling responses may happen from endosomal compartments (Geldner and Robatzek, 2008; Beck et al., 2012a).

LATE PTI RESPONSES

The final consequence of PTI is the induction of resistance responses that will prevent microbial colonization. Other late responses are the inhibition of seedling growth inhibition occurring within days of continuous PAMP treatment, and deposition of callose about 16 h after PAMP treatment (Boller and Felix, 2009). Callose is found during attempted fungal infections in cell wall fortifications termed papillae, structures that are assembled at fungal penetration sites (Underwood, 2012). The linear β-(1→3)-D-Glc*p* homo-polymer is produced by callose synthases; among these is the *Arabidopsis* POWDERY MILDEW RESISTANT 4 (PMR4), the predominant synthase responsible for defense-induced callose deposition (Vogel and Somerville, 2000; Nishimura et al., 2003; Ham et al., 2007; Chen and Kim, 2009). All the callose deposited in response to flg22, and most of the chitosan-induced depositions are synthesized by PMR4 (Luna et al., 2011). In addition to differences in their dependency on PMR4, callose depositions induced by these two PAMPs also differ in their requirements for other signaling components as only the flg22-induced callose depends on RbohD (Luna et al., 2011). Callose depositions like the papillae are an important feature of immunity, and are thought to reinforce the cell wall at fungal penetration sites to impede infections (Underwood, 2012). In agreement with this, overexpression of PMR4 in *Arabidopsis* results in enlarged callose deposits, and gain of penetration resistance against both virulent and non-adapted powdery mildew strains (Ellinger et al., 2013; Naumann et al., 2013). Absence of callose depositions also leads to susceptibility toward the non-adapted hemibiotrophic bacteria *Pseudomonas syringae* pv. *phaseolicola* (Ham et al., 2007). Surprisingly the *pmr4* mutant was identified by its increased powdery mildew resistance, a phenotype that is dependent on the defense phytohormone salicylic acid (Vogel and Somerville, 2000; Nishimura et al., 2003; Huibers et al., 2013). These observations are counterintuitive both with respect to the pattern of callose deposition during defense induction, and the recent evidence from overexpression lines, supporting a role for callose in the plants defensive strategy (Vogel and Somerville, 2000; Ellinger et al., 2013; Naumann et al., 2013). One explanation for such a discrepancy could be that PMR4 is guarded by a resistance gene. Accordingly, loss of PMR4 might be perceived as a breach on the plants defensive fences and thus set about induction of ETI via elevation of salicylic acid.

THE ROLE OF PLANT CELL WALLS IN MICROBIAL INTERACTIONS

Plant cell walls not only provide structure to the plant body but also act as barriers against biotic and abiotic stresses. The

cell wall, sometimes covered with a cuticle, is usually the first obstacle encountered by pathogens, and to penetrate this barrier microbes have evolved an arsenal of wall degrading enzymes which are key virulence factors (**Figure 3**; Nuhse, 2012; Davidsson et al., 2013).

Most plant cell walls are based on a co-extensive load-bearing network of cellulose micro-fibrils cross-linked with hemicelluloses (**Figure 3**; Fry, 2004; Scheller and Ulvskov, 2010; Pauly et al., 2013). In the primary walls of the growing parts of plants, this network is embedded in a matrix of pectic polysaccharides. In the secondary cell walls of mature, non-growing tissues pectin is less abundant but walls are reinforced with lignin (Endler and Persson, 2011). Although most cell walls are based on these main components they differ considerably in their fine structures and three dimensional architectures. This heterogeneity is reflected in the diversity of strategies that pathogens have evolved to breach them, including the secretion of numerous glycosyl hydrolases (Annis and Goodwin, 1997). In response to an attack, plants may deposit certain reinforcing polymers, notably callose, phenolic complexes, and employ toxic compounds (Huckelhoven, 2007). However, these physical and chemical responses are only one part of the cell walls role in defense

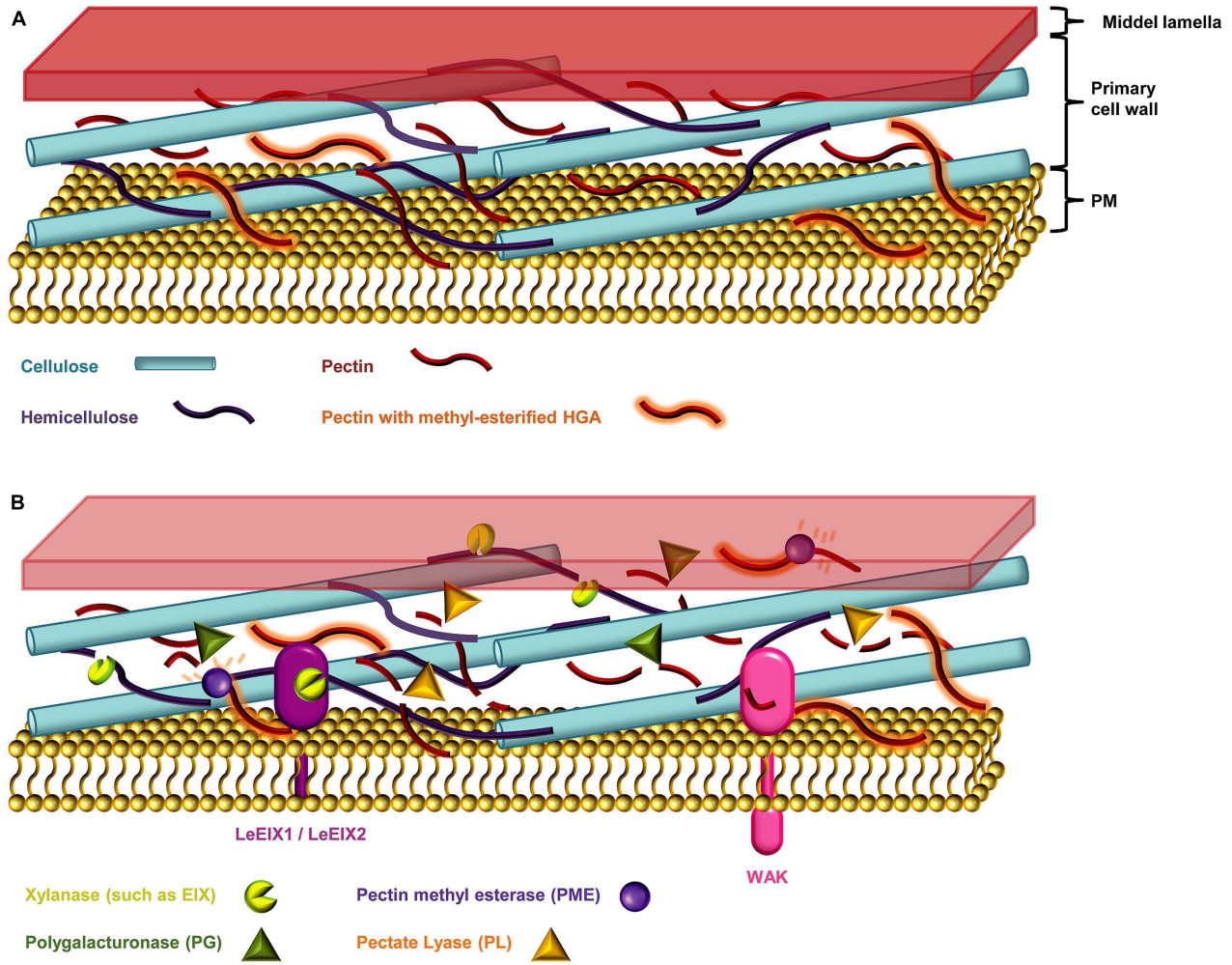


FIGURE 3 | The plant cell wall. (A) The primary cell wall is constructed of a web of cellulose micro-fibrils, hemicellulose polysaccharides, and the hetero-polysaccharide pectin (Endler and Persson, 2011).
(B) Necotrophic pathogens devour their hosts by degrading the plant cell wall, and secreting necrosis-inducing proteins and toxins (Davidsson et al., 2013). The host entry step of such microbes is thought to happen mainly via secretion of cell wall degrading enzymes such as cellulases, pectinases, and hemicellulases (Choquer et al., 2007; Davidsson et al., 2013). Xylanase, degrades the linear backbone of the predominant hemicellulose xylan into xylose residues (Belien et al., 2006; Scheller and Ulvskov, 2010). Pectinases such as polygalacturonases (PGs) and pectate lyases (PLs) degrades the pectic homogalacturonan (HGA) backbone. The HGA in newly synthesized pectin is methyl-esterified, which protects it from degradation by pectinases. De-esterification of HGA by the action of microbial pectin methyl esterases (PMEs) enables access PGs and PLs (Annis and Goodwin, 1997; Wolf et al., 2009; Lionetti et al., 2012). Endo-PGs cleave un-esterified regions while concomitantly releasing the oligogalacturonides (OGAs), oligomeric fragments of the HGA backbone (Annis and Goodwin, 1997; Ferrari et al., 2013). The wall-associated kinases (WAKs) are DAMP sensors that monitor the integrity of pectin by sensing the presence of OGAs (Ferrari et al., 2013). Plants can counter the cocktail of cell wall degrading enzymes by producing proteinaceous inhibitors such as the xylanase inhibitors and PG inhibitor proteins (PGIPs; Belien et al., 2006).

and the extensive ability of pathogens to degrade cell wall component has a cost. Disturbance of cell wall integrity (which may also cause deformation of adjacent portion of the plasma membrane) and the release of degradation fragments are monitored by plants and changed cell wall status is an important trigger for defense mechanisms (Hematy et al., 2009). Despite the undoubted importance of cell walls in plant defense there are many aspects that are poorly understood. For example, most work has focused on a limited set of cell wall polymers (notably callose, extensins, and lignin) and the potential roles of many other cell wall components are obscure. Another largely unexplored aspect of cell walls and defense is the evolution of and co-evolution of defense strategies (Sørensen et al., 2010). Discussed below are the major cell wall components in the context of pathogenic responses to them, and the prospects for advancing our understanding using emerging microarray technologies.

CELLULOSE

Tightly packed crystalline cellulose micro-fibrils serve a primary structural function in the cell wall (Endler and Persson, 2011; Nuhse, 2012). The fibrils are composed of hydrogen-bonded β-(1→4)-D-Glc*p* chains, and have a condensed nature that makes them generally resistant to degradation via glycosyl hydrolases. However, recent work has highlighted the role of another class of oxidative enzymes, the lytic polysaccharide monoxygenases that have the capacity to degrade recalcitrant crystalline cell wall components, including cellulose (Vaaje-Kolstad et al., 2010; Hemsworth et al., 2014). Cellulose micro-fibrils are synthesized by large multimeric complexes containing cellulose synthase catalytic subunits (CESAs; Endler and Persson, 2011). It is generally considered that in *Arabidopsis*, and probably other angiosperms, there are two CESA subfamilies with distinct roles. Whilst CESAs 1, 3, and 6 are responsible for the formation of cellulose in primary walls, CESAs 4, 7, and 8 synthesize cellulose in secondary walls (Endler and Persson, 2011). However, recent work has demonstrated that some functional cross over exists across these groups *in vitro* although it is not clear if these new CESA pairings are significant *in planta* (Carroll et al., 2012).

As in many cases where cell wall integrity is perturbed, compensatory mechanisms have evolved to limit the effects of disturbance to cellulose structure or deposition. Cellulose deficient mutants typically exhibit increased lignification (Cano-Delgado et al., 2003; Hamann, 2012). Interestingly, these changes appear to have effects beyond the purely structural. Such mutants also display enhanced defense responses (Hamann, 2012) as mutants in the primary wall *CESA3* are more resistant toward powdery mildew (Ellis and Turner, 2001; Cano-Delgado et al., 2003). Additionally defects in the secondary wall CESAs; CESA4, CESA7, and CESA8, also lead to elevated resistance against broad-host necrotrophic pathogens like the fungus *Plectosphaerella cucumerina* and the soil-borne bacterium *Ralstonia solanacearum* (Hernandez-Blanco et al., 2007). Consistent with the genetic evidence, similar results have also been described for treatment with the cellulose biosynthesis inhibitor isoxaben (Hamann et al., 2009; Hamann, 2012). In *Arabidopsis*, isoxaben-mediated wall damage and lignin production is induced via an RbohD-dependent mechanism, and fine-tuned through a jasmonic-acid-dependent negative feedback loop (Hamann et al., 2009; Denness et al., 2011). The defense associated cellulose deprivation phenotypes suggest that the cell wall damage triggers defense responses, and suggest the presence of a tight cell wall integrity monitoring system. The RLK THESEUS1 (THE1) seems to serve a role in this as it has been implicated in the response to cell wall damage. The *the1* mutant was identified as a suppressor of the *cesA6* null allele *procuste1-1*. Mutations in *THE1* reduce the ectopic lignification of several cellulose deficient mutants (Hematy et al., 2007).

HEMICELLULOSES

Hemicelluloses are a diverse group of polysaccharides usually characterized by having a β-(1→4)-linked backbone of mannose, glucose, or xylose. The central role of hemicelluloses is to fortify the cell wall by interaction with cellulose and sometimes lignin (Scheller and Ulvskov, 2010; Endler and Persson, 2011). Xylans are the predominant hemicelluloses in secondary plant cell walls. The common characteristic of xylans is a backbone of repeating β-(1→4)-D-Xyl*p* residues most often substituted by Ara*f* and Glc*p*A (Scheller and Ulvskov, 2010). The arabinosyl residues can in addition contain ferulic acid groups esterified to the O-5 position of the carboxyl group (Smith and Hartley, 1983). These ferulate esters can be oxidatively cross-linked to lignin possibly incorporating xylan into the lignin complex to advance strengthening of the network (Tanner and Morrison, 1983; Mcneil et al., 1984; Harris and Stone, 2009). Some phytopathogenic microbes secrete xylanases, which are enzymes that can degrade the linear xylan backbone into xylose units (Belien et al., 2006). Hemicellulose breakdown by xylanases weakens the cell wall and enables the microbe to breach it (Belien et al., 2006). Fungi of the *Trichoderma* spp. produce the ETHYLENE-INDUCING XYLANASE (EIX) that is recognized as a PAMP (Furman-Matarasso et al., 1999; Belien et al., 2006). In tomato (*Lycopersicum esculentum*), EIX is sensed by the cell wall-derived RLPs LeEix1 and LeEix2 (**Figure 3**; Ron and Avni, 2004).

In addition to feruloylation cell wall glycans can also be subjected to methylation and acetylation. REDUCED WALL ACETYLATION 2 (RWA2) has been shown to be responsible for the acetylation of numerous pectic and non-pectic polymers in *Arabidopsis*, and *rwa2* knock-out mutants have (Mcneil et al., 1984) increased resistance toward *Botrytis cinerea* (Manabe et al., 2011). Furthermore de-acetylation of xyloglucan and pectin in transgenic plants exhibit increased accessibility to degrading enzymes which could be part of a defense strategy to release oligosaccharides which can act as defense elicitors (Pogorelko et al., 2013).

PECTIN

The complex hetero-polysaccharide pectin is a major cell wall matrix component that is abundant in primary cell walls. It is composed of a series of structurally distinct domains acting as backbones or side chains to the pectic polysaccharides (Wolf et al., 2009). Back bones are comprised either of contiguous α-(1→4)-D-Gal*p*A residues (homogalacturonan or HGA) or

repeating dimers of α-(1→4)-D-Gal*p*A-α-(1→2)-L-Rha*p* residues (rhamogalacturonan). Within the pectin complex especially HGA appears to have special significance in the context of defense responses. HGA can be methyl esterified at C6 and acetylated at C2–C3 and regulation of these substitutions enable plants to fine-tune the functionality of HGA to suit prevailing local requirements. For example non-esterified HGA carrying negatively charged free carboxyl groups may be subject to cross-linking via calcium leading to the formation of gels that are required for cell adhesion and other structural roles (Cabrera et al., 2008).

Some of the first enzymes that phytopathogenic fungi secrete during infections are endo-polygalacturonases (PGs) which cleave HGA (**Figure 3**), thereby degrading cell wall integrity and aiding pathogen access (Annis and Goodwin, 1997). Moreover, HGA degradation releases oligogalacturonide (OGA) fragments from the backbone, which act as potent defense response elicitors (Galletti et al., 2009). The WALL-ASSOCIATED KINASES (WAKs) are thought to be DAMP sensors that monitor the integrity of pectin by detecting the presence of OGAs with a degree of polymerization between 10 and 15 (Ferrari et al., 2013). In the context of degradation by microbial enzymes the level of HGA methyl esterification is critically important. Endo-PGs and pectate lyases (PLs) preferentially cleave non-esterified HGA and these enzymes frequently act in concert with pectin methyl esterases (PMEs) that de-methyl esterify HGA to create endo-PG and PL cleavage sites (**Figure 3**). Moreover, another class of microbial enzymes – the pectin lyases act preferentially on highly methyl esterified HGA.

Homogalacturonan and the microbial enzymes that degrade it are good examples of how the high complexity of cell wall components is countered by sets of highly specific microbial enzymes. Nevertheless, the role of pectin in plant defense cannot only be viewed in straightforward terms of polysaccharide integrity versus enzymatic breakdown. An example is provided by the elevated activity of certain endogenous *Arabidopsis* PMEs in response to infection with the necrotrophic fungus *Alternaria brassicicola*, the hemibiotrophic bacterial pathogen *P. syringae pv. maculicola* ES4326, or by treatment with PAMPs (Bethke et al., 2014). The increase in PME activity in such infected plants leads to a concomitant decrease in methyl esterification of HGA, which doubtlessly renders the HGA more susceptible toward endo-PGs (Bethke et al., 2014). It seems counterintuitive to increase the activity of an endogenous wall degrading enzyme during a pathogen attack as this could aid microbial entry. One explanation is that the enhanced production of OGAs – and thereby an associated potential activation of DAMP signaling – is worth the price of a decrease in HGA integrity. It is also important to consider the broader picture of the 66 PMEs in *Arabidopsis* in terms of redundancy and spatiotemporal regulation. For example, in the case of *A. brassicicola* it is worth noting that the observed increase in PME activity occurred rather late after pathogen challenge. With this in mind it may be the case that the purpose of enhanced PME activity is localized HGA de-methyl esterification and the concomitant formation of gel structures to bolster damaged walls (Bethke et al., 2014).

Another noteworthy insight into the roles of pectin in defense is provided by the loss of function mutants of the endogenous plant enzyme POWDERY MILDEW RESISTANT 6 MUTANT (PMR6; Vogel et al., 2002). PMR6 has similarity to PLs, and loss of function leads to enhanced levels of intact pectin and but also lower levels of potential OGA release. One explanation for the enhanced resistance to powdery mildew in *pmr6* is that increased levels of inaccessible HGA *per se* provide more protection. But, the fact that *pmr6* only exhibits this elevated resistance against certain powdery mildew species (*Erysiphe cichoracearum* and *E. orontii*) implies a less generic and more subtle effect. For example, it is possible that alterations in *pmr6* cell wall composition make these mutants poor hosts specifically for *Erysiphe* spp. It certainly seems highly unlikely that a plant gene would have evolved to serve the needs of a pathogen, and the pleotropic effects observed in *pmr6* are consistent with a role as a susceptibility factor. It is plausible that *pmr6* resistance is a specialized form of disease resistance, possibly based on the loss of a host susceptibility gene required by the pathogen for growth and development (Vogel et al., 2002).

LIGNIN

Lignin is a phenolic polymer mainly deposited in secondary cell wall during the last stages of cell differentiation. It displaces the aqueous phase of the cell wall, encasing cellulose and matrix polysaccharides and providing enhanced mechanical strength and a water-impermeable barrier (Albersheim et al., 2011). Lignin is also required for reinforcing vascular cells that transport water under negative pressure as a result of transpiration. The importance of lignin in these tissues is demonstrated by vascular collapse in lignin deficient plants (Piquemal et al., 1998; Jones et al., 2001). Lignin is built from monolignols, with up to three different types in higher plants which appear to be incorporated into the lignin polymer in a non-predictable fashion (Martone et al., 2009; Vanholme et al., 2010). This apparently random pattern of synthesis may be significant in relation to microbial enzymes which have typically evolved to break polymers with structurally consistent cleavage sites (Sarkar et al., 2009).

Lignin has multiple roles in plant defense and lignin, or lignin-like phenolic polymers are often rapidly deposited in response to both biotic and abiotic stresses (Sattler and Funnell-Harris, 2013). Lignin not only acts as physical barrier to pathogen invasion, but the phenylpropanoid pathway responsible for lignin biosynthesis may also be recruited for defense purposes. For example, this pathway underpins the synthesis of other phenolic compounds including phytoalexins, stilbenes, coumarins, and flavonoids – some of which have been implicated in plant defense (Weiergang et al., 1996; Dicko et al., 2005; Lozovaya et al., 2007). Indeed, there is evidence that in some plants, salicylic acid which is known to be a key component of some defense pathways, may also be produced by the phenylpropanoid pathway (Ruuhola et al., 2003; Pan et al., 2006). Understanding the role of lignin in plant defense has received considerable attention in the context of engineering plants for use as bioenergy feedstocks. Lignin contributes substantially to the recalcitrance of cell walls to deconstruction into fermentable sugars and for this reason reducing lignin content has become an important goal for feedstock biotechnology research. However, since lignin is also required for plant defense there is probably a lower limit for lignin reduction beyond which

plant would become unacceptably vulnerable to pathogens (Sattler and Funnell-Harris, 2013). The roles of lignin in defense and the importance of this for bioenergy feedstocks are reviewed in (Sattler and Funnell-Harris, 2013).

INHIBITORS OF MICROBIAL WALL DEGRADING ENZYMES

Often, an infection involves a multifaceted strategy. In the case of *B. cinerea* the host entry step is thought to happen mainly via secretion of cell wall degrading enzymes such as a PMEs, endo-PGs, and endo-β-1,4-xylanase often encoded by multi-genic families (Choquer et al., 2007). Cellulases, pectinases, and hemicellulases are also secreted from bacteria like the *Pectobacterium* and *Dickeya* genera necrotrophic soft rot enterobacteria (Davidsson et al., 2013). At first glance, plants would seem defenseless against such savage enzyme cocktails, however, they can counter by producing proteinaceous inhibitors of cell wall degrading enzymes such as the xylanase inhibitors and PG inhibitor proteins (PGIPs; Belien et al., 2006). The cunning strategy of making PGIPs is not to inhibit pectin degradation entirely, but to shift it toward producing longer OGAs that can be sensed as DAMPs (Federici et al., 2006).

THE CHALLENGES OF MONITORING CELL WALL RESPONSES TO PATHOGENS

Even from a concise overview such as provided above, it is clear that analyzing the subtle, complex and often rapid changes that affect cell walls in response to pathogens is a formidable technical challenge. In contrast to proteins and nucleotides, the polysaccharides from which cell walls are primarily made cannot readily be sequenced, synthesized, or expressed. Although cell walls are usually physically tough, they are also typically highly plastic and can undergo rapid modifications in response to specific local conditions, even within cell wall micro-domains. The analytical difficulties are compounded by the sheer complexity and multilayered nature of host/pathogen interactions, and the differing nature of these interactions depending on developmental stage, organ, or tissue. Conventional biochemical techniques for carbohydrate analysis, for example methylation analysis and ion exchange chromatography, are powerful but low throughput and usually require large amounts of material. They also involve the complete or partial destruction of cell walls into their component parts – which inevitably means that information about three dimensional cell wall architectures is lost. Molecular probes, for example monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) have been invaluable for advancing our understanding of plant cell walls *per se*, and have immense potential for providing insight into host pathogen interactions at the cellular and subcellular levels. There is an extensive repertoire of probes directed against plant cell wall components (Lee et al., 2011), but this is not matched by probes for pathogens and their associated effectors. For example, our understanding of the precise role of chitosan (the de-acetylated form of chitin) during pathogenesis is hampered by a lack of suitable probes (Sorlier et al., 2003; Hoell et al., 2010; Baker et al., 2011).

One technique that appears to offer considerable promise for plant/pathogen interaction research is based on carbohydrate microarrays (Moller et al., 2007). This technique combines the specificity of mAbs and CBMs with the multiplexed analysis capacity of robotically produced microarrays, and simultaneously provides information about the relative abundance of multiple epitopes present in large sample sets (Tyler et al., 2014). This approach has been extensively used for cell wall analysis in numerous contexts (Tronchet et al., 2010; Sørensen et al., 2011; Fangel et al., 2012; Moore et al., 2014; Zhang et al., 2014), but has only been used in a very limited way for plant defense research (Nguema-Ona et al., 2013). Typically, at least 20 mAbs or CBMs are used in a single analysis, and several 100 samples can be processed in 2 days. Analysis starts with the preparation of cell wall material (although native plant material can also be used) which is then treated with a series of solvents to release cell wall components. For example, extraction with 1,2-diaminocyclohexanetetraacetic acid followed by sodium hydroxide is expected to release pectins and hemicellulose respectively. These extractions are then printed onto a suitable surface such as nitrocellulose membrane or modified glass slides using a microarray robot, and the resultant arrays probed with mAbs and/or CBMs and then quantified. The fact that relatively small amounts of cell wall material are needed (0.5–10 mg) and the ability to rapidly analyze large numbers of samples are attractive attributes in the context of plant/pathogen interaction research. Currently, the analysis of cell walls in the context of plant defense is often limited to polymers with well characterized roles, for example extensins and callose. The described technology can potentially provide the means to greatly increase the scope of cell wall analysis, both in terms of the number of cell wall components analyzed and the number of variables in an experimental set up. Our initial experiments indicate that glycan arrays are powerful tools for monitoring the progression of cell wall changes during defense responses and are well suited to the parallel analysis of, for example, multiple mutants, elicitors, or conditions. Furthermore we anticipate that analysis of cell wall glycomes will be integrated with transcriptomic analysis of defense related genes using the same material. Moreover, although the technique has mostly been applied to carbohydrate analysis, the same approach could be applied to investigate proteins and their conjugates of relevance to pathogenic interactions. So long as analytes can be extracted and immobilized, and providing that probes are available the scope of analysis can be extended.

CONCLUDING REMARKS

Our current knowledge of the interactions between plants and pathogens at the plant cell wall level, and how this affects downstream signaling is still limited. To obtain more insight we need to expand our understanding of the full repertoire of cell wall modifications that occurs during microbial interactions. Carbohydrate microarrays could be used to monitor the complex interplay between microbes and plants, and may reveal additional, perhaps more subtle, cell wall modifications to those documented so far. This technique enables rapid and multiplexed analysis of multiple changes in wall composition and could be used as a tool to provide new insight into the dynamic nature of host/pathogen interactions.

ACKNOWLEDGMENTS

We thank Dr. Milena Roux and Dr. Jacqueline Monaghan for critically reading and commenting on the manuscript.

REFERENCES

Albersheim, P., Darvill, A., Roberts, K., Sederoff, R., and Staehelin, A. (ed.). (2011). *Plant Cell Walls.* New York, NY: Garland Science, Taylor and Francis Publishing Group, LLC, 52–61.

Annis, S. L., and Goodwin, P. H. (1997). Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *Eur. J. Plant Pathol.* 103, 1–14. doi: 10.1023/A:1008656013255

Baker, L. G., Specht, C. A., and Lodge, J. K. (2011). Cell wall chitosan is necessary for virulence in the opportunistic pathogen *Cryptococcus neoformans*. *Eukaryot. Cell* 10, 1264–1268. doi: 10.1128/Ec.05138-11

Beck, M., Heard, W., Mbengue, M., and Robatzek, S. (2012a). The INs and OUTs of pattern recognition receptors at the cell surface. *Curr. Opin. Plant Biol.* 15, 367–374. doi: 10.1016/j.pbi.2012.05.004

Beck, M., Zhou, J., Faulkner, C., Maclean, D., and Robatzek, S. (2012b). Spatio-temporal cellular dynamics of the *Arabidopsis* flagellin receptor reveal activation status-dependent endosomal sorting. *Plant Cell* 24, 4205–4219. doi: 10.1105/tpc.112.100263

Belien, T., Van Campenhout, S., Robben, J., and Volckaert, G. (2006). Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? *Mol. Plant Microbe Interact.* 19, 1072–1081. doi: 10.1094/Mpmi-19-1072

Bethke, G., Grundman, R. E., Sreekanta, S., Truman, W., Katagiri, F., and Glazebrook, J. (2014). *Arabidopsis* PECTIN METHYLESTERASEs contribute to immunity against *Pseudomonas syringae*. *Plant Physiol.* 164, 1093–1107. doi: 10.1104/pp.113.227637

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Bolton, M. D., Van Esse, H. P., Vossen, J. H., De Jonge, R., Stergiopoulos, I., Stulemeijer, I. J. E., et al. (2008). The novel *Cladosporium fulvum* lysin motif effector Ecp6 is a virulence factor with orthologues in other fungal species. *Mol. Microbiol.* 69, 119–136. doi: 10.1111/j.1365-2958.2008.06270.x

Boudsocq, M., Willmann, M. R., Mccormack, M., Lee, H., Shan, L., He, P., et al. (2010). Differential innate immune signalling via Ca(2+) sensor protein kinases. *Nature* 464, 418–422. doi: 10.1038/nature08794

Bueter, C. L., Specht, C. A., and Levitz, S. M. (2013). Innate sensing of chitin and chitosan. *PLoS Pathog.* 9:e1003080. doi: 10.1371/journal.ppat.1003080

Cabrera, J. C., Boland, A., Messiaen, J., Cambier, P., and Van Cutsem, P. (2008). Egg box conformation of oligogalacturonides: the time-dependent stabilization of the elicitor-active conformation increases its biological activity. *Glycobiology* 18, 473–482. doi: 10.1093/glycob/cwn027

Cano-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003). Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *Plant J.* 34, 351–362. doi: 10.1046/j.1365-313X.2003.01729.x

Carroll, A., Mansoori, N., Li, S., Lei, L., Vernhettes, S., Visser, R. G., et al. (2012). Complexes with mixed primary and secondary cellulose synthases are functional in *Arabidopsis* plants. *Plant Physiol.* 160, 726–737. doi: 10.1104/pp.112.199208

Chen, X. Y., and Kim, J. Y. (2009). Callose synthesis in higher plants. *Plant Signal. Behav.* 4, 489–492. doi: 10.4161/psb.4.6.8359

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J. D., et al. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448, 497–500. doi: 10.1038/nature05999

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J. M., Simon, A., et al. (2007). *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. *FEMS Microbiol. Lett.* 277, 1–10. doi: 10.1111/j.1574-6968.2007.00930.x

Davidsson, P. R., Kariola, T., Niemi, O., and Palva, E. T. (2013). Pathogenicity of and plant immunity to soft rot pectobacteria. *Front. Plant Sci.* 4:191. doi: 10.3389/fpls.2013.00191

DeFalco, T. A., Bender, K. W., and Snedden, W. A. (2010). Breaking the code: Ca^{2+} sensors in plant signalling. *Biochem. J.* 425, 27–40. doi: 10.1042/BJ20091147

de Jonge, R., Van Esse, H. P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., et al. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science* 329, 953–955. doi: 10.1126/science.1190859

Denness, L., Mckenna, J. F., Segonzac, C., Wormit, A., Madhou, P., Bennett, M., et al. (2011). Cell wall damage-induced lignin biosynthesis is regulated by a reactive oxygen species- and jasmonic acid-dependent process in *Arabidopsis*. *Plant Physiol.* 156, 1364–1374. doi: 10.1104/pp.111.175737

Dicko, M. H., Gruppen, H., Barro, C., Traore, A. S., Van Berkel, W. J., and Voragen, A. G. (2005). Impact of phenolic compounds and related enzymes in sorghum varieties for resistance and susceptibility to biotic and abiotic stresses. *J. Chem. Ecol.* 31, 2671–2688. doi: 10.1007/s10886-005-7619-5

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812 x

Dubiella, U., Seybold, H., Durian, G., Komander, E., Lassig, R., Witte, C. P., et al. (2013). Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8744–8749. doi: 10.1073/pnas.1221294110

Ellinger, D., Naumann, M., Falter, C., Zwikowics, C., Jamrow, T., Manisseri, C., et al. (2013). Elevated early callose deposition results in complete penetration resistance to powdery mildew in *Arabidopsis*. *Plant Physiol.* 161, 1433–1444. doi: 10.1104/pp.112.211011

Ellis, C., and Turner, J. G. (2001). The *Arabidopsis* mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *Plant Cell* 13, 1025–1033. doi: 10.1105/tpc.13.5.1025

Endler, A., and Persson, S. (2011). Cellulose synthases and synthesis in *Arabidopsis*. *Mol. plant* 4, 199–211. doi: 10.1093/mp/ssq079

Fangel, J. U., Ulvskov, P., Knox, J. P., Mikkelsen, M. D., Harholt, J., Popper, Z. A., et al. (2012). Cell wall evolution and diversity. *Front. Plant Sci.* 3:152. doi: 10.3389/fpls.2012.00152

Federici, L., Di Matteo, A., Fernandez-Recio, J., Tsernoglou, D., and Cervone, F. (2006). Polygalacturonase inhibiting proteins: players in plant innate immunity? *Trends Plant Sci.* 11, 65–70. doi: 10.1016/j.tplants.2005.12.005

Ferrari, S., Savatin, D. V., Sicilia, F., Gramegna, G., Cervone, F., and Lorenzo, G. D. (2013). Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Front. Plant Sci.* 4:49. doi: 10.3389/fpls.2013.00049

Fry, S. C. (2004). Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. *New Phytol.* 161, 641–675. doi: 10.1111/j.1469-8137.2003.00980.x

Furman-Matarasso, N., Cohen, E., Du, Q., Chejanovsky, N., Hanania, U., and Avni, A. (1999). A point mutation in the ethylene-inducing xylanase elicitor inhibits the beta-1-4-endoxylanase activity but not the elicitation activity. *Plant Physiol.* 121, 345–351. doi: 10.1104/pp.121.2.345

Furukawa, T., Inagaki, H., Takai, R., Hirai, H., and Che, F. S. (2013). Two distinct EF-Tu epitopes induce immune responses in rice and *Arabidopsis*. *Mol. Plant Microbe Interact.* 27, 113–124. doi: 10.1094/MPMI-10-13-0304-R

Galletti, R., De Lorenzo, G., and Ferrari, S. (2009). Host-derived signals activate plant innate immunity. *Plant Signal. Behav.* 4, 33–34. doi: 10.4161/psb.4.1.7224

Geldner, N., and Robatzek, S. (2008). Plant receptors go endosomal: a moving view on signal transduction. *Plant Physiol.* 147, 1565–1574. doi: 10.1104/pp.108.120287

Gomez-Gomez, L., Felix, G., and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* 18, 277–284. doi: 10.1046/j.1365-313X.1999.00451.x

Hadwiger, L. A. (2013). Multiple effects of chitosan on plant systems: solid science or hype. *Plant Sci.* 208, 42–49. doi: 10.1016/j.plantsci.2013.03.007

Ham, J. H., Kim, M. G., Lee, S. Y., and Mackey, D. (2007). Layered basal defenses underlie non-host resistance of *Arabidopsis* to *Pseudomonas syringae* pv. *phaseolicola*. *Plant J.* 51, 604–616. doi: 10.1111/j.1365-313X.2007.03165.x

Hamann, T. (2012). Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms. *Front. Plant Sci.* 3:77. doi: 10.3389/fpls.2012.00077

Hamann, T., Bennett, M., Mansfield, J., and Somerville, C. (2009). Identification of cell-wall stress as a hexose-dependent and osmosensitive regulator of plant responses. *Plant J.* 57, 1015–1026. doi: 10.1111/j.1365-313X.2008.03744.x

Harris, P. J., and Stone, B. A. (eds). (2009). *Chemistry and Molecular Organization of Plant Cell Walls,* Chap. 4. Oxford: Blackwell Publishing Ltd.

Heese, A., Hann, D. R., Gimenez-Ibanez, S., Jones, A. M., He, K., Li, J., et al. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12217–12222. doi: 10.1073/pnas.0705306104

Hematy, K., Cherk, C., and Somerville, S. (2009). Host-pathogen warfare at the plant cell wall. *Curr. Opin. Plant Biol.* 12, 406–413. doi: 10.1016/j.pbi.2009.06.007

Hematy, K., Sado, P. E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., et al. (2007). A receptor-like kinase mediates the response of *Arabidopsis* cells to the inhibition of cellulose synthesis. *Curr. Biol.* 17, 922–931. doi: 10.1016/j.cub.2007.05.018

Hemsworth, G. R., Henrissat, B., Davies, G. J., and Walton, P. H. (2014). Discovery and characterization of a new family of lytic polysaccharide monooxygenases. *Nat. Chem. Biol.* 10, 122–126. doi: 10.1038/nchembio.1417

Hernandez-Blanco, C., Feng, D. X., Hu, J., Sanchez-Vallet, A., Deslandes, L., Llorente, F., et al. (2007). Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. *Plant Cell* 19, 890–903. doi: 10.1105/tpc.106.048058

Hoell, I. A., Vaaje-Kolstad, G., and Eijsink, V. G. H. (2010). Structure and function of enzymes acting on chitin and chitosan. *Biotechnol. Genet. Eng. Rev.* 27, 331–366. doi: 10.1080/02648725.2010.10648156

Huckelhoven, R. (2007). Cell wall-associated mechanisms of disease resistance and susceptibility. *Annu. Rev. Phytopathol.* 45, 101–127. doi: 10.1146/annurev.phyto.45.062806.094325

Huibers, R. P., Loonen, A. E., Gao, D., Van Den Ackerveken, G., Visser, R. G., and Bai, Y. (2013). Powdery mildew resistance in tomato by impairment of SlPMR4 and SlDMR1. *PLoS ONE* 8:e67467. doi: 10.1371/journal.pone.0067467

Iizasa, E., Mitsutomi, M., and Nagano, Y. (2010). Direct binding of a plant LysM receptor-like kinase, LysM RLK1/CERK1, to chitin in vitro. *J. Biol. Chem.* 285, 2996–3004. doi: 10.1074/jbc.M109.027540

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jones, L., Ennos, A. R., and Turner, S. R. (2001). Cloning and characterization of irregular xylem4 (irx4): a severely lignin-deficient mutant of *Arabidopsis. Plant J.* 26, 205–216. doi: 10.1046/j.1365-313x.2001.01021.x

Kimura, S., Kaya, H., Kawarazaki, T., Hiraoka, G., Senzaki, E., Michikawa, M., et al. (2012). Protein phosphorylation is a prerequisite for the Ca^{2+}-dependent activation of *Arabidopsis* NADPH oxidases and may function as a trigger for the positive feedback regulation of Ca^{2+} and reactive oxygen species. *Biochim. Biophys. Acta* 1823, 398–405. doi: 10.1016/j.bbamcr.2011.09.011

Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K., et al. (2007). Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. *Plant Cell* 19, 1065–1080. doi: 10.1105/tpc.106.048884

Kombrink, A., and Thomma, B. P. (2013). LysM effectors: secreted proteins supporting fungal life. *PLoS Pathog.* 9:e1003769. doi: 10.1371/journal.ppat.1003769

Krol, E., Mentzel, T., Chinchilla, D., Boller, T., Felix, G., Kemmerling, B., et al. (2010). Perception of the *Arabidopsis* danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. *J. Biol. Chem.* 285, 13471–13479. doi: 10.1074/jbc.M109.097394

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell* 16, 3496–3507. doi: 10.1105/tpc.104.026765

Lacombe, S., Rougon-Cardoso, A., Sherwood, E., Peeters, N., Dahlbeck, D., Van Esse, H. P., et al. (2010). Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. *Nat. Biotechnol.* 28, 365–369. doi: 10.1038/nbt.1613

Lee, K. J. D., Marcus, S. E., and Knox, J. P. (2011). Cell wall biology: perspectives from cell wall imaging. *Mol. Plant* 4, 212–219. doi: 10.1093/Mp/Ssq075

Li, J., Wen, J., Lease, K. A., Doke, J. T., Tax, F. E., and Walker, J. C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* 110, 213–222. doi: 10.1016/S0092-8674(02)00812-7

Lionetti, V., Cervone, F., and Bellincampi, D. (2012). Methyl esterification of pectin plays a role during plant-pathogen interactions and affects plant resistance to diseases. *Plant Physiol.* 169, 1623–1630. doi: 10.1016/j.jplph.2012.05.006

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., et al. (2012). Chitin-induced dimerization activates a plant immune receptor. *Science* 336, 1160–1164. doi: 10.1126/science.1218867

Liu, Z., Wu, Y., Yang, F., Zhang, Y., Chen, S., Xie, Q., et al. (2013). BIK1 interacts with PEPRs to mediate ethylene-induced immunity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6205–6210. doi: 10.1073/pnas.1215543110

Lozovaya, V. V., Lygin, A. V., Zernova, O. V., Ulanov, A. V., Li, S. X., Hartman, G. L., et al. (2007). Modification of phenolic metabolism in soybean hairy roots through down regulation of chalcone synthase or isoflavone synthase. *Planta* 225, 665–679. doi: 10.1007/s00425-006-0368-z

Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., et al. (2011). Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science* 332, 1439–1442. doi: 10.1126/science.1204903

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., and He, P. (2010). A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 496–501. doi: 10.1073/pnas.0909705107

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. (2011). Callose deposition: a multifaceted plant defense response. *Mol. Plant Microbe Interact.* 24, 183–193. doi: 10.1094/MPMI-07-10-0149

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., et al. (2011). Loss-of-function mutation of REDUCED WALL ACETYLATION2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea. Plant Physiol.* 155, 1068–1078. doi: 10.1104/pp.110.168989

Martone, P. T., Estevez, J. M., Lu, F. C., Ruel, K., Denny, M. W., Somerville, C., et al. (2009). Discovery of lignin in seaweed reveals convergent evolution of cell-wall architecture. *Curr. Biol.* 19, 169–175. doi: 10.1016/j.cub.2008.12.031

Medzhitov, R., and Janeway, C. A. Jr. (1997). Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol* 9, 4–9. doi: 10.1016/S0952-7915(97)80152-5

Moller, I., Sorensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Obro, J., et al. (2007). High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *Plant J.* 50, 1118–1128. doi: 10.1111/j.1365-313X.2007.03114.x

Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Curr. Opin. Plant Biol.* 15, 349–357. doi: 10.1016/j.pbi.2012.05.006

Moore, J. P., Nguema-Ona, E., Fangel, J. U., Willats, W. G. T., Hugo, A., and Vivier, M. A. (2014). Profiling the main cell wall polysaccharides of grapevine leaves using high-throughput and fractionation methods. *Carbohydr. Polym.* 99, 190–198. doi: 10.1016/j.carbpol.2013.08.013

Nam, K. H., and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* 110, 203–212. doi: 10.1016/S0092-8674(02)00814-0

Naumann, M., Somerville, S., and Voigt, C. (2013). Differences in early callose deposition during adapted and non-adapted powdery mildew infection of resistant *Arabidopsis* lines. *Plant Signal. Behav.* 8:e24408. doi: 10.4161/psb.24408

Nguema-Ona, E., Moore, J. P., Fagerstrom, A. D., Fangel, J. U., Willats, W. G. T., Hugo, A., et al. (2013). Overexpression of the grapevine PGIP1 in tobacco results in compositional changes in the leaf arabinoxyloglucan network in the absence of fungal infection. *BMC Plant Biol.* 13:46. doi: 10.1186/1471-2229-13-46

Mcneil, M., Darvill, A. G., Fry, S. C., and Albersheim, P. (1984). Structure and function of the primary-cell walls of plants. *Annu. Rev. Biochem.* 53, 625–663. doi: 10.1146/annurev.bi.53.070184.003205

Nishimura, M. T., Stein, M., Hou, B. H., Vogel, J. P., Edwards, H., and Somerville, S. C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* 301, 969–972. doi: 10.1126/science.1086716

Nuhse, T. S. (2012). Cell wall integrity signaling and innate immunity in plants. *Front. Plant Sci.* 3:280. doi: 10.3389/fpls.2012.00280

Ogasawara, Y., Kaya, H., Hiraoka, G., Yumoto, F., Kimura, S., Kadota, Y., et al. (2008). Synergistic activation of the *Arabidopsis* NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. *J. Biol. Chem.* 283, 8885–8892. doi: 10.1074/jbc.M708106200

Pan, Q. H., Zhan, J. C., Liu, H. T., Zhang, J. H., Chen, J. Y., Wen, P. F., et al. (2006). Salicylic acid synthesized by benzoic acid 2-hydroxylase participates in the development of thermotolerance in pea plants. *Plant Sci.* 171, 226–233. doi: 10.1016/j.plantsci.2006.03.012

Pauly, M., Gille, S., Liu, L. F., Mansoori, N., De Souza, A., Schultink, A., et al. (2013). Hemicellulose biosynthesis. *Planta* 238, 627–642. doi: 10.1007/s00425-013-1921-1

Petutschnig, E. K., Jones, A. M. E., Serazetdinova, L., Lipka, U., and Lipka, V. (2010). The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. *J. Biol. Chem.* 285, 28902–28911. doi: 10.1074/jbc.M110.116657

Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch, W., Grima-Pettenati, J., et al. (1998). Down-regulation of cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. *Plant J.* 13, 71–83. doi: 10.1046/j.1365-313X.1998.00014.x

Pogorelko, G., Lionetti, V., Fursova, O., Sundaram, R. M., Qi, M. S., Whitham, S. A., et al. (2013). *Arabidopsis* and *Brachypodium distachyon* transgenic plants expressing *Aspergillus nidulans* acetylesterases have decreased degree of polysaccharide acetylation and increased resistance to pathogens. *Plant Physiol.* 162, 9–23. doi: 10.1104/pp.113.214460

Raiborg, C., and Stenmark, H. (2009). The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* 458, 445–452. doi: 10.1038/nature07961

Rasmussen, M. W., Roux, M., Petersen, M., and Mundy, J. (2012). MAP kinase cascades in *Arabidopsis* innate immunity. *Front. Plant Sci.* 3:169. doi: 10.3389/fpls.2012.00169

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. *Genes Dev.* 20, 537–542. doi: 10.1101/gad.366506

Ron, M., and Avni, A. (2004). The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell* 16, 1604–1615. doi: 10.1105/Tpc.022475

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., et al. (2011). The *Arabidopsis* leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell* 23, 2440–2455. doi: 10.1105/tpc.111.084301

Ruuhola, T., Julkunen-Tiitto, R., and Vainiotalo, P. (2003). In vitro degradation of willow salicylates. *J. chem. ecol.* 29, 1083–1097.

Sanchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D. J., Thomma, B. P., et al. (2013). Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *Elife* 2:e00790. doi: 10.7554/eLife.00790

Sarkar, P., Bosneaga, E., and Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *J. Exp. Bot.* 60, 3615–3635. doi: 10.1093/Jxb/Erp245

Sattler, S. E., and Funnell-Harris, D. L. (2013). Modifying lignin to improve bioenergy feedstocks: strengthening the barrier against pathogens? *Front. Plant Sci.* 4:70. doi: 10.3389/Fpls.2013.00070

Scheller, H. V., and Ulvskov, P. (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289. doi: 10.1146/annurev-arplant-042809-112315

Schulze, B., Mentzel, T., Jehle, A. K., Mueller, K., Beeler, S., Boller, T., et al. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *J. Biol. Chem.* 285, 9444–9451. doi: 10.1074/jbc.M109.096842

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., et al. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* 64, 204–214. doi: 10.1111/j.1365-313X.2010.04324.x

Shinya, T., Motoyama, N., Ikeda, A., Wada, M., Kamiya, K., Hayafune, M., et al. (2012). Functional characterization of CEBiP and CERK1 homologs in *Arabidopsis* and rice reveals the presence of different chitin receptor systems in plants. *Plant Cell Physiol.* 53, 1696–1706. doi: 10.1093/pcp/pcs113

Smith, J. M., Salamango, D. J., Leslie, M. E., Collins, C. A., and Heese, A. (2014). Sensitivity to Flg22 is modulated by ligand-induced degradation and de novo synthesis of the endogenous flagellin-receptor FLAGELLIN-SENSING2. *Plant Physiol.* 164, 440–454. doi: 10.1104/pp.113.229179

Smith, M. M., and Hartley, R. D. (1983). Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. *Carbohydr. Res.* 118, 65–80. doi: 10.1016/0008-6215(83)88036-7

Sørensen, I., Domozych, D., and Willats, W. G. T. (2010). How have plant cell walls evolved? *Plant Physiol.* 153, 366–372. doi: 10.1104/pp.110.154427

Sørensen, I., Pettolino, F. A., Bacic, A., Ralph, J., Lu, F. C., O'Neill, M. A., et al. (2011). The charophycean green algae provide insights into the early origins of plant cell walls. *Plant J.* 68, 201–211. doi: 10.1111/j.1365-313X.2011.04686.x

Sorlier, P., Hartmann, D. J., Denuziere, A., Viton, C., and Domard, A. (2003). Preparation and development of anti-chitosan antibodies. *J. Biomed. Mater. Res. A* 67, 766–774. doi: 10.1002/Jbm.A.10132

Spallek, T., Beck, M., Ben Khaled, S., Salomon, S., Bourdais, G., Schellmann, S., et al. (2013). ESCRT-I mediates FLS2 endosomal sorting and plant immunity. *PLoS Genet.* 9:e1004035. doi: 10.1371/journal.pgen.1004035

Sun, Y., Li, L., Macho, A. P., Han, Z., Hu, Z., Zipfel, C., et al. (2013). Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. *Science* 342, 624–628. doi: 10.1126/science.1243825

Takken, F. L., Thomas, C. M., Joosten, M. H., Golstein, C., Westerink, N., Hille, J., et al. (1999). A second gene at the tomato Cf-4 locus confers resistance to *Cladosporium fulvum* through recognition of a novel avirulence determinant. *Plant J.* 20, 279–288. doi: 10.1046/j.1365-313X.1999.00601.x

Tanner, G. R., and Morrison, I. M. (1983). Phenolic-carbohydrate complexes in the cell-walls of *Lolium perenne*. *Phytochemistry* 22, 1433–1439. doi: 10.1016/S0031-9422(00)84030-3

Thomas, C. M., Jones, D. A., Parniske, M., Harrison, K., Balint-Kurti, P. J., Hatzixanthis, K., et al. (1997). Characterization of the tomato Cf-4 gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. *Plant Cell* 9, 2209–2224. doi: 10.1105/tpc.9.12.2209

Torres, M. A., Dangl, J. L., and Jones, J. D. (2002). *Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl. Acad. Sci. U.S.A.* 99, 517–522. doi: 10.1073/pnas.012452499

Tronchet, M., Balague, C., Kroj, T., Jouanin, L., and Roby, D. (2010). Cinnamyl alcohol dehydrogenases-C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in *Arabidopsis*. *Mol. Plant Pathol.* 11, 83–92. doi: 10.1111/j.1364-3703.2009.00578.X

Tyler, L., Fangel, J. U., Fagerstrom, A. D., Steinwand, M. A., Raab, T. K., Willats, W. G., et al. (2014). Selection and phenotypic characterization of a core collection of *Brachypodium distachyon* inbred lines. *BMC Plant Biol.* 14:25. doi: 10.1186/1471-2229-14-25

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Front. Plant Sci.* 3:85. doi: 10.3389/fpls.2012.00085

Vaaje-Kolstad, G., Westereng, B., Horn, S. J., Liu, Z., Zhai, H., Sorlie, M., et al. (2010). An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* 330, 219–222. doi: 10.1126/science.1192231

van den Burg, H. A., Harrison, S. J., Joosten, M. H., Vervoort, J., and De Wit, P. J. (2006). *Cladosporium fulvum* Avr4 protects fungal cell walls against hydrolysis by plant chitinases accumulating during infection. *Mol. Plant Microbe Interact.* 19, 1420–1430. doi: 10.1094/MPMI-19-1420

van der Hoorn, R. A., and Jones, J. D. (2004). The plant proteolytic machinery and its role in defence. *Curr. Opin. Plant Biol.* 7, 400–407. doi: 10.1016/j.pbi.2004.04.003

van Esse, H. P., Bolton, M. D., Stergiopoulos, I., de Wit, P. J., and Thomma, B. P. (2007). The chitin-binding *Cladosporium fulvum* effector protein Avr4 is a virulence factor. *Mol. Plant Microbe Interact.* 20, 1092–1101. doi: 10.1094/Mpmi-20-9-1092

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., and Boerjan, W. (2010). Lignin biosynthesis and structure. *Plant Physiol.* 153, 895–905. doi: 10.1104/pp.110.155119

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. C. (2002). PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis*. *Plant Cell* 14, 2095–2106. doi: 10.1105/tpc.003509

Vogel, J., and Somerville, S. (2000). Isolation and characterization of powdery mildew-resistant *Arabidopsis* mutants. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1897–1902. doi: 10.1073/pnas.030531997

Weiergang, I., Hipskind, J. D., and Nicholson, R. L. (1996). Synthesis of 3-deoxyanthocyanidin phytoalexins in sorghum occurs independent of light. *Physiol. Mol. Plant Pathol.* 49, 377–388. doi: 10.1006/pmpp.1996.0060

Wolf, S., Mouille, G., and Pelloux, J. (2009). Homogalacturonan methyl-esterification and plant development. *Mol. Plant.* 2, 851–860. doi: 10.1093/mp/ssp066

Wurzinger, B., Mair, A., Pfister, B., and Teige, M. (2011). Cross-talk of calcium-dependent protein kinase and MAP kinase signaling. *Plant Signal. Behav.* 6, 8–12. doi: 10.4161/psb.6.1.14012

Xu, J., Xie, J., Yan, C., Zou, X., Ren, D., and Zhang, S. (2014). A chemical genetic approach demonstrates that MPK3/MPK6 activation and NADPH oxidase-mediated oxidative burst are two independent signaling events in plant immunity. *Plant J.* 77, 222–234. doi: 10.1111/tpj.12382

Yamaguchi, Y., Huffaker, A., Bryan, A. C., Tax, F. E., and Ryan, C. A. (2010). PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in *Arabidopsis*. *Plant Cell* 22, 508–522. doi: 10.1105/tpc.109.068874

Zhang, H., Fangel, J. U., Willats, W. G. T., Selig, M. J., Lindedam, J., Jorgensen, H., et al. (2014). Assessment of leaf/stem ratio in wheat straw feedstock and impact on enzymatic conversion. *Glob. Change Biol. Bioenergy* 6, 90–96. doi: 10.1111/Gcbb.12060

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., et al. (2010). Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. *Cell Host Microbe* 7, 290–301. doi: 10.1016/j.chom.2010.03.007

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 February 2014; accepted: 14 April 2014; published online: 06 May 2014.

*Citation: Malinovsky FG, Fangel JU and Willats WGT (2014) The role of the cell wall in plant immunity. Front. Plant Sci. **5**:178. doi: 10.3389/fpls.2014.00178*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 03 June 2013
doi: 10.3389/fpls.2013.00157

Overexpression of a citrus NDR1 ortholog increases disease resistance in *Arabidopsis*

***Hua Lu*[1]*, *Chong Zhang*[1], *Ute Albrecht*[2], *Rena Shimizu*[1], *Guanfeng Wang*[1] and *Kim D. Bowman*[2]**

[1] *Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD, USA*
[2] *United States Horticultural Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Fort Pierce, FL, USA*

Edited by:
Vincenzo Lionetti, "Sapienza" Università di Roma, Italy

Reviewed by:
Brad Day, Michigan State University, USA
Robin K. Cameron, McMaster University, Canada

***Correspondence:**
Hua Lu, Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250, USA
e-mail: hualu@umbc.edu

Emerging devastating diseases, such as Huanglongbing (HLB) and citrus canker, have caused tremendous losses to the citrus industry worldwide. Genetic engineering is a powerful approach that could allow us to increase citrus resistance against these diseases. The key to the success of this approach relies on a thorough understanding of defense mechanisms of citrus. Studies of *Arabidopsis* and other plants have provided a framework for us to better understand defense mechanisms of citrus. Salicylic acid (SA) is a key signaling molecule involved in basal defense and resistance (R) gene-mediated defense against broad-spectrum pathogens. The *Arabidopsis* gene *NDR1* (NON-RACE-SPECIFIC DISEASE RESISTANCE 1) is a positive regulator of SA accumulation and is specifically required for signaling mediated by a subset of R genes upon recognition of their cognate pathogen effectors. Our bioinformatic analysis identified an ortholog of *NDR1* from citrus, *CsNDR1*. Overexpression of *CsNDR1* complemented susceptibility conferred by the *Arabidopsis ndr1-1* mutant to *Pseudomonas syringae* strains and also led to enhanced resistance to an oomycete pathogen *Hyaloperonospora arabidopsidis*. Such heightened resistance is associated with increased SA production and expression of the defense marker gene *PATHOGENESIS RELATED 1* (*PR1*). In addition, we found that expression of *PR1* and accumulation of SA were induced to modest levels in citrus infected with *Candidatus* Liberibacter asiaticus, the bacterial pathogen associated with HLB disease. Thus, our data suggest that *CsNDR1* is a functional ortholog of *Arabidopsis NDR1*. Since *Ca.* L. asiaticus infection only activates modest levels of defense responses in citrus, we propose that genetically increasing SA/NDR1-mediated pathways could potentially lead to enhanced resistance against HLB, citrus canker, and other destructive diseases challenging global citrus production.

Keywords: *Pseudomonas syringae*, salicylic acid, citrus canker, Huanglongbing, greening disease, *Candidatus* Liberibacter asiaticus, genetic engineering

INTRODUCTION

Huanglongbing (HLB; also called citrus greening disease), citrus canker, and other emerging diseases have imposed serious threats to the citrus industry worldwide (Bove, 2006; Gottwald, 2007). Citrus canker is caused by the gram negative bacterium *Xanthomonas axonopodis* pv. *citri*. Wind and rain facilitate the dispersal of the pathogen and spreading of the disease. More than 16 million citrus trees have been destroyed in Florida in an effort to restrict the disease (Gaskalla, 2006). HLB is even more devastating than citrus canker since it is highly contagious and lethal to afflicted plants (Bove, 2006; Brlansky and Rogers, 2007; Gottwald, 2007). The parasitic bacterium *Candidatus* Liberibacter that lives in the phloem tissue of a citrus tree is believed to be the associating agent of HLB. The disease is transmitted by a small insect vector of the family Psyllidae. The growth of psyllids cannot yet be reliably controlled by conventional insecticide application (Ichinose et al., 2010). Without successful measures to control the causal pathogen and its transmission vector, HLB is endemic to a variety of citrus species and related plants. Therefore, it is imperative to develop strategies to contain and eventually eradicate HLB and other diseases challenging the production of citrus worldwide.

Successful manipulation of citrus disease resistance relies on a thorough understanding of defense mechanisms of the plant. Although not well understood yet in citrus, defense mechanisms are well studied in other plants, in particular the model plant *Arabidopsis thaliana*, and have been shown to be relatively conserved among plants (Nishimura and Dangl, 2010). Therefore, prior knowledge of defense mechanisms from other plants should help us to better understand citrus defense and ultimately develop effective strategies to combat devastating diseases challenging the citrus industry.

Plant defense can be preformed or induced. The preformed defense includes some existing physical structures and chemical compounds produced by plants before infection that can restrict pathogen invasion. The induced defense can be activated upon pathogen invasion, involving sophisticated surveillance systems to recognize general elicitors from pathogens and subsequently to activate basal defense. Much stronger defense can be induced

when host resistance (R) proteins specifically recognize their cognate pathogen effectors; thus such defense is also termed as R protein-mediated defense. The largest class of R proteins is represented by a family of proteins that have two conserved domains, nucleotide binding site (NBS) and leucine-rich repeat (LRR; Martin et al., 2003; McDowell and Simon, 2006). This class of R proteins can be further divided into two groups according to the N-terminal sequence, coiled-coil (CC)–NBS–LRR and Toll-interleukin-1 receptor (TIR)–NBS–LRR. Some CC–NBS–LRR type proteins are found to signal through NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1). For instance, an *ndr1* mutation compromises resistance conferred by the CC–NBS–LRR proteins RPS2, RPM1, or RPS5 to *Pseudomonas syringae* expressing the avirulence effectors *avrRpt2*, *avrB* and *avrRpm1*, or *avrPph3*, respectively (Century et al., 1995; Aarts et al., 1998). On the other hand, some TIR–NBS–LRR type proteins functionally require ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1). For instance, an *eds1* mutant is immune-compromised to *P. syringae avrRps4*, resistance to which is conferred by the TIR–NBS–LRR protein RPS4 but not by TIR–NBS–LRR type R proteins (Aarts et al., 1998; Falk et al., 1999). These observations suggest a general rule that these two subgroups of R proteins can activate distinct downstream signaling pathways. However, exceptions to this rule also exist for some other NBS–LRR R proteins (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001; Xiao et al., 2001).

The small phenolic molecule salicylic acid (SA) plays a key role in signaling both basal and R protein-mediated defense (Hammond-Kosack and Jones, 1996; Tsuda et al., 2008) and is involved in resistance against diverse pathogens and in response to various stress conditions (Malamy et al., 1990; Rassmussen et al., 1991; Sharma and Davis, 1997; Tsuda et al., 2008). While increased SA accumulation and/or signaling lead to enhanced disease resistance, disrupting these processes by gene mutations or transgene expression result in compromised defense against pathogens (Durrant and Dong, 2004; Lu, 2009). Genes involved in SA-mediated defense can affect SA biosynthesis, accumulation, and/or signaling (Lu, 2009). For instance, *SA INDUCTION-DEFICIENT 2*/*EDS16*, encodes isochorismate synthase contributing to the majority of SA biosynthesis (Wildermuth et al., 2001). Both *NDR1* and *EDS1* are known to act upstream of SA to regulate SA accumulation (Falk et al., 1999; Shapiro and Zhang, 2001). Downstream of SA signaling, *NONEXPRESSOR OF PR GENES 1* (*NPR1*) acts as a signal transducer that regulates systemic acquired resistance, a long-lasting defense against broad-spectrum pathogens at the whole plant level (Cao et al., 1997; Ryals et al., 1997; Shah et al., 1997; Dong, 2004). Overexpression of *NDR1*, *EDS1*, *NPR1*, or several other SA regulators confers enhanced disease resistance to a range of pathogens in *Arabidopsis* and/or in other plants (Chern et al., 2001; Fitzgerald et al., 2004; Lin et al., 2004; Makandar et al., 2006; Malnoy et al., 2007; Pegadaraju et al., 2007; Sandhu et al., 2009; Gao et al., 2010). Therefore, manipulation of SA-mediated defense has the potential to introduce broad-spectrum disease resistance in plants.

NDR1 encodes a glycosyl-phosphatidyl inositol-anchored plasma membrane protein that belongs to a large protein family (Dormann et al., 1995; Varet et al., 2002; Coppinger et al., 2004; Zheng et al., 2004). A recent study implicates the function of *NDR1* in mediating plasma membrane-cell wall adhesion (Knepper et al., 2011). *NDR1*-like genes widely exist in different plants (Lee et al., 2006; Chong et al., 2008; Cacas et al., 2011). Besides *NDR1*, some *Arabidopsis* homologs of *NDR1* were shown to be highly induced by pathogen infection and/or to confer enhanced disease resistance to *P. syringae* when overexpressed (Varet et al., 2002, 2003; Coppinger et al., 2004; Zheng et al., 2004). Thus *NDR1* and some members in the family are critical components of plant defense.

In this study, we report the isolation and characterization of a functional ortholog of *NDR1* in citrus, named *CsNDR1*. We found that overexpression of *CsNDR1* complements the susceptibility of *Arabidopsis ndr1-1* mutant to *P. syringae avrRpt2* and further confers enhanced disease resistance to *P. syringae avrRps4*, which normally is not affected by the endogenous *NDR1*. Overexpression of *CsNDR1* also led to increased resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) isolate Noco2. *CsNDR1*-induced disease resistance is associated with increased SA accumulation and expression of the defense marker gene *PATHOGENESIS RELATED 1* (*PR1*) in the transgenic *Arabidopsis* plants. In addition, we found that citrus infected with *Candidatus* Liberibacter asiaticus, a pathogen associated with the HLB disease, expressed modestly increased *CsNDR1* and SA levels, compared with mock-treated plants. We propose that genetic engineering to enhance SA/NDR1 signaling pathway in citrus could potentially enhance its resistance to HLB, citrus canker, and other emerging diseases.

MATERIALS AND METHODS

PLANT MATERIALS

Arabidopsis plants were grown in growth chambers with a 12 h light/12 h dark cycle, light intensity at 200 μmol m^{-2} s^{-1}, 60% humidity, and 22°C. The *ndr1-1* mutant was previously described (Century et al., 1995, 1997). Citrus plants, "Valencia" (*Citrus sinensis* [L.] Osbeck), were grown on the greenhouse bench and kept at 24°C under natural light conditions. Plants were irrigated as needed and fertilized every 3 weeks using a water-soluble fertilizer mix, 20N–10P–20K (Peters Professional, The Scotts Company, Marysville, OH, USA).

BIOINFORMATIC ANALYSIS

Basic Local Alignment Search Tools (BLAST) was used to search protein sequence databases for *Arabidopsis*[1] and *Citrus sinensis*[2], using appropriate query sequences. Sequence alignment and phylogenetic analysis were performed with the MEGA program (version 5.05). To construct the phylogenetic tree, the neighbor-joining method with 1000 bootstrap replications was used.

PATHOGEN INFECTIONS

Pseudomonas syringae strains used in this study were previously described (Lee et al., 2008; Wang et al., 2011b). Bacteria were cultured at 28°C with King's B medium (10 g proteose peptone, 1.5 g K_2HPO_4, 3.2 ml 1 M $MgSO_4$, and 5 g glycerol/l) containing the appropriate antibiotics. Freshly cultured bacteria at the optical

[1] http://blast.ncbi.nlm.nih.gov/Blast.cgi

[2] http://www.phytozome.net/search.php?method=Org_Csinensis

density of 0.5–0.8 were harvested, washed once, and resuspended in 10 mM $MgSO_4$ to make the infection solution at the desired concentrations. The fifth to seventh leaves of 25-day-old *Arabidopsis* plants were infected by infiltration with a 1-ml needleless syringe. For bacterial growth assay, six leaves selected from over 10 plants of each genotype were collected 3 days post-infiltration, bored with a core borer (6 mm in diameter), and ground for bacterial growth measurement as described previously (Lu et al., 2003). For hypersensitive response (HR) test, one-half of a leaf at the fifth, sixth, or seventh position was infiltrated with *P. syringae* pv. *maculicola* ES4326 *avrRpt2* (*Pma avrRpt2*; $OD_{600} = 0.1$) and scored 16–24 h post-infiltration for leaf collapse. Leaves infiltrated with 10 mM $MgSO_4$ or the isogenic virulent strain *Pma* ($OD_{600} = 0.1$) were used as controls. At least 16 leaves from different plants of each genotype were scored for the HR symptoms. The rate of HR was expressed by the percentile of the number of leaves that developed HR symptoms out of the total number of inoculated leaves.

Hyaloperonospora arabidopsidis isolate Noco2 was a kind gift from S. Xiao at University of Maryland College Park. Strain propagation and preparation were conducted as previously described (Song et al., 2004; McDowell et al., 2010). *Hpa Noco2* spores (5×10^4 spores/ml in water) were sprayed on 7-day-old soil-grown seedlings. Sporangiophores on both sides of cotyledons were counted 7 days post-inoculation. At least 50 cotyledons from each genotype were counted to derive the average number of sporangiophores per genotype.

For *Ca.* L. asiaticus infection, 15-month-old "Valencia" plants were inoculated by grafting with two bark- or bud-pieces and two leaf pieces from infected greenhouse-grown "Valencia" plants, which were tested PCR-positive for *Ca.* L. asiaticus and demonstrated symptoms for HLB. Plants similarly inoculated but with disease-free tissue pieces obtained from healthy greenhouse-grown "Valencia" plants were used as controls. Plants were pruned immediately after graft-inoculation to promote new leaf growth and HLB disease development. The inoculated plants were randomized periodically on the greenhouse bench to minimize the effect of environment on their defense responses to *Ca.* L. asiaticus.

PCR-DETECTION OF *Ca.* L. asiaticus IN CITRUS

Ca. L. asiaticus-infected "Valencia" plants began to show typical HLB symptoms, yellowing and blotchy mottling around 11 weeks after inoculation (wai), which progressed more severely later. The symptomatic leaves were collected at 11 and 16 wai and extracted for DNA followed by PCR-detection of *Ca.* L. asiaticus (Albrecht and Bowman, 2012). Specifically, 100 mg leaf tissue was ground for DNA extraction, using the Plant DNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For detection of *Ca.* L. asiaticus, real-time PCR assays were performed using primers HLBas and HLBr and the probe HLBp as described (Li et al., 2006). Amplifications were performed over 40 cycles using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the QuantiTect Probe PCR Kit (Qiagen) according to the manufacturer's instructions. All reactions were carried out in a 20-μl reaction volume using 5 μl DNA. Once a branch was confirmed positive for *Ca.* L. asiaticus, symptomatic leaves on the branch were harvested for further RNA and SA analyses.

RNA EXTRACTION AND ANALYSIS

Twenty-five-day-old *Arabidopsis* plants were harvested for RNA extraction and northern blotting as described (Ng et al., 2011). Radioactive probes were made by PCR with an antisense primer specific for a gene fragment in the presence of [32P] dCTP. Primers used for making the *CsNDR1* probe were CsNDR1-F1 (ATGTCAGAAAACGCCGGTG) and CsNDR1-R1 (TTAAGCAAAAATCAAGACAAAAAAATAC). Primers for the *PR1* and *18S rRNA* probes were described previously (Ng et al., 2011).

Fully expanded leaves from *Ca.* L. asiaticus-infected "Valencia" plants showing HLB symptoms were collected 16 wai. One gram leaf tissue was ground in liquid nitrogen with a mortar and pestle and resuspended in 10 ml guanidinium isothiocyanate buffer (Chomczynski and Sacci, 1987). Total RNA was extracted as previously described (Strommer et al., 1993) with slight modifications. Phenol/chloroform/isoamyl alcohol (25:24:1) extraction was followed by two extractions with chloroform/isoamyl alcohol and precipitation of RNA with isopropanol at −20°C overnight. RNA was pelleted by centrifugation at 10,000 *g* and 4°C for 1 h, resuspended in 5 ml water and precipitated overnight at 0°C with an equal volume of 8 M LiCl. After centrifugation at 10,000 *g* and 4°C for 1 h, RNA was washed twice with 70% ethanol, air-dried, and dissolved in 500 μl of water. RNA was further purified, using the RNeasy® MinElute Cleanup kit (Qiagen) according to the manufacturer's instructions. Total RNA was DNase-treated using the TURBO DNA-*free*-Kit™ (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Quantitative reverse transcription real-time PCR (qRT-PCR) was performed using an ABI 7500 real-time PCR system (Applied Biosystems) and the QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Sixty nanograms of DNase-treated RNA were used in a total volume of 20 μl. For detection of *CsNDR1* transcripts, forward primer 5′-TGCTGCAGCTTCATCTTCAC-3′ and reverse primer 5′-TGTCGTGTTGTTTCGGTTGT-3′ were used. For detection of *18S rRNA* transcripts, forward primer 5′-GCTTAGGCCAAGGAAGTTTG-3′ and reverse primer 5′-TCTATCCCCATCACGATGAA-3′ were used. Melting curve analysis was performed to ensure amplification of a single product and the absence of primer-dimers. For relative quantification of gene expression, the $2^{-\Delta\Delta CT}$ method was applied as previously described (Livak and Schmittgen, 2001), using cycle threshold (Ct) values of *18S rRNA* for normalization.

cDNA AMPLIFICATION, DNA CONSTRUCTION, AND PLANT TRANSFORMATION

To obtain the full-length *CsNDR1* cDNA sequence, we used the SMARTer™ RACE cDNA Amplification Kit (Clontech) to make a cDNA library from RNA extracted from *Ca.* L. asiaticus-infected 15-month-old "Valencia" plants. Nested primers, NDR1-5R-P1 (CACTTTCTGATCGGTCAGCGCAG) and NDR1-5R-P2 (CAATCACGGACGTGCCGATG), were used to amplify the 5′ end missing sequence while NDR1-3R-P1 (CATCGGCACGTCCGTGATTG) and NDR1-3R-P2 (CTGCGCTGACCGATCAGAAAGTG) were used to amplify the 3′ end missing sequence. The amplified fragments were cloned in the pJET cloning vector, using the CloneJET™ PCR Cloning Kit (Thermo

Scientific), and sequenced to obtain the full-length cDNA sequence. The full-length *CsNDR1* cDNA was further amplified from the library with NDR1-F1 (ATGTCAGAAAACGCCGGTG) and NDR1-R1 (TTAAGCAAAAATCAAGACAAAAAAATAC) and cloned into the *pJET* vector. At least 10 individual colonies were prepared for DNA and analyzed by sequencing. The sequence with fewer polymorphisms, compared with the reference sequence, and a correct open reading frame was used as the template for further cloning into the binary vector *pBINplusARS* under the control of the *CAMV 35s* promoter. The construct was confirmed by sequencing and transferred to *Agrobacterium tumefaciens* for *ndr1-1* transformation, using the floral dipping method (Clough and Bent, 1998). T_0 seeds were selected for T_1 plants on MS plates containing kanamycin, resistance to which was conferred by the binary vector. T_1 transgenic plants were collected for seeds, which were further selected for homozygous T_2 plants.

ION LEAKAGE MEASUREMENT

Leaves of 25-day-old *Arabidopsis* plants were infiltrated with a bacterial suspension *Pma avrRpt2* ($OD_{600} = 0.1$) with a blunt-end syringe, using 10 mM $MgSO_4$ treatment as a control. At 0, 4, and 7 h post-inoculation, five leaf disks, cut with a 6-mm core borer, were collected, washed in de-ionized water, and placed in a 15-ml tube with 5 ml of de-ionized water. Triplicate tubes for each sample were gently shaken for 15 min followed by the measurement of solution conductivity, using an electrical conductivity meter (The London Company, Welwyn International Inc. Cleveland, OH, USA). Each tube was measured three times to derive average conductivity.

SA MEASUREMENT

Free and total SA (glucosylated SA) were extracted from 25-day-old Arabidopsis plants (Ng et al., 2011; Wang et al., 2011a). The same protocol was used to extract SA from leaves of *Ca.* L. asiaticus-positive "Valencia" plants that demonstrated HLB symptoms. SA separation and detection were conducted with a high-performance liquid chromatography (HPLC) instrument as previously described (Ng et al., 2011; Wang et al., 2011a).

RESULTS

IDENTIFICATION OF A CITRUS NDR1 ORTHOLOG

Since NDR1 plays a critical role in *Arabidopsis* defense, we set out to identify the citrus ortholog and investigate its role in defense regulation. In *Arabidopsis*, NDR1 belongs to a large protein family with over 40 members, named NDR1/HIN1-like (NHL) proteins (Dörmanna et al., 2000). BLAST searching of the sequence database of *Citrus sinensis*[3] with the NDR1 protein sequence revealed a citrus protein (CsNDR1; orange1.1g028712m) with the highest similarity to NDR1 (the *E*-value is $2.4e^{-53}$) and three other top hits with *E*-values below 1.0 e^{-5}. We further used CsNDR1 as the query to search the *Arabidopsis* protein database and retrieved sequences of NDR1 and 14 NHL proteins as the top hits. To determine the extent of similarity among these proteins, phylogenetic analysis was conducted, using the MEGA program (version 5.0; Tamura et al., 2011). **Figure 1** shows that CsNDR1 is in the same cluster with NDR1 with 99% bootstrap support. Two other citrus NHL proteins (orange1.1g041808m and orange1.1g08713m) are also in the same cluster with NDR1 but with lower confidence levels in bootstrap support. Thus, bioinformatic analysis suggests that CsNDR1 is an ortholog of NDR1.

ECTOPIC EXPRESSION OF *CsNDR1* COMPLEMENTS *Arabidopsis ndr1-1* MUTANT

To test if *CsNDR1* shares conserved function with its *Arabidopsis* correspondence, we used a genetic complementation approach. The full-length *CsNDR1* cDNA was amplified via RT-PCR from a cDNA library made from *Ca.* L. asiaticus-infected "Valencia" plants and was cloned initially to the pJET vector and then to the binary vector *pBINplusARS* under the control of the CAMV 35s promoter. The *CsNDR1*/*pBINplusARS* construct was used to transform *ndr1-1* via the standard floral dipping method (Clough and Bent, 1998). The presence of the transgene was confirmed by PCR with gene-specific primers. Initial infection of the T_1 transgenic plants with a virulent strain *P. syringae* pv. *maculicola* ES4326 (*Pma*) indicated that some of the transgenic plants were more resistant than *ndr1-1* (data not shown). We further isolated homozygous plants for eight independently transformed lines (*ndr1-1* + *CsNDR1)*. Infection of these plants with *Pma* showed that all lines were more resistant than *ndr1-1* and some were even more resistant than Col-0 (**Figure 2A**). Total RNA was isolated from these plants and northern blotting indicated that the level of disease resistance in some transgenic plants was correlated with the degree of transgene expression (**Figure 2B**). Thus, our results suggest that *CsNDR1* positively regulates *Arabidopsis* defense.

The *Arabidopsis* RPS2 is a CC–NBS–LRR type R protein. When recognizing the avirulent strain *Pma avrRpt2*, RPS2 activates strong defense responses. Such defense activation requires the function of NDR1 and sometimes leads to HR, a rapid programed cell death in the infected region (Aarts et al., 1998). We found that all transgenic plants showed enhanced disease resistance to *Pma avrRpt2* ($OD_{600} = 0.0004$), compared with *ndr1-1* (**Figure 3A**). In addition, the *ndr1-1* mutant showed compromised HR in response to a high dose of *Pma avrRpt2* ($OD_{600} = 0.1$), as indicated by the lack of leaf collapse (**Figure 3B**, top panel; Century et al., 1995). We found that all transgenic plants showed partial to full rescue of HR-defect of *ndr1-1* (**Figure 3B**, bottom panel).

Interestingly, line 15 that showed the highest level of *CsNDR1* expression, had a low frequency of leaf collapse in the HR assay, albeit still more than the *ndr1-1* mutant. We also noticed that this line is smaller than other lines (**Figure 4A**). Thus we suspected that the small leaf size might obscure the HR scoring. To better quantify the HR cell death, we performed ion leakage measurement with this line and another line (#9) that showed medium *CsNDR1* expression (**Figure 2B**). When challenged with *Pma avrRpt2* ($OD_{600} = 0.1$), *ndr1-1* had the lowest level of ion leakage (**Figure 4B**; Zhang et al., 2004), consistent with its HR-deficit. The ion leakage level was highest in line 15 and medium in line 9, compared with Col-0. Together disease resistance and HR assays suggest that *CsNDR1* functions similarly as

[3]http://www.phytozome.net

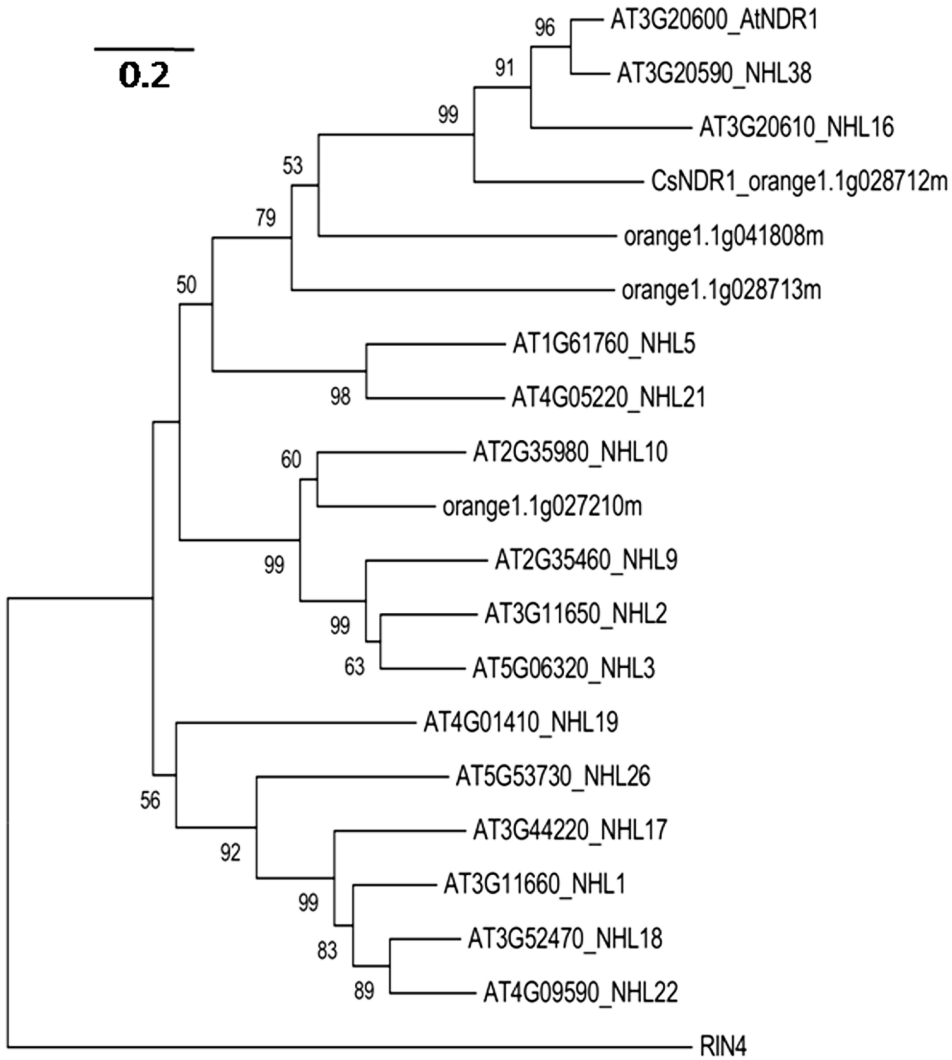


FIGURE 1 | Neighbor-joining phylogenetic tree to show relationship among NDR1 homologs from *Arabidopsis* and citrus. The protein sequences were retrieved from BLAST search of *Citrus sinensis* protein database with NDR1 sequence as a query or from BLAST search of *Arabidopsis* protein database with CsNDR1 sequence as a query, using an *E*-value cutoff e^{-4}. The MEGA program (version 5.05) was used to construct the tree. Protein sequences of the indicated genes were aligned with the ClustalW method and the tree was generated with the neighbor-joining method, using 1000 bootstrap replications. Numbers on the tree indicate bootstrap support (values <50% not shown). Branch lengths were drawn to scale; size bar represents number of amino acid substitutions per site. RIN4 protein sequence was used as an outgroup to root the tree.

Arabidopsis NDR1 in both basal and resistance protein-mediated defense.

ECTOPIC EXPRESSION OF *CsNDR1* LEADS TO ACTIVATION OF SA-MEDIATED DEFENSE AND BROAD-SPECTRUM DISEASE RESISTANCE

Salicylic acid is a key signaling molecule regulating defense pathways including basal defense, R gene-mediated resistance, and systemic acquired resistance (Durrant and Dong, 2004; Lu, 2009). To test whether SA-mediated defense is activated in the transgenic plants, we quantified SA levels. We found that line 15 but not line 9 accumulated much higher levels of both free and total SA (glucosylated SA; **Figure 4C**). Consistent with its high SA levels, line 15 also showed higher expression of the SA marker gene *PR1* (**Figure 4D**). These results suggest that overexpression of *CsNDR1* to a certain level activates SA signaling.

To further investigate how overexpressing *CsNDR1* affects disease resistance, we challenged line 9 and 15 with additional *P. syringae* strains. Ectopic expression of *CsNDR1* complemented susceptibility conferred by *ndr1-1* to the virulent strain *P. syringae* pv. *tomato* DC3000 (*Pto*; **Figure 5A**, left). Line 15 is also more resistant to the isogenic avirulent strain *Pto avrRps4* (**Figure 5A**, right), which is recognized by RPS4 (a TIR–NBS–LRR type of R protein) independently of NDR1 (Aarts et al., 1998). Thus, these results indicate that ectopic expression of *CsNDR1* leads to activation of resistance to a pathogen that the endogenous gene otherwise does not have an effect on. In addition, we found that line 9 and 15 showed increased resistance to the virulent oomycete

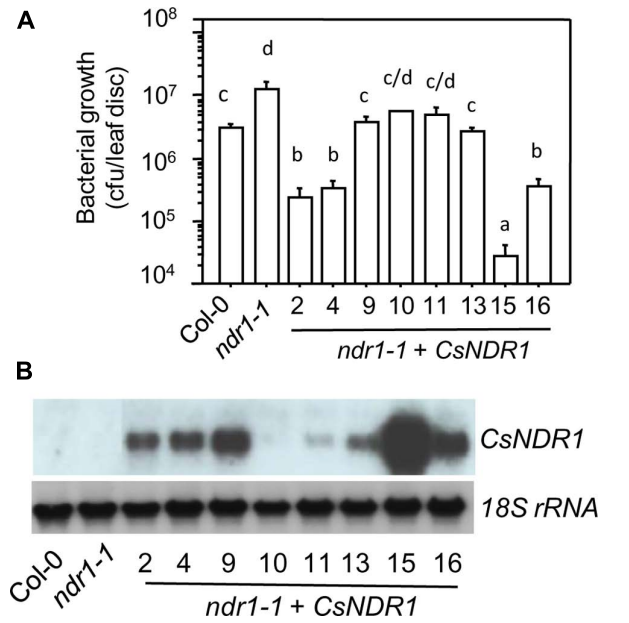


FIGURE 2 | *CsNDR1* rescues enhanced disease susceptibility conferred by *Arabidopsis ndr1-1* to a virulent *P. syringae* strain. (A) Bacterial growth assay. Twenty-five-day-old plants were infected with the virulent strain *P. syringae* pv. *maculicola* ES4326 (*Pma*; $OD_{600} = 0.0001$). Bacterial growth was assessed 3 days after infection. Data represent the average of bacterial numbers in six samples ± standard error. Statistical analysis was performed with log transformed data, using one-way analysis of variance (ANOVA) Fisher's protected least significant difference (PLSD) tests (StatView 5.0.1). Different letters indicate significant difference among the samples ($P < 0.05$). **(B)** Expression analysis of transgenic plants carrying *CsNDR1*. Total RNA was extracted from 25-day-old plants and analyzed by northern blotting. A probe specific to *CsNDR1* was used to detect expression of the transgene. The *18S rRNA* probe was used to indicate equal loading in the samples. These experiments were repeated two times with similar results.

pathogen *Hpa* Noco2, compared with *ndr1-1* (**Figure 5B**). Thus, overexpressing *CsNDR1* could lead to broad-spectrum disease resistance.

SA ACCUMULATION AND EXPRESSION OF *CsNDR1* ARE MODESTLY INDUCED BY *Ca.* L. asiaticus INFECTION

To see how SA signaling is affected by HLB in citrus, we infected 15-month-old "Valencia" plants with *Ca.* L. asiaticus, using mock-treated plants as a control. We began to observe at 11 wai HLB symptoms, chlorosis and/or blotchy mottling of leaves, which increased in severity by 16 wai (**Figure 6A**). We did qPCR with the symptomatic and control leaves, using primers specific to *Ca.* L. asiaticus. The average Ct values of the symptomatic leaves were 21.8 at 11 wai and 20.7 at 16 wai. No *Ca.* L. asiaticus was detected in the control. Thus these symptomatic leaves were confirmed to be *Ca.* L. asiaticus positive. The symptomatic leaves were further collected for SA measurement and RNA analysis. Compared with the mock control, the symptomatic leaves from infected plants had about twofold more total SA levels (**Figure 6B**). Similarly, expression of *CsNDR1* was also induced about twofold more in the infected leaves (**Figure 6C**). These data suggest that SA and/or

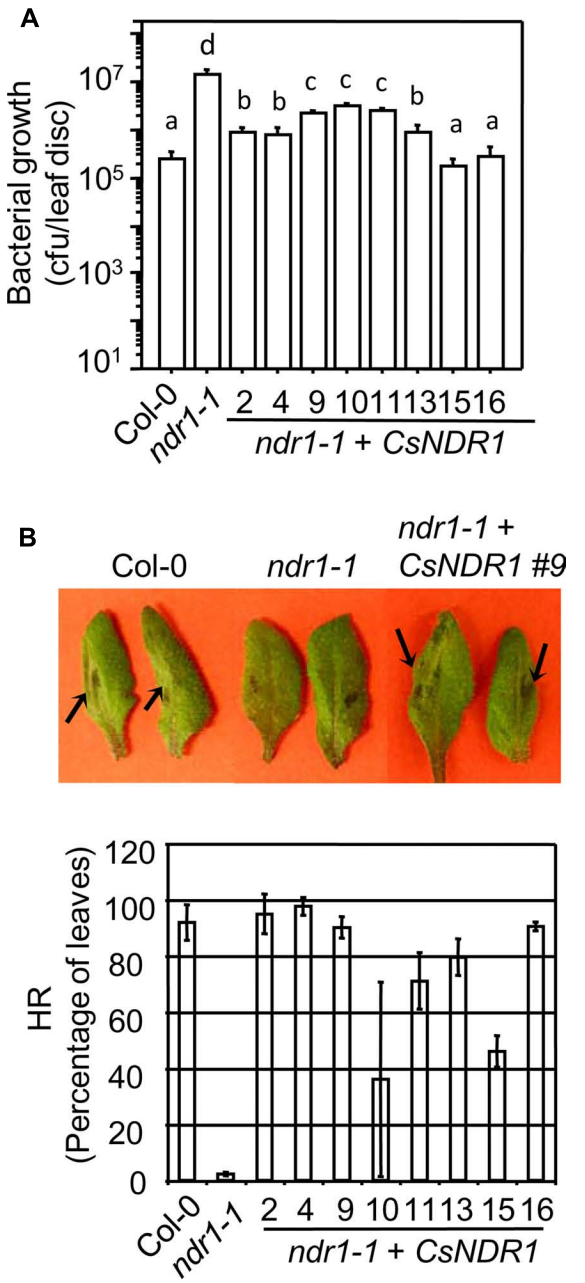


FIGURE 3 | *CsNDR1* rescues enhance disease susceptibility and HR-defect conferred by *ndr1-1* in response to *Pma avrRpt2*. (A) Bacterial growth assay. Twenty-five-day-old plants were infected with *Pma avrRpt2* ($OD_{600} = 0.0004$). Bacterial growth was assessed 3 days after infection. Data represent the average of bacterial numbers in six samples ± standard error. Statistical analysis was performed with log transformed data, using one-way ANOVA Fisher's PLSD tests (StatView 5.0.1). Different letters indicate significant difference among the samples ($P < 0.05$). **(B)** HR test. Half leaves of the fourth to seventh leaves of 25-day-old plants were infiltrated with *Pma avrRpt2* ($OD_{600} = 0.1$) and scored for the HR symptoms 16 h post-infection. At least 16 leaves were infected in each sample. *Top panel*: picture of infected leaves. The arrowheads indicate leaf collapse, a typical HR symptom, in Col-0 and a representative complemented plant (line 9) but not in *ndr1-1*. *Bottom panel*: the percentage of the infected leaves of each genotype that showed HR. These experiments were repeated two times with similar results.

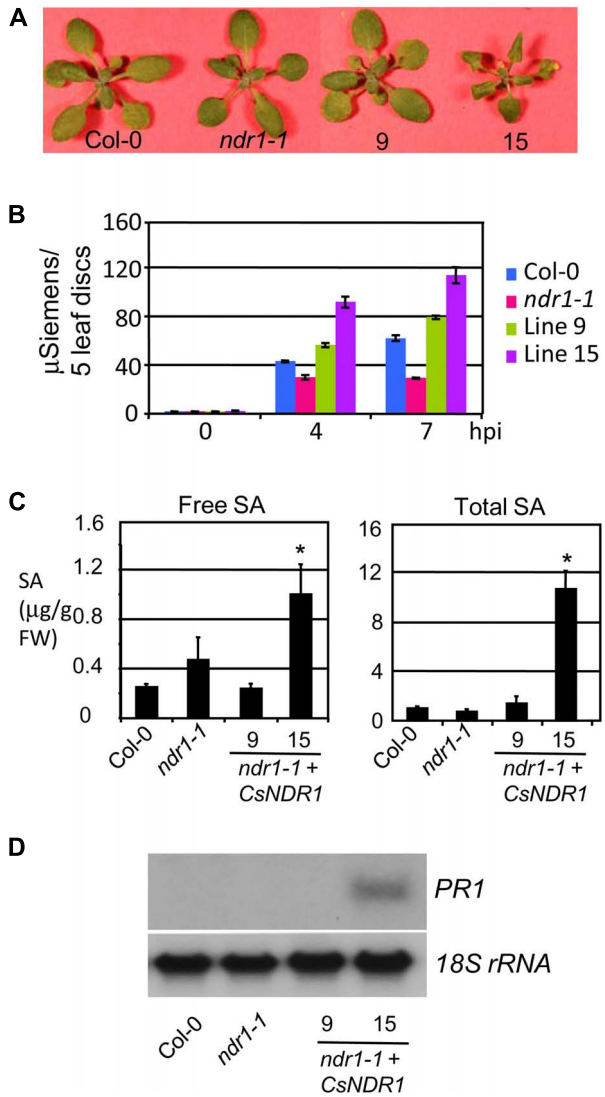


FIGURE 4 | *CsNDR1* overexpression leads to increased ion leakage, SA accumulation, and *PR1* expression. (A) Picture of 25-day-old plants. Other transgenic lines resemble Col-0 (not shown). **(B)** Ion leakage measurement. The fourth to seventh leaves of 25-day-old plants were infiltrated with *Pma avrRpt2* ($OD_{600} = 0.1$) and collected at the indicated times post-infection for ion leakage measurement. The data represent average of triplicate samples, each of which contained five individual leaf disks of 6 mm in diameter. **(C)** SA quantitation. Twenty-five-day-old plants were harvested for SA extraction followed by HPLC analysis. Asterisks indicate significant difference between the samples and Col-0 ($P < 0.05$; $n = 3$). **(D)** Northern blotting. DNA fragments specific to *PR1* and *18s rRNA*, respectively, were used to probe blots containing RNA samples from Col-0, *ndr1-1*, and transgenic lines 9 and 15. These experiments were repeated two times with similar results.

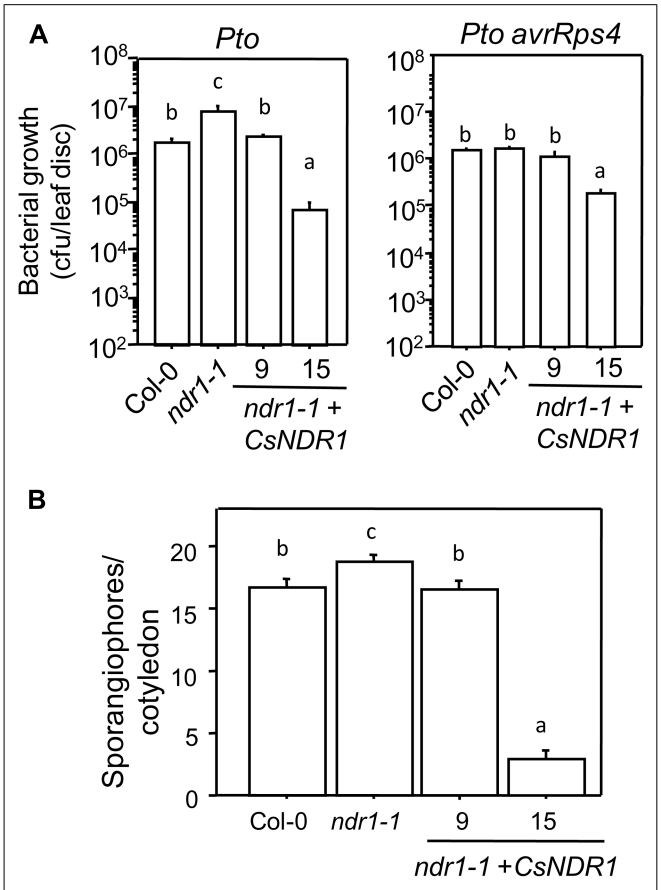


FIGURE 5 | *CsNDR1* confers broad-spectrum disease resistance. (A) Bacterial growth assay. Twenty-five-day-old plants were infected with *Pto* (left panel; $OD_{600} = 0.0002$) or *Pto avrRps4* (right panel; $OD_{600} = 0.0004$). Bacterial growth was assessed 3 days after infection. Data represent the average of bacterial numbers in six samples ± standard error. **(B)** *Hpa* sporangiophore measurement. Seven-day-old seedlings were spray-infected with *Hpa* Noco2 (5×10^4 spores/ml in water). Statistical analysis was performed with one-way ANOVA Fisher's PLSD tests (StatView 5.0.1). Different letters indicate significant difference among the samples ($P < 0.05$). These experiments were repeated twice with similar results.

CsNDR1-mediated signaling are activated modestly upon *Ca.* L. asiaticus infection.

DISCUSSION

In this study, we presented bioinformatic and experimental evidence suggesting that the citrus gene *CsNDR1* is an *Arabidopsis NDR1* ortholog. Overexpression of *CsNDR1* in *Arabidopsis* rescues *ndr1-1*-conferred susceptibility to *P. syringae* infection and leads to broad-spectrum disease resistance to the oomycete pathogen *Hpa* Noco2. We also found that both SA accumulation and *CsNDR1* expression are induced to modest levels in citrus upon infection with *Ca.* L. asiaticus, the agent associated with HLB. Our data suggest a possibility that manipulation of SA/*CsNDR1*-mediated defense may lead to enhanced resistance to HLB and other devastating diseases in citrus.

SA plays a critical role in regulating plant resistance against various pathogens. Broad-spectrum disease resistance has been successfully introduced into several economically important plants via manipulation of the SA pathway. Much of the previous studies have been focused on NPR1, a key SA signal transducer. For instance, overexpression of *Arabidopsis* NPR1 and/or its homologs from other plants confers resistance against diverse bacterial and fungal pathogens in *Arabidopsis*, apple, citrus, soybean, tomato,

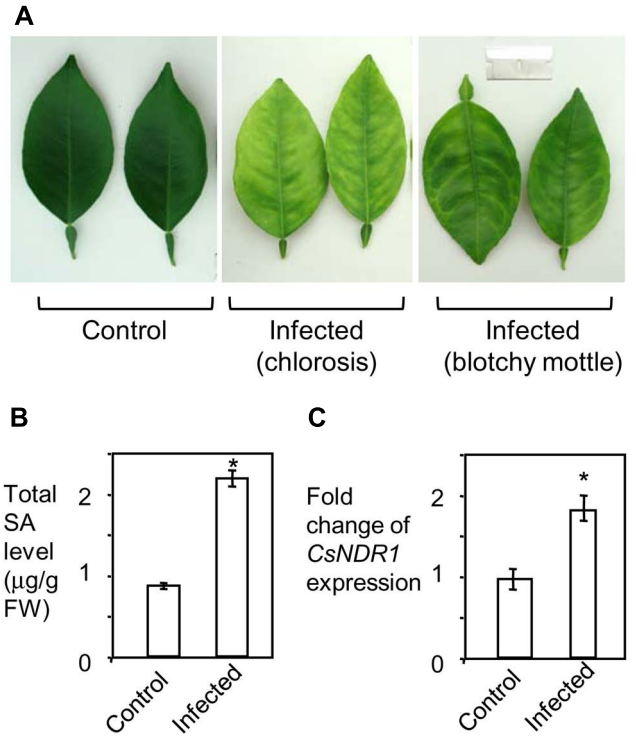


FIGURE 6 | *Ca.* L. asiaticus infection induces modest increase of SA accumulation and *CsNDR1* expression in citrus. Fifteen-month-old "Valencia" plants were infected with *Ca.* L. asiaticus or mock treated. **(A)** Photo of leaves collected at 16 wai. Note the infected leaves showing chlorosis and blotchy mottling, typical HLB symptoms. **(B)** SA quantitation. **(C)** qRT-PCR analysis. *CsNDR1* expression was normalized to the expression of *18S rRNA* and is presented as mean fold differences relative to control. Results are based on six biological replications. Statistical analysis was performed with one-way ANOVA Fisher's PLSD tests (StatView 5.0.1). Asterisks indicate significant different between the samples ($P < 0.05$). These experiments were repeated two times with similar results.

rice, and/or wheat (Fitzgerald et al., 2004; Li et al., 2004; Makandar et al., 2006; Malnoy et al., 2007; Sandhu et al., 2009; Zhang et al., 2010). These observations suggest that SA-mediated defense is conserved in monocots and dicots, and can be activated to against a range of pathogens (Campbell et al., 2002). Consistent with this notion, reports showed that exogenous application of SA agonists, such as benzothiadiazole and its commercial forms, acibenzolar-*S*-methyl (ASM, Actigard®, Syngenta Crop Protection, Inc.) and imidacloprid (Imid, Admire®, Bayer Crop Science), to citrus and other plants could induce defense marker gene expression and/or activate some protections of plants to a variety of viral, bacterial, and fungal pathogens (Campbell et al., 2002; Maxson-Stein et al., 2002; Dekkers et al., 2004; Francis et al., 2009; Graham and Myers, 2009).

Arabidopsis NDR1 is a positive SA regulator, which is known to be specifically required for defense activated by some CC–NBS–LRR R proteins but not by some TIR–NBS–LRR type of R proteins (Aarts et al., 1998; Coppinger et al., 2004). Although not much is known about R-avr recognition in many non-*Arabidopsis* plants, the fact that *NDR1* homologs widely exist in diverse plants (Lee et al., 2006; Chong et al., 2008; Cacas et al., 2011) suggests conserved defense signaling involving *NDR1* homologs. Recently a coffee *NDR1* homolog was shown to complement the *ndr1-1* mutant for its susceptibility to *P. syringae* strains (Cacas et al., 2011). Here we also showed a complementation of *ndr1-1* by *CsNDR1*. The transgenic plants showed varying levels of *CsNDR1* expression, which is not uncommon for transgenes (Lu et al., 2003). We noticed that there is a degree of correlation between the level of transgene expression and the level of disease resistance in some transgenic plants (**Figure 2**). One line highly expressing *CsNDR1* (line 15) constitutively activates SA-mediated defense, associated with enhanced disease resistance to *Pto avrRps4* (that is not affected by the endogenous *Arabidopsis* NDR1) and to the oomycete isolate *Hpa Noco2* (**Figure 5**). It is known that signals induced by different R genes upon recognition of their cognate effectors from pathogens can converge at downstream steps, involving SA-mediated defense (Martin et al., 2003; Chisholm et al., 2006). Our data suggest that hyper-activation of one branch of R-gene pathways, such as the *CsNDR1* branch, could potentially activate SA signaling, leading to broad-spectrum disease resistance.

In *Arabidopsis*, both resistant and susceptible responses to pathogen infection are characterized by elevated SA accumulation and defense gene induction but with differences in the speed and amplitude of the responses (Zhou et al., 1998; Maleck et al., 2000; Tao et al., 2003; Song et al., 2004). Compared with a resistance response in *Arabidopsis*, induction of SA levels in citrus infected with *Ca.* L. asiaticus is quite small (**Figure 6B**). In addition, our gene expression data (**Figure 6C**) and microarray analyses (Albrecht and Bowman, 2008, 2012) indicate that the spectrum and intensity of defense genes induced by *Ca.* L. asiaticus are also quite limited. These observations suggest that when infected by *Ca.* L. asiaticus, citrus plants do not activate considerable host defense. This can be explained with at least two possible reasons: (1) a lack of recognition of effector proteins from *Ca.* L. asiaticus by citrus; and/or (2) a suppression of host defense by *Ca.* L. asiaticus. Thus, the interaction between citrus and *Ca.* L. asiaticus can be viewed as a compatible interaction, leading to disease symptom development in the host. The relatively low level of host defense in response to *Ca.* L. asiaticus also suggests a possibility that HLB resistance can be achieved if we could manipulate the host to enhance its defense levels.

Genetic engineering is a particularly attractive approach to introduce disease resistance traits into citrus because citrus has long juvenile growth – it typically takes 5–15 years for a citrus plant to flower. In addition, most commercial citrus cultivars produce polyembryonic seeds asexually, which complicates the process of introducing novel traits into citrus via traditional breeding (Koltunow et al., 1996). Recently the *Arabidopsis NPR1* gene was shown to increase resistance to the canker disease when overexpressed in citrus (Zhang et al., 2010). Thus, *CsNDR1*, citrus *NPR1*, and other citrus homologs of SA regulatory genes are ideal candidates that can be genetically manipulated to increase their expression in order to test if these genes confer resistance to HLB in citrus. Engineering such genes could yield citrus plants with enhanced disease resistance

that are also more acceptable to the consumers than those engineered with similar genes from other plants. Moreover, the newly released citrus genome sequence has greatly facilitated the identification of additional citrus defense genes. We anticipate that large-scale functional genomic analysis could uncover defense genes that play critical roles in resistance against HLB, citrus canker, and/or other emerging diseases challenging the citrus industry worldwide.

ACKNOWLEDGMENTS

We thank members in the Lu laboratory for assistance with this work. We thank Dr. Kevin Omland for assistance in phylogenetic analysis, Dr. William LaCourse for sharing his HPLC instrument, and Mr. Tim Ford for taking pictures for this publication. This work was supported by a grant from Citrus Research and Development Foundation to Hua Lu and a scholarship from China Scholarship Council to Chong Zhang.

REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B. J., Daniels, M. J., and Parker, J. E. (1998). Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10306–10311.

Albrecht, U., and Bowman, K. (2008). Gene expression in *Citrus sinensis* (L.) Osbeck following infection with the bacterial pathogen *Candidatus* Liberibacter asiaticus causing Huanglongbing in Florida. *Plant Sci.* 175, 291–306.

Albrecht, U., and Bowman, K. D. (2012). Transcriptional response of susceptible and tolerant citrus to infection with *Candidatus* Liberibacter asiaticus. *Plant Sci.* 185–186, 118–130.

Bittner-Eddy, P. D., and Beynon, J. L. (2001). The *Arabidopsis* downy mildew resistance gene, *RPP13-Nd*, functions independently of *NDR1* and *EDS1* and does not require the accumulation of salicylic acid. *Mol. Plant Microbe Interact.* 14, 416–421.

Bove, J. M. (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol.* 88, 7–37.

Brlansky, R. H., and Rogers, M. E. (2007). Citrus Huanglongbing: understanding the vector–pathogen interaction for disease management. *Plant Health Progr.* doi: 10.1094/APSnetFeature-2007-1207 (published online).

Cacas, J. L., Petitot, A. S., Bernier, L., Estevan, J., Conejero, G., Mongrand, S., et al. (2011). Identification and characterization of the Non-race specific Disease Resistance 1 (NDR1) orthologous protein in coffee. *BMC Plant Biol.* 11:144. doi: 10.1186/1471-2229-11-144

Campbell, M. A., Fitzgerald, H. A., and Ronald, P. C. (2002). Engineering pathogen resistance in crop plants. *Transgenic Res.* 11, 599–613.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., and Dong, X. (1997). The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88, 57–63.

Century, K. S., Holub, E. B., and Staskawicz, B. J. (1995). NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6597–6601.

Century, K. S., Shapiro, A. D., Repetti, P. P., Dahlbeck, D., Holub, E., and Staskawicz, B. J. (1997). NDR1, a pathogen-induced component required for *Arabidopsis* disease resistance. *Science* 278, 1963–1965.

Chern, M. S., Fitzgerald, H. A., Yadav, R. C., Canlas, P. E., Dong, X., and Ronald, P. C. (2001). Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in *Arabidopsis*. *Plant J.* 27, 101–113.

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host–microbe interactions: shaping the evolution of the plant immune response. *Cell* 124, 803–814.

Chomczynski, P., and Sacci, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* 162, 156–159.

Chong, J., Le Hananff, G., Bertsch, C., and Walter, B. (2008). Identification, expression analysis, and characterization of defense and signaling genes in *Vitis vinifera*. *Plant Physiol. Biochem.* 46, 469–481.

Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743.

Coppinger, P., Repetti, P. P., Day, B., Dahlbeck, D., Mehlert, A., and Staskawicz, B. J. (2004). Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. *Plant J.* 40, 225–237.

Dekkers, M. G. H., Graham, J. H., Burns, J. K., Cubero, J., and Colburn, G. C. (2004). Evaluation of chemical inducers and PR protein reporters for induced systemic resistance to citrus bacterial diseases. *Phytophathology*, S25.

Dong, X. (2004). NPR1, all things considered. *Curr. Opin. Plant. Biol.* 7, 547–552.

Dormann, P., Hoffman-Benning, S., Balbo, I., and Benning, C. (1995). Isolation and characterization of an *Arabidopsis* mutant deficient in the thylakoid lipid digalactosyl diacylglycerol. *Plant Cell* 7, 1801–1810.

Dörmanna, P., Gopalanb, S., He, S.-Y., and Benning, C. (2000). A gene family in *Arabidopsis thaliana* with sequence similarity to *NDR1* and *HIN1*. *Plant Physiol. Biochem.* 38, 789–796.

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209.

Falk, A., Feys, B. J., Frost, L. N., Jones, J. D., Daniels, M. J., and Parker, J. E. (1999). EDS1, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3292–3297.

Fitzgerald, H. A., Chern, M. S., Navarre, R., and Ronald, P. C. (2004). Overexpression of (*At*)*NPR1* in rice leads to a BTH- and environment-induced lesion-mimic/cell death phenotype. *Mol. Plant Microbe Interact.* 17, 140–151.

Francis, M. I., Redondo, A., Burns, J. K., and Graham, J. H. (2009). Soil application of imidacloprid and related SAR inducing compounds produces effective and persistent control of citrus canker. *Eur. J. Plant Pathol.* 124, 283–292.

Gao, F., Shu, X., Ali, M. B., Howard, S., Li, N., Winterhagen, P., et al. (2010). A functional EDS1 ortholog is differentially regulated in powdery mildew resistant and susceptible grapevines and complements an *Arabidopsis eds1* mutant. *Planta* 231, 1037–1047.

Gaskalla, R. (2006). *Comprehensive Report on Citrus Canker Eradication Program in Florida Through 14 January 2006 Revised.* Tallahassee, FL: Division of Plant Industry, Florida Department of Agriculture and Consumer Services, 25 p.

Gottwald, T. R. (2007). Citrus canker and citrus huanglongbing, two exotic bacterial diseases threatening the citrus industries of the western hemisphere. *Outlooks Pest Manage.* 18, 274–279.

Graham, J. H., and Myers, M. (2009). Soil drenches of imidacloprid, thiamethoxam and acibenzolar-S-methyl for induction of SAR to control citrus canker in young citrus trees. *Phytopathology* 99, S46.

Hammond-Kosack, K. E., and Jones, J. D. (1996). Resistance gene-dependent plant defense responses. *Plant Cell* 8, 1773–1791.

Ichinose, K., Miyazi, K., Matsuhira, K., Yasuda, K., Sadoyama, Y., Do, H. T., et al. (2010). Unreliable pesticide control of the vector psyllid *Diaphorina citri* (Hemiptera: Psyllidae) for the reduction of microorganism disease transmission. *J. Environ. Sci. Health B* 45, 466–472.

Knepper, C., Savory, E. A., and Day, B. (2011). *Arabidopsis* NDR1 is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. *Plant Physiol.* 156, 286–300.

Koltunow, A. M., Hidaka, T., and Robinson, S. P. (1996). Polyembryony in citrus accumulation of seed storage proteins in seeds and in embryos cultured *in vitro*. *Plant Physiol.* 599–609.

Lee, M. W., Jelenska, J., and Greenberg, J. T. (2008). *Arabidopsis* proteins important for modulating defense responses to *Pseudomonas syringae* that secrete HopW1-1. *Plant J.* 54, 452–465.

Lee, S. B., Ham, B. K., Park, J. M., Kim, Y. J., and Kh, P. (2006). BnNHL18A shows a localization change by stress-inducing chemical treatments. *Biochem. Biophys. Res. Comm.* 339, 399–406.

Li, J., Brader, G., and Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16, 319–331.

Li, W., Hartung, J. S., and Levy, L. (2006). Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus huanglongbing. *J. Microbiol. Methods* 66, 104–115.

Lin, W. C., Lu, C. F., Wu, J. W., Cheng, M. L., Lin, Y. M., Yang, N. S., et al. (2004).

Transgenic tomato plants expressing the *Arabidopsis NPR1* gene display enhanced resistance to a spectrum of fungal and bacterial diseases. *Transgenic Res.* 13, 567–581.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 25, 402–408.

Lu, H. (2009). Dissection of salicylic acid-mediated defense signaling networks. *Plant Signal. Behav.* 4, 713–717.

Lu, H., Rate, D. N., Song, J. T., and Greenberg, J. T. (2003). ACD6, a novel ankyrin protein, is a regulator and an effector of salicylic acid signaling in the *Arabidopsis* defense response. *Plant Cell* 15, 2408–2420.

Makandar, R., Essig, J. S., Schapaugh, M. A., Trick, H. N., and Shah, J. (2006). Genetically engineered resistance to *Fusarium* head blight in wheat by expression of *Arabidopsis* NPR1. *Mol. Plant Microbe Interact.* 19, 123–129.

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250, 1002–1004.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K. A., et al. (2000). The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* 26, 403–410.

Malnoy, M., Jin, Q., Borejsza-Wysocka, E. E., He, S. Y., and Aldwinckle, H. S. (2007). Overexpression of the apple *MpNPR1* gene confers increased disease resistance in *Malus* × *domestica*. *Mol. Plant Microbe Interact.* 20, 1568–1580.

Martin, G. B., Bogdanove, A. J., and Sessa, G. (2003). Understanding the functions of plant disease resistance proteins. *Annu. Rev. Plant Biol.* 54, 23–61.

Maxson-Stein, K., He, S., Hammerschmidt, R., and Jones, A. (2002). Effect of treating apple trees with acibenzolar-S-methyl on fire blight and expression of pathogenesis-related protein genes. *Plant Disease* 86, 785–790.

McDowell, J. M., Cuzick, A., Can, C., Beynon, J., Dangl, J. L., and Holub, E. B. (2000). Downy mildew (*Peronospora parasitica*) resistance genes in *Arabidopsis* vary in functional requirements for NDR1, EDS1, NPR1 and salicylic acid accumulation. *Plant J.* 22, 523–529.

McDowell, J. M., Hoff, T., Anderson, R. G., and Deegan, D. (2010). Propagation, storage, and assays with *Hyaloperonospora arabidopsidis*: a model oomycete pathogen of *Arabidopsis*. *Methods Mol. Biol.*712, 137–151.

McDowell, J. M., and Simon, S. A. (2006). Recent insights into *R* gene evolution. *Mol. Plant Pathol.* 7, 437–448.

Ng, G., Seabolt, S., Zhang, C., Salimian, S., Watkins, T. A., and Lu, H. (2011). Genetic dissection of salicylic acid-mediated defense signaling networks in *Arabidopsis*. *Genetics* 189, 851–859.

Nishimura, M. T., and Dangl, J. L. (2010). *Arabidopsis* and the plant immune system. *Plant J.* 61, 1053–1066.

Pegadaraju, V., Louis, J., Singh, V., Reese, J. C., Bautor, J., Feys, B. J., et al. (2007). Phloem-based resistance to green peach aphid is controlled by *Arabidopsis* PHYTOALEXIN DEFICIENT4 without its signaling partner ENHANCED DISEASE SUSCEPTIBILITY1. *Plant J.* 52, 332–341.

Rassmussen, J. B., Hammerschmidt, R., and Zook, M. N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.* 97, 1342–1347.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H. Y., et al. (1997). The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *Plant Cell* 9, 425–439.

Sandhu, D., Tasma, I. M., Frasch, R., and Bhattacharyya, M. K. (2009). Systemic acquired resistance in soybean is regulated by two proteins, orthologous to *Arabidopsis* NPR1. *BMC Plant Biol.* 9:105. doi: 10.1186/1471-2229-9-105

Shah, J., Tsui, F., and Klessig, D. F. (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. *Mol. Plant Microbe Interact.* 10, 69–78.

Shapiro, A. D., and Zhang, C. (2001). The role of NDR1 in avirulence gene-directed signaling and control of programmed cell death in *Arabidopsis*. *Plant Physiol.* 127, 1089–1101.

Sharma, Y. K., and Davis, K. R. (1997). The effects of ozone on antioxidant responses in plants. *Free Radic. Biol. Med.* 23, 480–488.

Song, J. T., Lu, H., Mcdowell, J. M., and Greenberg, J. T. (2004). A key role for ALD1 in activation of local and systemic defenses in *Arabidopsis*. *Plant J.* 40, 200–212.

Strommer, J., Gregerson, R., and Vayda, M. (1993). "Isolation and characterization of plant mRNA," in *Methods in Plant Molecular Biology and Biotechnology*, eds B. M. Greenberg and B. R. Glick (Boca Raton, FL: CRC Press Inc.) 49–65.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H. S., Han, B., et al. (2003). Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15, 317–330.

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J. D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. *Plant J.* 53, 763–775.

Varet, A., Hause, B., Hause, G., Scheel, D., and Lee, J. (2003). The *Arabidopsis NHL3* gene encodes a plasma membrane protein and its overexpression correlates with increased resistance to *Pseudomonas syringae* pv. *tomato* DC3000. *Plant Physiol.* 132, 2023–2033.

Varet, A., Parker, J., Tornero, P., Nass, N., Nurnberger, T., Dangl, J. L., et al. (2002). *NHL25* and *NHL3*, two *NDR1*/*HIN1*-like genes in *Arabidopsis thaliana* with potential role(s) in plant defense. *Mol. Plant Microbe Interact.* 15, 608–616.

Wang, G. F., Seabolt, S., Hamdoun, S., Ng, G., Park, J., and Lu, H. (2011a). Multiple roles of WIN3 in regulating disease resistance, cell death, and flowering time in *Arabidopsis*. *Plant Physiol.* 156, 1508–1519.

Wang, G. Y., Shi, J. L., Ng, G., Battle, S. L., Zhang, C., and Lu, H. (2011b). Circadian clock-regulated phosphate transporter PHT4;1 plays an important role in *Arabidopsis* defense. *Mol. Plant* 4, 516–526.

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565.

Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M., et al. (2001). Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by RPW8. *Science* 291, 118–120.

Zhang, C., Gutsche, A. T., and Shapiro, A. D. (2004). Feedback control of the *Arabidopsis* hypersensitive response. *Mol. Plant Microbe Interact.* 17, 357–365.

Zhang, X., Francis, M. I., Dawson, W. O., Graham, J. H., Orbovic, V., Triplett, E. W., et al. (2010). Over-expression of the *Arabidopsis NPR1* gene in citrus increases resistance to citrus canker. *Eur. J. Plant Pathol.* 128, 91–100.

Zheng, M. S., Takahashi, H., Miyazaki, A., Hamamoto, H., Shah, J., Yamaguchi, I., et al. (2004). Up-regulation of *Arabidopsis thaliana* NHL10 in the hypersensitive response to Cucumber mosaic virus infection and in senescing leaves is controlled by signalling pathways that differ in salicylate involvement. *Planta* 218, 740–750.

Zhou, N., Tootle, T. L., Tsui, F., Klessig, D. F., and Glazebrook, J. (1998). PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell* 10, 1021–1030.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 February 2013; accepted: 07 May 2013; published online: 03 June 2013.

Citation: Lu H, Zhang C, Albrecht U, Shimizu R, Wang G and Bowman KD (2013) Overexpression of a citrus NDR1 ortholog increases disease resistance in Arabidopsis. Front. Plant Sci. ***4****:157. doi: 10.3389/fpls.2013.00157*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 18 March 2014
doi: 10.3389/fpls.2014.00101

Cell wall methanol as a signal in plant immunity

Tatiana V. Komarova[1,2], *Ekaterina V. Sheshukova*[2] *and Yuri L. Dorokhov*[1,2]*

[1] A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia
[2] N. I. Vavilov Institute of General Genetics, Russian Academy of Science, Moscow, Russia

Edited by:
Vincenzo Lionetti, Sapienza Università di Roma, Italy

Reviewed by:
Simone Ferrari, Sapienza Università di Roma, Italy
Alessandro Raiola, University of Padova, Italy

***Correspondence:**
Yuri L. Dorokhov, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119991, Russia
e-mail: dorokhov@genebee.msu.ru

Cell wall pectin forms a matrix around the cellulose–xyloglucan network that is composed of rhamnogalacturonan I, rhamnogalacturonan II, and homogalacturonan (HG), a major pectic polymer consisting of α-1,4-linked galacturonic acids. HG is secreted in a highly methyl-esterified form and selectively de-methyl-esterified by pectin methylesterases (PMEs) during cell growth and pathogen attack. The mechanical damage that often precedes the penetration of the leaf by a pathogen promotes the activation of PME, which in turn leads to the emission of methanol (MeOH), an abundant volatile organic compound, which is quickly perceived by the intact leaves of the damaged plant, and the neighboring plants. The exposure to MeOH may result in a "priming" effect on intact leaves, setting the stage for the within-plant, and neighboring plant immunity. The emission of MeOH by a wounded plant enhances the resistance of the non-wounded, neighboring "receiver" plants to bacterial pathogens and promotes cell-to-cell communication that facilitates the spread of viruses in neighboring plants.

Keywords: cell wall, methanol, pectin, pectin methylesterase, plant immunity, priming

INTRODUCTION

Plant cells are covered with a dense extracellular matrix that prevents direct contact between adjacent cells and pathogens. Damage to the plant epidermis caused by abiotic (wind, hail, and rain) and biotic (insects) factors may allow the penetration of pathogens (bacteria, fungi, oomycetes, and nematodes) into the intercellular space of the leaf and virus particles into the cell. Thus, plant wounding is one of the conditions for pathogen entry. However, mechanical damage to the leaf promotes the emission of volatile organic compounds (VOCs), including the green leaf volatiles (GLVs), and methanol (MeOH), which are quickly perceived by the intact leaves of the damaged plant and the intact neighboring plants (Dorokhov et al., 2014). The transport of VOCs is much faster compared to the transport of infectious viral entities and bacterial effectors through the phloem. Thus, exposure to VOCs may result in a "priming" effect on intact leaves, setting the stage for subsequent plant immunity. GLVs are associated with the smell of a freshly mown lawn and are derived from C18 fatty acids released from damaged membranes. MeOH, quantitatively the most important plant volatile after CO_2, is a product of the demethylation of pectin by the pectin methylesterases (PMEs) during cell wall (CW) formation and modification (Pelloux et al., 2007).

PME-MEDIATED PLANT IMMUNITY

Cell wall pectin forms a matrix around the cellulose–xyloglucan network that is composed of three main components called rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGA II), and homogalacturonan (HG), a major pectic polymer consisting of α-1,4-linked galacturonic acids (Peaucelle et al., 2012). HG is secreted in a highly methyl-esterified form and selectively de-methyl-esterified by PMEs, resulting in MeOH formation. The *PME* genes encode a pro-PME precursor with an *N*-terminal extension of variable length that is essential for protein targeting to the endoplasmic reticulum (Dorokhov et al., 1999). PME maturation requires removal of the PME leader including the transmembrane domain and spacer sequence (Dorokhov et al., 2006a). It was hypothesized that the spacer sequence plays a role in subcellular targeting and acts as an intramolecular chaperone for folding of the mature enzyme or as an autoinhibitor during transport through the endomembrane system (Pelloux et al., 2007). PME participates in CW modulation during general plant growth as it is involved in cell expansion and CW modification (Pelletier et al., 2010). The synthesis of PME is one of the aspects of plant growth that leads to the demethylesterification of the elastic "soft" pectins that accompanies MeOH generation (Komarova et al., 2014) as part of the natural division and maturation of the plant cell. After demethylesterification, pectate can form Ca^{2+}-pectate cross-linked complexes of rigid "hard" pectin, referred to as "egg boxes" (Peaucelle et al., 2012).

The important role of PME in the resistance of plants to fungi and bacteria has been demonstrated (Pelloux et al., 2007). A higher degree of pectin methyl esterification in certain plants induces resistance to pathogenic fungi (Lionetti et al., 2012). CW pectin methyl esterification may have an impact on plant resistance because highly methyl-esterified pectin can be less susceptible to hydrolysis by pectic enzymes such as fungal endopolygalacturonases. This view is supported by experiments performed with plants that were stably transformed with the *PME inhibitor (PMEI)* gene. The PMEI transgenic *Arabidopsis* (Lionetti et al., 2007) and durum wheat (Volpi et al., 2011) plants exhibited high levels of resistance to fungal and bacterial pathogens. Moreover, PME-mediated pectin methyl de-esterification may influence the polygalacturonase-mediated release of pectin-derived compounds, which in turn elicits a defense response (Pelloux et al., 2007; Lionetti et al., 2012).

The role of PME in viral infection is more complicated. PME interacts with the movement protein (MP) of the *Tobacco mosaic virus* (TMV; Dorokhov et al., 1999; Chen et al., 2000), suggesting that PME may be involved in the cell-to-cell movement of plant viruses (Chen and Citovsky, 2003). Interestingly, PMEI also interacts with PME to negatively affect viral infection (Lionetti et al., 2014), most likely by interfering with PME and TMV MP binding. The complex role of PME in viral infection is also underscored by the effects of PME on nuclear protein transport (Komarova et al., 2011) and gene silencing mediated by the activation of siRNA and miRNA production (Dorokhov et al., 2006b).

MeOH AND PLANT IMMUNITY

The PME-mediated conversion of HG methoxyl groups into carboxyl groups results in MeOH release. In humans, MeOH is considered to be a poison because alcohol dehydrogenase metabolizes MeOH into toxic formaldehyde. However, recent data have indicated that MeOH is actually a naturally occurring compound in normal, healthy human individuals. MeOH is not toxic to plant cells and has long been assumed to be a metabolic waste product. Recently, it has been shown that MeOH may regulate plant growth (Komarova et al., 2014) and serve an alarm function (Dorokhov et al., 2012a). The effects of PME-generated MeOH emitted from plants ("emitters") on the defensive reactions of other plants ("receivers") were studied (Dorokhov et al., 2012a). The results of this study led to the conclusion that MeOH is a signaling molecule that is involved in within-plant and plant-to-plant communication (Dorokhov et al., 2012a).

Mechanical damage to plants drastically increases MeOH and GLVs emission. GLVs that are rapidly released from wounded leaves may in turn stimulate PME-generated MeOH production (Dorokhov et al., 2012a). Herbivore attacks also increase MeOH emission levels: *Manduca sexta* caterpillars enhance wound-induced MeOH emission in *Nicotiana attenuate* (von Dahl et al., 2006). The over-expression of PME, derived from *Arabidopsis thaliana* and *Aspergillus niger*, in transgenic tobacco plants enhances resistance to polyphagous insect pests (Dixit et al., 2013). Transgenic plants with a silenced *PME* gene exhibited a 50% reduction in PME activity in their leaves and a 70% reduction in herbivore-induced MeOH emissions compared to wild type plants. This result demonstrates that herbivore-induced MeOH emissions originate from pectin demethylation by PME (Körner et al., 2009). The emission of MeOH is very fast and can be detected immediately following mechanical damage. Thus, the MeOH emitted from wounded leaves is produced by two forms of PME: pre-existing PME deposited in the CW before wounding, which allows rapid MeOH release (Körner et al., 2009), and PME that is synthesized *de novo* after wounding (Dorokhov et al., 2012a), which likely generates MeOH for an extended period.

Unlike longer-chain alcohols, the MeOH emitted by a wounded plant attracts insects and bark beetles. Moreover, mice prefer the odor of MeOH to the odors of other plant volatiles under laboratory conditions, and MeOH exposure alters the accumulation of mRNA in the mouse brain (Dorokhov et al., 2012b). This finding led to the conclusion that the MeOH emitted by wounded plants may have a role in plant-animal signaling.

Investigations demonstrated (Dorokhov et al., 2012a) that increased MeOH emissions from *PME*-transgenic or mechanically wounded non-transgenic plants retarded the growth of the bacterial pathogen *Ralstonia solanacearum* in neighboring "receiver" plants. The suppression of *R. solanacearum* growth observed in the "receiver" plants could be caused by gaseous MeOH or/and by GLVs. Indeed, *cis*-3-hexen-1-ol evaporated in a desiccator also resulted in decreased bacterial growth in the target plants. However, GLVs rapidly released from wounded leaves stimulated PME-generated MeOH production (Dorokhov et al., 2012a), suggesting that their influence on bacterial growth may be indirect. MeOH-stimulated antibacterial resistance was preceded by the upregulation of genes that control stress response and cell-to-cell communication in the "receiver". Antibacterial resistance accompanied by MeOH-induced genes (MIGs) upregulation was most likely related to the transcriptional induction of the *type II proteinase inhibitor (PI-II)* gene. PI-IIs are powerful inhibitors of serine endopeptidases in animals and microorganisms (Turra and Lorito, 2011). The *PI-II* gene is not expressed in the leaves of healthy plants, but it is induced in leaves that have been subjected to different types of stress, including wounding and bacterial infection. *PME*-transgenic tobacco with high levels of *PI-II* expression exhibited increased resistance to *R. solanacearum* (Dorokhov et al., 2012a). This finding supports the role of *PI-II* in the suppression of bacterial proteases.

Experiments with gaseous MeOH provided examples of priming in intact plants (**Figure 1**), which led to conditions conducive for viral infection (Dorokhov et al., 2012a). This effect could be explained by the enhancement of cell-to-cell communication by the MIGs, such as *β-1,3-glucanase* (*BG*; Zavaliev et al., 2011) and *non-cell-autonomous pathway protein* (*NCAPP*; Lee et al., 2003).

A model (**Figure 1**) proposing that MeOH-triggered PD dilation should enhance viral spread within the plant was confirmed in experiments in which BG and NCAPP activated cell-to-cell communication and TMV RNA accumulation. Moreover, gaseous MeOH or the vapors from wounded plants increased TMV reproduction in the "receivers" (Dorokhov et al., 2012a).

Thus, MeOH has a contradictory effect on the sensitivity of the leaves of the "receiver" plant to bacteria and viruses. The mechanisms that underlie this phenomenon are not clear; however, we can consider two factors that may explain this inconsistency in the MeOH-induced effects. First, there is a fundamental difference between bacteria and viruses with respect to their modes of intercellular transport. Bacterial pathogens do not cross the plant CW boundaries because they inhabit the intercellular spaces. In contrast, viral pathogens require cell-to-cell movement for local and systemic spread. Second, the most abundant MIGs can be divided into two groups according to their ability to participate in either bacterial or viral pathogenesis. The first, including *PI-II* and *PME* inhibitor, are involved in immunity against non-viral pathogens. The second group of genes, including *NCAPP* and *MIG-21* (Dorokhov et al., 2012a), is involved in the PD-mediated intercellular transport and reproduction of viruses. The most

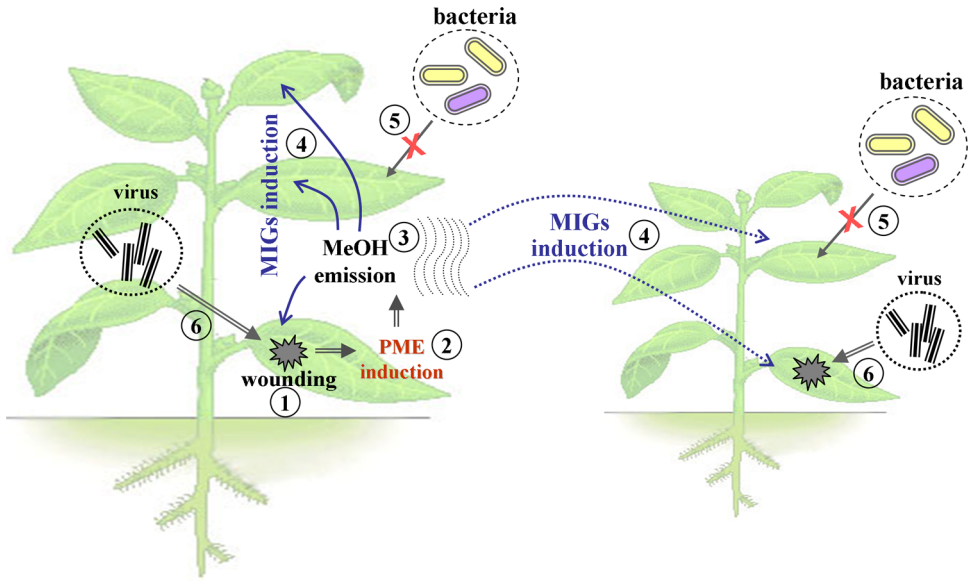


FIGURE 1 | The model of the effects of methanol emitted by the damaged plant. Mechanical damage to the leaves of the plant (1), leads to an increase in the expression level of PME (2), and induction of the release of gaseous MeOH (3). Emitted MeOH causes priming of adjacent leaves and neighboring plants, including activation of the MIGs (4), the emergence of bacterial immunity (5), the opening of plasmodesmata, and increased sensitivity to the virus penetration and intercellular distribution of viral pathogens (6).

abundant MIG, the *BG* gene, is involved in antibacterial immunity; however, the BG protein also accelerates PD-mediated intercellular transport.

CONCLUSION

Based on the available data, we can conclude that wounding-stimulated MeOH that is released into the air by damaged plants or plants compromised by herbivorous insects serves as an alarm to help neighboring plants or adjacent leaves prepare for a defense. The MeOH provides protection against herbivorous insects and plant pathogens such as bacteria. However, considering the role of MeOH in the relationship between viruses and plants, we do not find a negative, or even a neutral, influence of MeOH on viruses. On the contrary, the findings described (Dorokhov et al., 2012a) indicate that MeOH sensitizes the plant to allow the entry and spread of a virus through the plant and between plants by insect vectors. Therefore, MeOH promotes viral propagation. The positive impact of MeOH on viral infection may be explained by several factors. First, plant viruses differ from other types of pathogens as they inhabit the symplast. Furthermore, the survival of a virus depends on its ability to move from cell-to-cell exploiting PD to accumulate to sufficient levels and in enough tissues to guarantee survival despite using a very limited amount of genetic material. Thus, a virus, with its small but highly variable genome, spends its entire life in the cell symplast, while other pathogens occupy the apoplast. Second, the symplast is not only the space in which viruses reproduce, but it is also the site of RNA interference mechanisms that serve to eliminate foreign RNA. The specific degradation of RNA by RNA interference allows the host plant to effectively control viruses and other pathogens. It is known that the intracellular and intercellular transport of silencing factors is necessary for effective RNA interference. Therefore, a MeOH-mediated increase in viral replication may be regarded as compensation for the acquisition of antimicrobial resistance.

ACKNOWLEDGMENTS

This work was supported by the Russian Science Foundation, the Russian Foundation for Basic Research (grants Nos. 11-04-01152, 12-04-33016, 14-04-00109), and stipend of the President of the Russian Federation for young scientists.

REFERENCES

Chen, M. H., and Citovsky, V. (2003). Systemic movement of a tobamovirus requires host cell pectin methylesterase. *Plant J.* 35, 386–392. doi: 10.1046/j.1365-313X.2003.01818.x

Chen, M. H., Sheng, J., Hind, G., Handa, A. K., and Citovsky, V. (2000). Interaction between the tobacco mosaic virus movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. *EMBO J.* 19, 913–920. doi: 10.1093/emboj/19.5.913

Dixit, S., Upadhyay, S. K., Singh, H., Sidhu, O. P., Verma, P. C., and K, C. (2013). Enhanced methanol production in plants provides broad spectrum insect resistance. *PLoS ONE* 8:e79664. doi: 10.1371/journal.pone.0079664

Dorokhov, Y. L., Komarova, T. V., Petrunia, I. V., Frolova, O. Y., Pozdyshev, D. V., and Gleba, Y. Y. (2012a). Airborne signals from a wounded leaf facilitate viral spreading and induce antibacterial resistance in neighboring plants. *PLoS Pathog.* 8:e1002640. doi: 10.1371/journal.ppat.1002640

Dorokhov, Y. L., Komarova, T. V., Petrunia, I. V., Kosorukov, V. S., Zinovkin, R. A., Shindyapina, A. V., et al. (2012b). Methanol may function as a cross-kingdom signal. *PLoS ONE* 7:e36122. doi: 10.1371/journal.pone.0036122

Dorokhov, Y. L., Komarova, T. V., and Sheshukova, E. V. (2014). "Volatile organic compounds and plant virus-host interaction," in *Plant Virus-Host Interaction*, eds R. K. Gaur, T. Hohn, and P. Sharma (Amsterdam: Elsevier).

Dorokhov, Y. L., Makinen, K. M., Frolova, O. Y., Merits, A., Kalkkinen, N., Saarinen, J., et al. (1999). A novel function for a ubiquitous plant enzyme

pectin methylesterase: the host-cell receptor for the tobacco mosaic virus movement protein. *FEBS Lett.* 461, 223–228. doi: 10.1016/S0014-5793(99)01447-7

Dorokhov, Y. L., Skurat, E. V., Frolova, O. Y., Gasanova, T. V., Ivanov, P. A., Ravin, N. V., et al. (2006a). Role of the leader sequences in tobacco pectin methylesterase secretion. *FEBS Lett.* 580, 3329–3334. doi: 10.1016/j.febslet.2006.04.090

Dorokhov, Y. L., Frolova, O. Y., Skurat, E. V., Ivanov, P. A., Gasanova, T. V., Sheveleva, A.S., et al. (2006b). A novel function for a ubiquitous plant enzyme pectin methylesterase: the enhancer of RNA silencing. *FEBS Lett.* 580, 3872–3878. doi: 10.1016/j.febslet.2006.06.013

Komarova, T. V., Citovsky, V., and Dorokhov, Y. L. (2011). "Pectin methylesterase enhances tomato bushy stunt virus P19 RNA silencing suppressor effects," in *RNAi Technology*, eds R. K. Gaur, Y. Gafni, P. Sharma, and V. K. Gupta (Boca Raton, FL: CRC Press, Taylor & Francis Group), 125–134.

Komarova, T. V., Pozdyshev, D. V., Petrunia, I. V., Sheshukova, E. V., and Dorokhov, Y. L. (2014). Pectin methylesterase-generated methanol may be involved in tobacco leaf growth. *Biochemistry (Mosc.)* 79, 102–110. doi: 10.1134/S0006297914020035

Körner, E., von Dahl, C. C., Bonaventure, G., and Baldwin, I. T. (2009). Pectin methylesterase NaPME1 contributes to the emission of methanol during insect herbivory and to the elicitation of defence responses in *Nicotiana attenuata*. *J. Exp. Bot.* 60, 2631–2640. doi: 10.1093/jxb/erp106

Lee, J. Y., Yoo, B. C., Rojas, M. R., Gomez-Ospina, N., Staehelin, L. A., and Lucas, W. J. (2003). Selective trafficking of non-cell-autonomous proteins mediated by NtNCAPP1. *Science* 299, 392–326. doi: 10.1126/science.1077813

Lionetti, V., Cervone, F., and Bellincampi, D. (2012). Methyl esterification of pectin plays a role during plant-pathogen interactions and affects plant resistance to diseases. *J. Plant Physiol.* 169, 1623–1630. doi: 10.1016/j.jplph.2012.05.006

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., et al. (2007). Overexpression of pectin methylesterase inhibitors in *Arabidopsis* restricts fungal infection by *Botrytis cinerea*. *Plant Physiol.* 143, 1871–1880. doi: 10.1104/pp.106.090803

Lionetti, V., Raiola, A., Cervone, F., and Bellincampi, D. (2014). Transgenic expression of pectin methylesterase inhibitors limits tobamovirus spread in tobacco and *Arabidopsis*. *Mol. Plant Pathol.* 15, 265–274. doi: 10.1111/mpp.12090

Peaucelle, A., Braybrook, S., and Höfte, H. (2012). Cell wall mechanics and growth control in plants: the role of pectins revisited. *Front. Plant Sci.* 3:121. doi: 10.3389/fpls.2012.00121

Pelletier, S., Van Orden, J., Wolf, S., Vissenberg, K., Delacourt, J., Ndong, Y. A. et al. (2010). A role for pectin de-ethylesterification in a developmentally regulated growth acceleration in dark-grown *Arabidopsis hypocotyls*. *New Phytol.* 188, 726–739. doi: 10.1111/j.1469-8137.2010.03409.x

Pelloux, J., Rusterucci, C., and Mellerowicz, E. J. (2007). New insights into pectin methylesterase structure and function. *Trends Plant Sci.* 12, 267–277. doi: 10.1016/j.tplants.2007.04.001

Turra, D., and Lorito, M. (2011). Potato type I and II proteinase inhibitors: modulating plant physiology and host resistance. *Curr. Protein Pept. Sci.* 12, 374–385. doi: 10.2174/138920311796391151

Volpi, C., Janni, M., Lionetti, V., Bellincampi, D., Favaron, F., and D'Ovidio, R. (2011). The ectopic expression of a pectin methyl esterase inhibitor increases pectin methylesterification and limits fungal diseases in wheat. *Mol. Plant Microbe Interact.* 24, 1012–1019. doi: 10.1094/MPMI-01-11-0021

von Dahl, C. C., Hävecker, M., Schlögl, R., and Baldwin, I. T. (2006). Caterpillar-elicited methanol emission: a new signal in plant-herbivore interactions? *Plant J.* 46, 948–960. doi: 10.1111/j.1365-313X.2006.02760.x

Zavaliev, R., Ueki, S., Epel, B. L., and Citovsky, V. (2011). Biology of callose (β-1,3-glucan) turnover at plasmodesmata. *Protoplasma* 248, 117–130. doi: 10.1007/s00709-010-0247-0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2014; accepted: 02 March 2014; published online: 18 March 2014.
Citation: Komarova TV, Sheshukova EV and Dorokhov YL (2014) Cell wall methanol as a signal in plant immunity. Front. Plant Sci. ***5****:101. doi: 10.3389/fpls.2014.00101*
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 23 June 2014
doi: 10.3389/fpls.2014.00293

Sucrose and invertases, a part of the plant defense response to the biotic stresses

***Alexandra S. Tauzin*[†‡] *and Thierry Giardina**[*‡]

CNRS, Centrale Marseille, iSm2 UMR 7313, Aix Marseille Université, Marseille, France

Edited by:
Vincenzo Lionetti, Sapienza "Università di Roma," Italy

Reviewed by:
Zuhua He, Chinese Academy of Sciences, China
Ingo Bergmann, Leibniz Institute for Agricultural Engineering Potsdam-Bornim e.V. (ATB), Germany

***Correspondence:**
Thierry Giardina, CNRS, Centrale Marseille, iSm2 UMR 7313, Aix Marseille Université, Avenue Escadrille Normandie-Niemen, 13397 Marseille, France
e-mail: thierry.giardina@univ-amu.fr

[†]***Present address:***
Michael Smith, Laboratories and Department of Chemistry, University of British Columbia, Vancouver, Canada

[‡]*These authors have contributed equally to this work.*

Sucrose is the main form of assimilated carbon which is produced during photosynthesis and then transported from source to sink tissues *via* the phloem. This disaccharide is known to have important roles as signaling molecule and it is involved in many metabolic processes in plants. Essential for plant growth and development, sucrose is engaged in plant defense by activating plant immune responses against pathogens. During infection, pathogens reallocate the plant sugars for their own needs forcing the plants to modify their sugar content and triggering their defense responses. Among enzymes that hydrolyze sucrose and alter carbohydrate partitioning, invertases have been reported to be affected during plant-pathogen interactions. Recent highlights on the role of invertases in the establishment of plant defense responses suggest a more complex regulation of sugar signaling in plant-pathogen interaction.

Keywords: sucrose, cell wall invertase, vacuolar invertase, alkaline/neutral invertase, plant defense response

INTRODUCTION

Cash and subsistence crops are susceptible to a large number of diseases caused by plant pathogens. Among pathogenic organisms: fungi, oomycetes, viruses and bacteria are the most important ones. The direct consequence of pathogen attack is the decrease of the crop yield. In addition to economic loss, consumer health may be compromised due to risks in ingesting toxins produced from secondary metabolites of these pathogens. Mycotoxins are probably the most known factors produced by fungi, which are not only poisonous but also carcinogenic for human (Maresca, 2013).

The plant response is mediated by a sophisticated immune system divided into two different pathways. The first is microbial-associated molecular-patterns-triggered immunity (MTI), constituted by elicitors recognized by the plant innate immune systems *via* pattern recognition receptors (PRRs) (Ausubel, 2005; Katagiri and Tsuda, 2010). The second is the effector-triggered immunity (ETI) stimulated on the basis of the perception of pathogen effectors by plant disease resistance proteins (Dangl and Jones, 2001; Jones and Dangl, 2006).

Pathogens modify the host metabolism which results in an energy increase and production of carbon sources (Thines et al., 2000) including sucrose and its cleavage products, glucose and fructose (Roitsch and Gonzalez, 2004; Rolland et al., 2006). Sucrose hydrolysis is catalyzed by invertases, and the consequence is the shifts of the apoplastic sucrose/hexose ratio in favor of hexoses. The aim of this paper is to review recent evidence on the crucial roles of invertases during plant pathogen attacks and how the invertase activity is regulated.

FROM CARBOHYDRATE PARTITIONING TO PLANT DEFENSE RESPONSE

SUCROSE SIGNAL MOLECULE

In higher plants, sucrose is the major transport form of carbohydrates. Sucrose is produced during photosynthesis in source tissues (leaves), and then transported *via* the phloem to the different sink tissues (roots, stem, reproductive organs and vegetative storage organs) to provide the carbon and energy needed for growth and synthesis of storage reserves.

The role of sucrose as signaling molecule is well established (for reviews see Koch, 2004; Rolland et al., 2006; Wind et al., 2010; Tognetti et al., 2013). It affects plant development processes such as plant growth, regulation of flowering, differentiation of vascular tissue and development of storage organs (for review see Tognetti et al., 2013). Sucrose cleavage products, glucose and fructose, also act as signaling molecules. Of the two hexoses, glucose has been better described in relation with the hexokinase signaling pathway (Moore et al., 2003; Cho et al., 2009) while for fructose a specific pathway has been proposed involving the abscisic acid (ABA)- and ethylene-signaling pathway (Cho and Yoo, 2011; Li et al., 2011).

Gomez-Ariza et al. (2007) observed that the pre-treatment of rice plants with sucrose drastically reduced symptoms of fungal *Magnaporthe oryzae* infection and they proposed sucrose as a signal molecule in plant immunity.

PLANT INVERTASES

Invertases (EC.3.2.1.26) hydrolyze irreversibly sucrose into glucose and fructose. Three groups were identified: alkaline/neutral invertases (A/NInv) localized in the cytosol, mitochondria and/or in plastids, and two types of acid invertases, insoluble bound to the cell wall (cell wall invertase, CWI) and soluble found in the vacuole space (vacuolar invertase, VI), respectively.

ACID INVERTASES AND PROTEINACEOUS INHIBITORS

Acid invertases, CWIs and VIs, belong to the GH32 family. CWIs play a key role in sucrose partitioning, plant development and cell differentiation while VIs are involved in cell expansion, sugar storage and regulation of cold induced sweetening (Roitsch and Gonzalez, 2004). Both are post-translationally regulated by proteinaceous inhibitors (INHs) which belong, with pectin methylesterase inhibitors (PMEIs), to the pectin methylesterase inhibitor related protein (PMEI-RP) family (Pfam 04043) (Hothorn et al., 2004).

During plant infection, the level of VI modulation is poorly understood with contradictory reports in the literature that leads to an unclear functional assignment (**Table 1**). On the one hand, a reduction of VI expression has been observed during the infection of *Vicia faba* by *Uromyces fabae* and *Vitis vinifera* by *Erysiphe necator* and *Plasmopora viticola* (Voegele et al., 2006; Hayes et al., 2010). This down-regulation was attributed to a decrease in the availability of sucrose in the storage compartment (Voegele et al., 2006; Hayes et al., 2010). By contrast, a high VI activity was observed during the first stage of infection of castor beans by *Agrobacterium tumefaciens* that might suggest a supportive function during invasion (Wachter et al., 2003). Moreover, the expression of a VI (TIV-1) is not affected in tomato infected by *Botrytis cinerea* (Hyun et al., 2011). Finally, when Essmann et al. compared wild type tobacco plants and transgenic plants silenced for CWI after infection by *Phytophthora nicotianae*, they noticed no significant changes in the VI activity (Essmann et al., 2008a,b) suggesting that the VI is not involved in the plant defense response. These results reinforce the doubts concerning the exact role of VIs in plant immunity.

By contrast, the link between plant response against pathogen and CWI activity has been widely studied (**Table 1**). A common trend is observed for the rapid increase of the CWI mRNA level after infection by bacterial, fungal, viruses, oomycetes and nematodes (for detailed references see **Table 1**). Indeed, the up-regulation of CWI activity is essential to modulate sugar partitioning and provide the sugars which are necessary for the pathogen development. A clear example has been demonstrated for gall development in *A. thaliana* (Siemens et al., 2011). Moreover, it was shown that during infection CWI activity also triggers plant defense responses such as induction of defense-related gene expression, callose deposition and reduction of photosynthesis or cell death. CWI silencing disrupts the ability of transgenic plants to answer correctly to the pathogen attacks and impairs the defense induced reaction (Essmann et al., 2008a). In rice, the loss-of-function mutant of the CWI gene GRAIN INCOMPLETE FILLING 1 (GIF1) has been demonstrated to be hypersusceptible to postharvest pathogens while the constitutive expression of GIF1 enhances the resistance to pathogens by activating the plant defense response (Sun et al., 2013). In the particular case of symbiosis (such as arbuscular mycorrhiza), the expression of CWI is finely controlled by the partner to prevent the induction of *pathogenesis-related* (PR) genes and promote "long-term" interaction (Schaarschmidt et al., 2006, 2007).

Invertase activity is potentially modulated by proteinaceous inhibitors (INHs) in a pH-dependent manner (Tauzin et al., 2014). Greiner et al. (1998) demonstrated that tobacco INH didn't affect invertases purified from two fungi, *Candida utilis* and *Saccharomyces cerevisiae*, supporting the idea that INHs are not involved in plant defense mechanisms. However, a strong repression of the expression of one of the three INHs from *A. thaliana* after infection by *Pseudomonas syringae* pv. tomato DC3000 was documented (Bonfig et al., 2010). The invertase activity was detectable only in infected plants while the enzyme was present in infected and uninfected crude extract cells, indicating that the enzyme activity was repressed by a specific inhibitor. This result was corroborated by the utilization of the pseudo tetrasaccharide acarbose which inhibits invertase activity *in planta* resulting in an increased susceptibility of the infected plant compared to the wild type (Bonfig et al., 2010).

ALKALINE/NEUTRAL INVERTASES

A/NInvs are non-glycosylated proteins and they belong to the GH100 family (Lammens et al., 2009). They have different subcellular localizations such as cytosol, mitochondria, chloroplast and nuclei (Vargas and Salerno, 2010). A/NInvs are involved in plant growth and development, flowering and seed germination (Jia et al., 2008; Barratt et al., 2009; Welham et al., 2009). Xiang et al. (2011) demonstrated that A/NInvs are part of the antioxidant system involved in cellular reactive oxygen species homeostasis. Moreover, exogenous application of gibberellic acid (GA) rescued the delay of germination in the seeds of the A/NInv mutants suggesting a communication between A/NInv and phytohormones (Xiang et al., 2011; Martin et al., 2013).

Correlated with the increase of the CWI activity, an increase of the A/NInv activity has been observed in *Pisum sativum*, tobacco and *A. thaliana* during infection by powdery mildew (Storr and Hall, 1992), oomycetes (Essmann et al., 2008a), and the beet curly top virus (Park et al., 2013), respectively. Interestingly, in transgenic tobacco plants silenced for CWI, the A/NInv activity remained unchanged during the interaction with the oomycetic phytopathogen (Essmann et al., 2008a). The authors suggested that the CWI activity increased first and by consequence the availability of carbohydrate changes and triggers the A/NInvs activity as a secondary phenomenon in the plant immunity (Essmann et al., 2008a). By contrast, the infections of *A. thaliana* by two different nematodes *Heterodera schachtii* and *Meloidogyne javanica* led to the down-regulation of *A/NInv* gene (*AtCINV1*) reflected by a decrease of activity (Cabello et al., 2013). Thus, the importance of A/NInv might vary depending on the pathosystem.

Table 1 | Summary of plant pathogen interaction studies referring to invertase modulations.

Microorganism	Plant	Effects on invertase	Additional features	References
BACTERIA				
Erwinia carotovora	Carrot	CWI (+)	Induction of *PAL*	Sturm and Chrispeels, 1990
Agrobacterium tumefaciens	*Ricinus communis*	CWI (+) VI (+)	Change in sugar content, ABA synthesis	Wachter et al., 2003
Xanthomonas campestris pv *vesicatoria*	Tomato	CWI (+)	Change in sugar content, induction of senescence-associated and *PR* genes	Kocal et al., 2008
Xanthomonas campestris pv *vesicatoria*	Pepper	CWI (+)	Induction of defense response *PR-Q*	Sonnewald et al., 2012
Bois noir	Grapevine	CWI (+)	Callose deposition, modulation of *SUC* genes	Santi et al., 2013a,b
Xanthomonas oryzae pv. *oryzae*	Rice	CWI (+)	Change in sugar content, callose deposition, induction of *PR* genes, ROS accumulation	Sun et al., 2013
FUNGI				
Biotrophic				
Erysiphe pisi	*Pisum sativum*	CWI/VI (+) A/NInv (+)	Decrease of starch content	Storr and Hall, 1992
Puccinia hordei	Barley	CWI/VI (+)	ND	Tetlow and Farrar, 1992
Blumeria graminis	Barley	CWI (+) VI (+)	Change in sugar content, down-regulation of photosynthesis, callose deposition, induction of defense response *PR-1*	Scholes et al., 1994; Wright et al., 1995; Swarbrick et al., 2006
Blumeria graminis	Wheat	CWI (+) VI (+)	ND	Greenshields et al., 2004
Blumeria graminis	Wheat	CWI (+) VI (+) A/NInv (+)	Change in sugar content	Sutton et al., 2007
Albugo candida	*A. thaliana*	CWI (+) VI (/)	Change in sugar content, decrease of starch content, down-regulation of photosynthesis, decrease chloropyll content, induction of defense proteins	Chou et al., 2000
Erysiphe cichoracearum	*A. thaliana*	CWI (+)	Induction of *HXT* genes	Fotopoulos et al., 2003
Uromyces fabae	*Vicia faba*	CWI (+) VI (−)	ND	Voegele et al., 2006
Erysiphe necator	*Vitis vinifera*	CWI (+) VI (−)	Induction of *HXT* and ABA biosynthesis-associated genes	Hayes et al., 2010
Hemibiotrophic				
Magnaporthe grisea	Rice	CWI (+)	Change in sugar content, callose deposition, induction of *PR* genes, ROS accumulation	Cho et al., 2005; Sun et al., 2013
Necrotrophic				
Fusarium oxysporum	Tomato	CWI (+)	ND	Benhamou et al., 1991
Botrytis cinerea	Tomato	CWI (+) VI (/)	ND	Hyun et al., 2011
Symbiotic				
Glomus intraradices	Tomato	CWI (+)	ND	Schaarschmidt et al., 2006
Glomus intraradices	Tobacco	CWI (+)	Change in sugar content, exchange of nutrients, decrease chloropyll content, induction of *PR* genes	Schaarschmidt et al., 2007
OOMYCETES				
Phytophthora nicotianae	Tobacco	CWI (+) VI (/) A/NInv (+)	Down-regulation of photosynthesis, callose deposition, induction of *PR* and *PAL* genes	Scharte et al., 2005; Essmann et al., 2008a,b
Plasmopara viticola	*Vitis vinifera*	CWI (+)	Induction of *HXT* and ABA biosynthesis-associated genes	Hayes et al., 2010
RHIZARIA				
Plasmodiophora brassicae	*A. thaliana*	CWI (+) VI (+)	ND	Siemens et al., 2011
NEMATODE				
Heterodera schachtii	*A. thaliana*	CWI (−) VI (−) A/NInv (−)	Change in sugar content	Cabello et al., 2013
Meloidogyne javanica	*A. thaliana*	CWI (−) VI (−) A/NInv (+/−)	Change in sugar content	
VIRUS				
Potato virus Y	Tobacco	CWI (+) VI (/)	Down-regulation of photosynthesis, induction of *PR* genes, callose deposition	Herbers et al., 2000
Beet severe curly top virus	*A. thaliana*	CWI (+)	Callus-like structures, induction cell cycle-related genes	Park et al., 2013

Abbreviations: (+), up-regulation; (−), down-regulation; (/), no change; ABA, abscisic acid; HXT, hexose transporter; PR, pathogenesis-related; ROS, reactive oxygen species; SUC, sucrose transporter; ND, not described.

DEFENSE-INDUCED FEATURES AFFECTED BY SUCROSE AND INVERTASES

CLOCK, PHOTOSYNTHESIS, AND SUGAR CONTENT

The connections between the clock, the sugars and the immunity have been previously presented (Roden and Ingle, 2009; Bolouri Moghaddam and Van Den Ende, 2013) and here we discuss the latest updates on this interconnectivity. Exogenous sucrose is able to stimulate the circadian clock by inhibiting photosynthesis and to coordinate answers during the light-dark cycles (Knight et al., 2008; Dalchau et al., 2011; Haydon et al., 2013). A new metabolic feedback loop involving the morning-expressed *pseudo response regulator 7* (*prr7*) gene was proposed by Haydon et al. (2013). At dawn, the light activates PRR7 and photosynthesis, then the photosynthetically produced derived sugars accumulate and repress the PRR7 promoter which causes the de-repression of the molecular oscillator component circadian clock associated 1 (CCA1) (Haydon et al., 2013). The clock-related genes (*cca1* and *lhy*) affect stomatal aperture after pathogen infection and suggest a crucial role of circadian clock in plant defense response (Wang et al., 2011; Zhang et al., 2013). Diurnal rhythm has been shown to regulate a CWI (LIN6) from tomato and that both CCA1 and LHY activate the *Lin6* promoter (Proels and Roitsch, 2009). During pathogen attack, the increase of CWI activity leading to an accumulation of hexoses is associated with a down-regulation of photosynthesis and expression of genes-related to photosynthesis (**Table 1**). It is noteworthy that transgenic infected plants silenced for CWI showed a delay in the reduction of photosynthesis (Kocal et al., 2008). Thus, the cross-talk between clock, sucrose and invertases tends to illustrate that a fine regulation of the sucrose/hexose ratio is crucial in defense regulation (Haydon et al., 2013).

During the day, both sucrose and starch are produced during photosynthesis. During the night, the starch, accumulated in the chloroplasts, is subsequently degraded to provide substrates for sucrose synthesis. Starch synthesis can be regulated by sucrose and clock by modulating the expression of starch synthase (Wang et al., 2001). After pathogen infection, a decrease in the starch content is observed in the infected region suggesting that the degradation of starch provides more substrates to sucrose synthesis. Interestingly, Engelsdorf et al. tested the susceptibility of starch-free *A. thaliana* mutants against biotrophic, hemibiotrophic and necrotrophic pathogens and pointed out that depending on the studied pathosystem the diurnal carbon availability is a susceptibility factor (Engelsdorf et al., 2013). Their results imply that sugar availability might impact the ability of plants to trigger defense responses.

One of the other possibilities for changing the sugar content is the regulation of the expression of the sucrose transporter. Sucrose acts on carbohydrate partitioning and phloem loading by modulating the sucrose transporter expression, such as inducing the expression of *SUT2* in tomatoes or repressing the expression of *BvSUT1* in beet (Barker et al., 2000; Vaughn et al., 2002). Depending on the stage of infection, the expression of sucrose transporters can be altered and as a consequence the sucrose partitioning can be modified. In rice infected by *Xanthomonas oryzae* pv. *Oryzae*, SWEET proteins are upregulated and sucrose accumulates in apoplast ready to be used for the pathogen growth (Chen et al., 2010, 2012). Santi et al. reported a sequential regulation of sucrose transporter genes which are first downregulated during infection of grapevine by stolbur to limit the spread and then upregulated during the recovery stage providing necessary nutrients (Santi et al., 2013a,b). It is noteworthy that during fungal infection the expression of CWI and hexose transporters displayed a correlation enhancing the hexoses supply from the phloem to the surrounding tissues during the transition from source to sink (Fotopoulos et al., 2003; Hayes et al., 2010). Moreover, Hayes et al. reported a relationship between CWI, hexose transporters and ABA biosynthesis during the transition from source to sink after infection (Hayes et al., 2010).

PHYTOHORMONES

For different phytohormones such as ABA, gibberellins, ethylene and jasmonate, it was shown that they interact with the sucrose signaling pathway (Finkelstein et al., 2002; Leon and Sheen, 2003; Gibson, 2004; Heil et al., 2012). Their implication in plant defense response and the relationship with sugars have been widely discussed in various reviews (Bolouri Moghaddam and Van Den Ende, 2012, 2013).

PATHOGENESIS RELATED PROTEINS

PR proteins are synthesized in response to plant pathogen attack. Their classification and their properties have been well described (for reviews see Kitajima and Sato, 1999; Van Loon et al., 2006; Sels et al., 2008). As reported in several studies, the up-regulation of CWI due to the infection goes along with the induction of *PR* genes (**Table 1**) such as PR-1a, PR-1b, PR3, PR10, WRKY45, and NPR1 in rice (Sun et al., 2013), PR-1b and PR-Q in tobacco (Herbers et al., 1996; Schaarschmidt et al., 2007; Essmann et al., 2008b) and PR-Q, Pin-II and GluB in tomato (Kocal et al., 2008). During transgenic approaches the overexpression of CWI in tobacco or in rice presented constitutively high levels of *PR* transcripts compared to the wild type plants (Herbers et al., 1996; Sun et al., 2013). To support this idea, in different cases of infected transgenic plants silenced for CWI, the induction of *PR* genes was abolished (Schaarschmidt et al., 2007; Essmann et al., 2008b; Kocal et al., 2008). Thus CWI activity is required to enhance the expression of *PR* genes mediated by the accumulated hexoses which act as signal molecules. Besides, exogenous sucrose induced the expression of *PR* genes (Thibaud et al., 2004; Gomez-Ariza et al., 2007) confirming the idea of sucrose as an important signal molecule for plant defense response.

PHENYLPROPANOID PATHWAY

The phenylalanine ammonia-lyase (PAL), a key enzyme which is involved in the phenylpropanoid pathway, leads to the biosynthesis of lignin and the production of many other important compounds such as the flavonoids, coumarins and lignans (for review see Dixon and Paiva, 1995). During infection of lupine by *Fusarium oxysporum*, sucrose induced the phenylpropanoid metabolism by stimulating the activity of PAL (Morkunas et al., 2005, 2011). Sturm and Chrispeels showed an accumulation of PAL mRNA subsequently to the increase of CWI mRNA in carrot infected by *Erwinia carotovora* (Sturm and Chrispeels, 1990). Moreover when tobacco plants are silenced for CWI, the PAL

activity is delayed after infection compared with the wild type plants (Essmann et al., 2008b). Hence, the regulation of PAL is mediated by the variation of the sucrose/hexose ratio. All in all, these results demonstrate that the regulation of the expression of PAL is sugar-related.

Anthocyanin (a flavonoid) has an antimicrobial potential reducing the spread of the pathogens. The synthesis of anthocyanin is regulated by sucrose signaling pathway (Solfanelli et al., 2006) through the induction of the *PAP1/MYB75* transcription factor (Teng et al., 2005) and ABA and jasmonate pathways have a synergic effect (Loreti et al., 2008). This induction is repressed by gibberellins. At concentrations of sucrose higher than 2% the anthocyanin synthesis is induced independently of the ABA signaling pathway (Dai et al., 2014). Recently, a key positive regulator in the sucrose signaling pathway controlling the anthocyanin synthesis has been identified as the DELLA protein which targets *PAP1/MYB75* (Li et al., 2014).

In potato tubers, a transcription factor (*AN1*) was proposed to up-regulate the phenylpropanoid pathway. The authors suggested that *PAL* might be induced by *AN1* after sucrose feeding. Moreover they proposed a loop in which sucrose increases *AN1* expression while *AN1* induces sucrolytic enzymes which release hexoses used by the phenylpropanoid pathway (Payyavula et al., 2013). By the synthesis of secondary metabolites such as phenolic compounds or later on lignin, plants produce chemical and physical barriers against pathogens.

CELL WALL REINFORCEMENT

As another physical barrier, there is the deposition of callose, a β-(1,3)-glucan cell wall polymer, which is a stress related process limiting invasion by regulating the plasmodesmata and the sieve plates permeability (Chen and Kim, 2009; Luna et al., 2011). In tobacco plants overexpressing a yeast invertase in the apoplast or in the vacuole, the increase of callose deposition was comparable to that observed in wild type plants infected with potato virus Y (Herbers et al., 1996). These results were consistent with a positive regulation of callose deposition by GIF1 in rice after infection by both, bacterial and fungal pathogens (Sun et al., 2013), leading to a regulation mediated by CWI activity. Increasing concentrations of the exogenous sucrose repressed the callose deposition in *A. thaliana* cells (Luna et al., 2011) suggesting that hexose cleavage products of sucrose are responsible for the formation of the physical barrier against invading pathogens through cell wall reinforcement.

CONCLUSION AND PERSPECTIVES

Due to a high demand in carbohydrates during infection, plants evolved strategies to modulate their carbohydrate availability and trigger to defense responses. In most of the studied pathosystems, sucrose seems to act as a "priming" agent activating a cascade of signaling pathways such as the modulation of circadian clock genes, phytohormones, cell wall strength and cellular signaling pathways.

A rapid induction of CWIs after infection increases the hexose content and modulates sink strength. It has been demonstrated that CWIs are essential for triggering an appropriate answer during pathogen invasion. The accumulation of hexoses leads to an induction of the *PR* genes, a down-regulation of the photosynthesis, and an establishment of the chemical and physical barriers. A/NInvs, which are induced afterwards, might be involved in providing more energy during infection. The exact function of the VIs remains unclear but they might release stored carbohydrates and allow reserves mobilization. Moreover, the specificity of plant response depending on the studied pathosystem might be interesting points to investigate.

A better understanding of the "sweet immunity" and the complex network between sucrose, circadian clock and phytohormones might be useful to avoid substantial losses in yield and quality of crops every year. Recently, these biotic elicitors were proposed as interesting elements to generate ready-to-eat cruciferous vegetables and maximize their health-promoting compounds (Baenas et al., 2014).

AUTHOR CONTRIBUTIONS

Alexandra S. Tauzin and Thierry Giardina contribute equally to the writing of this review.

ACKNOWLEDGMENTS

We would like to gratefully thank Dr. Tyler Yin for assistance in manuscript preparation. This work was supported by the Ministère de l'Enseignement Supérieur et de la Recherche Scientifique.

REFERENCES

Ausubel, F. M. (2005). Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* 6, 973–979. doi: 10.1038/ni1253

Baenas, N., Garcia-Viguera, C., and Moreno, D. A. (2014). Biotic elicitors effectively increase the glucosinolates content in brassicaceae sprouts. *J. Agric. Food. Chem.* 62, 1881–1889. doi: 10.1021/jf404876z

Barker, L., Kuhn, C., Weise, A., Schulz, A., Gebhardt, C., Hirner, B., et al. (2000). SUT2, a putative sucrose sensor in sieve elements. *Plant Cell* 12, 1153–1164. doi: 10.1105/tpc.12.7.1153

Barratt, D. H., Derbyshire, P., Findlay, K., Pike, M., Wellner, N., Lunn, J., et al. (2009). Normal growth of Arabidopsis requires cytosolic invertase but not sucrose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13124–13129. doi: 10.1073/pnas.0900689106

Benhamou, N., Grenier, J., and Chrispeels, M. J. (1991). Accumulation of beta-fructosidase in the cell walls of tomato roots following infection by a fungal wilt pathogen. *Plant Physiol.* 97, 739–750. doi: 10.1104/pp.97.2.739

Bolouri Moghaddam, M. R., and Van Den Ende, W. (2012). Sugars and plant innate immunity. *J. Exp. Bot.* 63, 3989–3998. doi: 10.1093/jxb/ers129

Bolouri Moghaddam, M. R., and Van Den Ende, W. (2013). Sweet immunity in the plant circadian regulatory network. *J. Exp. Bot.* 64, 1439–1449. doi: 10.1093/jxb/ert046

Bonfig, K. B., Gabler, A., Simon, U. K., Luschin-Ebengreuth, N., Hatz, M., Berger, S., et al. (2010). Post-translational derepression of invertase activity in source leaves via down-regulation of invertase inhibitor expression is part of the plant defense response. *Mol. Plant* 3, 1037–1048. doi: 10.1093/mp/ssq053

Cabello, S., Lorenz, C., Crespo, S., Cabrera, J., Ludwig, R., Escobar, C., et al. (2013). Altered sucrose synthase and invertase expression affects the local and systemic sugar metabolism of nematode-infected *Arabidopsis thaliana* plants. *J. Exp. Bot.* 65, 201–212. doi: 10.1093/jxb/ert359

Chen, L. Q., Hou, B. H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X. Q., et al. (2010). Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* 468, 527–532. doi: 10.1038/nature09606

Chen, L. Q., Qu, X. Q., Hou, B. H., Sosso, D., Osorio, S., Fernie, A. R., et al. (2012). Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Science* 335, 207–211. doi: 10.1126/science.1213351

Chen, X. Y., and Kim, J. Y. (2009). Callose synthesis in higher plants. *Plant Signal. Behav.* 4, 489–492. doi: 10.4161/psb.4.6.8359

Cho, J. I., Lee, S. K., Ko, S., Kim, H. K., Jun, S. H., Lee, Y. H., et al. (2005). Molecular cloning and expression analysis of the cell-wall invertase gene family in rice (*Oryza sativa* L.). *Plant Cell Rep.* 24, 225–236. doi: 10.1007/s00299-004-0910-z

Cho, J. I., Ryoo, N., Eom, J. S., Lee, D. W., Kim, H. B., Jeong, S. W., et al. (2009). Role of the rice hexokinases OsHXK5 and OsHXK6 as glucose sensors. *Plant Physiol.* 149, 745–759. doi: 10.1104/pp.108.131227

Cho, Y. H., and Yoo, S. D. (2011). Signaling role of fructose mediated by FINS1/FBP in Arabidopsis thaliana. *PLoS Genet.* 7:e1001263. doi: 10.1371/journal.pgen.1001263

Chou, H. M., Bundock, N., Rolfe, S. A., and Scholes, J. D. (2000). Infection of Arabidopsis thaliana leaves with Albugo candida (white blister rust) causes a reprogramming of host metabolism. *Mol. Plant Pathol.* 1, 99–113. doi: 10.1046/j.1364-3703.2000.00013.x

Dai, Z. W., Meddar, M., Renaud, C., Merlin, I., Hilbert, G., Delrot, S., et al. (2014). Long-term *in vitro* culture of grape berries and its application to assess the effects of sugar supply on anthocyanin accumulation. *J. Exp. Bot*. doi: 10.1093/jxb/ert489. [Epub ahead of print].

Dalchau, N., Baek, S. J., Briggs, H. M., Robertson, F. C., Dodd, A. N., Gardner, M. J., et al. (2011). The circadian oscillator gene GIGANTEA mediates a long-term response of the Arabidopsis thaliana circadian clock to sucrose. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5104–5109. doi: 10.1073/pnas.1015452108

Dangl, J. L., and Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411, 826–833. doi: 10.1038/35081161

Dixon, R. A., and Paiva, N. L. (1995). Stress-induced phenylpropanoid metabolism. *Plant Cell* 7, 1085–1097. doi: 10.1105/tpc.7.7.1085

Engelsdorf, T., Horst, R. J., Prols, R., Proschel, M., Dietz, F., Huckelhoven, R., et al. (2013). Reduced carbohydrate availability enhances the susceptibility of Arabidopsis toward Colletotrichum higginsianum. *Plant Physiol.* 162, 225–238. doi: 10.1104/pp.112.209676

Essmann, J., Bones, P., Weis, E., and Scharte, J. (2008a). Leaf carbohydrate metabolism during defense: intracellular sucrose-cleaving enzymes do not compensate repression of cell wall invertase. *Plant Signal Behav.* 3, 885–887. doi: 10.4161/psb.3.10.6501

Essmann, J., Schmitz-Thom, I., Schon, H., Sonnewald, S., Weis, E., and Scharte, J. (2008b). RNA interference-mediated repression of cell wall invertase impairs defense in source leaves of tobacco. *Plant Physiol.* 147, 1288–1299. doi: 10.1104/pp.108.121418

Finkelstein, R. R., Gampala, S. S., and Rock, C. D. (2002). Abscisic acid signaling in seeds and seedlings. *Plant Cell* 14(Suppl.), S15–S45. doi: 10.1105/tpc.010441

Fotopoulos, V., Gilbert, M. J., Pittman, J. K., Marvier, A. C., Buchanan, A. J., Sauer, N., et al. (2003). The monosaccharide transporter gene, AtSTP4, and the cell-wall invertase, Atbetafruct1, are induced in Arabidopsis during infection with the fungal biotroph *Erysiphe cichoracearum*. *Plant Physiol.* 132, 821–829. doi: 10.1104/pp.103.021428

Gibson, S. I. (2004). Sugar and phytohormone response pathways: navigating a signalling network. *J. Exp. Bot.* 55, 253–264. doi: 10.1093/jxb/erh048

Gomez-Ariza, J., Campo, S., Rufat, M., Estopa, M., Messeguer, J., San Segundo, B., et al. (2007). Sucrose-mediated priming of plant defense responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. *Mol. Plant Microbe Interact.* 20, 832–842. doi: 10.1094/MPMI-20-7-0832

Greenshields, D. L., Wang, F., Selvaraj, G., and Wei, Y. D. (2004). Activity and gene expression of acid invertases in einkorn wheat (Triticum monococcum) infected with powdery mildew. *Can. J. Plant Pathol. Rev. Can. Phytopathol.* 26, 506–513. doi: 10.1080/07060660409507171

Greiner, S., Krausgrill, S., and Rausch, T. (1998). Cloning of a tobacco apoplasmic invertase inhibitor. Proof of function of the recombinant protein and expression analysis during plant development. *Plant Physiol.* 116, 733–742. doi: 10.1104/pp.116.2.733

Haydon, M. J., Mielczarek, O., Robertson, F. C., Hubbard, K. E., and Webb, A. A. (2013). Photosynthetic entrainment of the Arabidopsis thaliana circadian clock. *Nature* 502, 689–692. doi: 10.1038/nature12603

Hayes, M. A., Feechan, A., and Dry, I. B. (2010). Involvement of abscisic acid in the coordinated regulation of a stress-inducible hexose transporter (VvHT5) and a cell wall invertase in grapevine in response to biotrophic fungal infection. *Plant Physiol.* 153, 211–221. doi: 10.1104/pp.110.154765

Heil, M., Ibarra-Laclette, E., Adame-Alvarez, R. M., Martinez, O., Ramirez-Chavez, E., Molina-Torres, J., et al. (2012). How plants sense wounds: damaged-self recognition is based on plant-derived elicitors and induces octadecanoid signaling. *PLoS ONE* 7:e30537. doi: 10.1371/journal.pone.0030537

Herbers, K., Meuwly, P., Frommer, W. B., Metraux, J. P., and Sonnewald, U. (1996). Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. *Plant Cell* 8, 793–803. doi: 10.1105/tpc.8.5.793

Herbers, K., Takahata, Y., Melzer, M., Mock, H. P., Hajirezaei, M., and Sonnewald, U. (2000). Regulation of carbohydrate partitioning during the interaction of potato virus Y with tobacco. *Mol. Plant Pathol.* 1, 51–59. doi: 10.1046/j.1364-3703.2000.00007.x

Hothorn, M., Wolf, S., Aloy, P., Greiner, S., and Scheffzek, K. (2004). Structural insights into the target specificity of plant invertase and pectin methylesterase inhibitory proteins. *Plant Cell* 16, 3437–3447. doi: 10.1105/tpc.104.025684

Hyun, T. K., Eom, S. H., Rim, Y., and Kim, J. S. (2011). Alteration of the expression and activation of tomato invertases during Botrytis cinerea infection. *Plant Omics* 4, 413–417.

Jia, L., Zhang, B., Mao, C., Li, J., Wu, Y., Wu, P., et al. (2008). OsCYT-INV1 for alkaline/neutral invertase is involved in root cell development and reproductivity in rice (*Oryza sativa* L.). *Planta* 228, 51–59. doi: 10.1007/s00425-008-0718-0

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Katagiri, F., and Tsuda, K. (2010). Understanding the plant immune system. *Mol. Plant Microbe. Interact.* 23, 1531–1536. doi: 10.1094/MPMI-04-10-0099

Kitajima, S., and Sato, F. (1999). Plant pathogenesis-related proteins: molecular mechanisms of gene expression and protein function. *J. Biochem.* 125, 1–8. doi: 10.1093/oxfordjournals.jbchem.a022244

Knight, H., Thomson, A. J., and McWatters, H. G. (2008). Sensitive to freezing6 integrates cellular and environmental inputs to the plant circadian clock. *Plant Physiol.* 148, 293–303. doi: 10.1104/pp.108.123901

Kocal, N., Sonnewald, U., and Sonnewald, S. (2008). Cell wall-bound invertase limits sucrose export and is involved in symptom development and inhibition of photosynthesis during compatible interaction between tomato and Xanthomonas campestris pv vesicatoria. *Plant Physiol.* 148, 1523–1536. doi: 10.1104/pp.108.127977

Koch, K. (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Curr. Opin. Plant Biol.* 7, 235–246. doi: 10.1016/j.pbi.2004.03.014

Lammens, W., Le Roy, K., Schroeven, L., Van Laere, A., Rabijns, A., and Van Den Ende, W. (2009). Structural insights into glycoside hydrolase family 32 and 68 enzymes: functional implications. *J. Exp. Bot.* 60, 727–740. doi: 10.1093/jxb/ern333

Leon, P., and Sheen, J. (2003). Sugar and hormone connections. *Trends Plant Sci.* 8, 110–116. doi: 10.1016/S1360-1385(03)00011-6

Li, P., Wind, J. J., Shi, X., Zhang, H., Hanson, J., Smeekens, S. C., et al. (2011). Fructose sensitivity is suppressed in Arabidopsis by the transcription factor ANAC089 lacking the membrane-bound domain. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3436–3441. doi: 10.1073/pnas.1018665108

Li, Y., Van Den Ende, W., and Rolland, F. (2014). Sucrose induction of anthocyanin biosynthesis is mediated by della. *Mol. Plant* 7, 570–572. doi: 10.1093/mp/sst161

Loreti, E., Povero, G., Novi, G., Solfanelli, C., Alpi, A., and Perata, P. (2008). Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in Arabidopsis. *New Phytol.* 179, 1004–1016. doi: 10.1111/j.1469-8137.2008.02511.x

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. (2011). Callose deposition: a multifaceted plant defense response. *Mol. Plant Microbe Interact.* 24, 183–193. doi: 10.1094/MPMI-07-10-0149

Maresca, M. (2013). From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. *Toxins (Basel)* 5, 784–820. doi: 10.3390/toxins5040784

Martin, M. L., Lechner, L., Zabaleta, E. J., and Salerno, G. L. (2013). A mitochondrial alkaline/neutral invertase isoform (A/N-InvC) functions in developmental energy-demanding processes in Arabidopsis. *Planta* 237, 813–822. doi: 10.1007/s00425-012-1794-8

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W. H., Liu, Y. X., et al. (2003). Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* 300, 332–336. doi: 10.1126/science.1080585

Morkunas, I., Marczak, L., Stachowiak, J., and Stobiecki, M. (2005). Sucrose-induced lupine defense against Fusarium oxysporum. Sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to Fusarium

oxysporum. *Plant Physiol. Biochem.* 43, 363–373. doi: 10.1016/j.plaphy.2005.02.011

Morkunas, I., Narozna, D., Nowak, W., Samardakiewicz, S., and Remlein-Starosta, D. (2011). Cross-talk interactions of sucrose and Fusarium oxysporum in the phenylpropanoid pathway and the accumulation and localization of flavonoids in embryo axes of yellow lupine. *J. Plant Physiol.* 168, 424–433. doi: 10.1016/j.jplph.2010.08.017

Park, J., Kim, S., Choi, E., Auh, C. K., Park, J. B., Kim, D. G., et al. (2013). Altered invertase activities of symptomatic tissues on Beet severe curly top virus (BSCTV) infected Arabidopsis thaliana. *J. Plant Res.* 126, 743–752. doi: 10.1007/s10265-013-0562-6

Payyavula, R. S., Singh, R. K., and Navarre, D. A. (2013). Transcription factors, sucrose, and sucrose metabolic genes interact to regulate potato phenylpropanoid metabolism. *J. Exp. Bot.* 64, 5115–5131. doi: 10.1093/jxb/ert303

Proels, R. K., and Roitsch, T. (2009). Extracellular invertase LIN6 of tomato: a pivotal enzyme for integration of metabolic, hormonal, and stress signals is regulated by a diurnal rhythm. *J. Exp. Bot.* 60, 1555–1567. doi: 10.1093/jxb/erp027

Roden, L. C., and Ingle, R. A. (2009). Lights, rhythms, infection: the role of light and the circadian clock in determining the outcome of plant-pathogen interactions. *Plant Cell* 21, 2546–2552. doi: 10.1105/tpc.109.069922

Roitsch, T., and Gonzalez, M. C. (2004). Function and regulation of plant invertases: sweet sensations. *Trends Plant Sci.* 9, 606–613. doi: 10.1016/j.tplants.2004.10.009

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.* 57, 675–709. doi: 10.1146/annurev.arplant.57.032905.105441

Santi, S., De Marco, F., Polizzotto, R., Grisan, S., and Musetti, R. (2013a). Recovery from stolbur disease in grapevine involves changes in sugar transport and metabolism. *Front Plant Sci.* 4:171. doi: 10.3389/fpls.2013.00171

Santi, S., Grisan, S., Pierasco, A., Marco, F., and Musetti, R. (2013b). Laser microdissection of grapevine leaf phloem infected by stolbur reveals site-specific gene responses associated to sucrose transport and metabolism. *Plant Cell Environ.* 36, 343–355. doi: 10.1111/j.1365-3040.2012.02577.x

Schaarschmidt, S., Kopka, J., Ludwig-Muller, J., and Hause, B. (2007). Regulation of arbuscular mycorrhization by apoplastic invertases: enhanced invertase activity in the leaf apoplast affects the symbiotic interaction. *Plant J.* 51, 390–405. doi: 10.1111/j.1365-313X.2007.03150.x

Schaarschmidt, S., Roitsch, T., and Hause, B. (2006). Arbuscular mycorrhiza induces gene expression of the apoplastic invertase LIN6 in tomato (*Lycopersicon esculentum*) roots. *J. Exp. Bot.* 57, 4015–4023. doi: 10.1093/jxb/erl172

Scharte, J., Schon, H., and Weis, E. (2005). Photosynthesis and carbohydrate metabolism in tobacco leaves during an incompatible interaction with Phytophthora nicotianae. *Plant Cell Environ.* 28, 1421–1435. doi: 10.1111/j.1365-3040.2005.01380.x

Scholes, J. D., Lee, P. J., Horton, P., and Lewis, D. H. (1994). Invertase - understanding changes in the photosynthetic and carbohydrate-metabolism of barley leaves infected with powdery mildew. *New Phytol.* 126, 213–222. doi: 10.1111/j.1469-8137.1994.tb03939.x

Sels, J., Mathys, J., De Coninck, B. M., Cammue, B. P., and De Bolle, M. F. (2008). Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiol. Biochem.* 46, 941–950. doi: 10.1016/j.plaphy.2008.06.011

Siemens, J., Gonzalez, M. C., Wolf, S., Hofmann, C., Greiner, S., Du, Y., et al. (2011). Extracellular invertase is involved in the regulation of clubroot disease in Arabidopsis thaliana. *Mol. Plant Pathol.* 12, 247–262. doi: 10.1111/j.1364-3703.2010.00667.x

Solfanelli, C., Poggi, A., Loreti, E., Alpi, A., and Perata, P. (2006). Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. *Plant Physiol* 140, 637–646. doi: 10.1104/pp.105.072579

Sonnewald, S., Priller, J. P., Schuster, J., Glickmann, E., Hajirezaei, M. R., Siebig, S., et al. (2012). Regulation of cell wall-bound invertase in pepper leaves by Xanthomonas campestris pv. vesicatoria type three effectors. *PLoS ONE* 7:e51763. doi: 10.1371/journal.pone.0051763

Storr, T., and Hall, J. L. (1992). The Effect of Infection by Erysiphe-Pisi Dc on Acid and Alkaline Invertase Activities and Aspects of Starch Biochemistry in Leaves of Pisum-Sativum L. *New Phytol.* 121, 535–543. doi: 10.1111/j.1469-8137.1992.tb01123.x

Sturm, A., and Chrispeels, M. J. (1990). cDNA cloning of carrot extracellular beta-fructosidase and its expression in response to wounding and bacterial infection. *Plant Cell* 2, 1107–1119.

Sun, L., Yang, D. L., Kong, Y., Chen, Y., Li, X. Z., Zeng, L. J., et al. (2013). Sugar homeostasis mediated by cell wall invertase GRAIN INCOMPLETE FILLING 1 (GIF1) plays a role in pre-existing and induced defence in rice. *Mol. Plant. Pathol.* 15, 161–173. doi: 10.1111/mpp.12078

Sutton, P. N., Gilbert, M. J., Williams, L. E., and Hall, J. L. (2007). Powdery mildew infection of wheat leaves changes host solute transport and invertase activity. *Physiol. Plant.* 129, 787–795. doi: 10.1111/j.1399-3054.2007.00863.x

Swarbrick, P. J., Schulze-Lefert, P., and Scholes, J. D. (2006). Metabolic consequences of susceptibility and resistance (race-specific and broad-spectrum) in barley leaves challenged with powdery mildew. *Plant Cell Environ.* 29, 1061–1076. doi: 10.1111/j.1365-3040.2005.01472.x

Tauzin, A. S., Sulzenbacher, G., Lafond, M., Desseaux, V., Reca, I. B., Perrier, J., et al. (2014). Functional characterization of a vacuolar invertase from Solanum lycopersicum: post-translational regulation by N-glycosylation and a proteinaceous inhibitor. *Biochimie* 101, 39–49. doi: 10.1016/j.biochi.2013.12.013

Teng, S., Keurentjes, J., Bentsink, L., Koornneef, M., and Smeekens, S. (2005). Sucrose-specific induction of anthocyanin biosynthesis in Arabidopsis requires the MYB75/PAP1 gene. *Plant Physiol.* 139, 1840–1852. doi: 10.1104/pp.105.066688

Tetlow, I. J., and Farrar, J. F. (1992). Sucrose-Metabolizing Enzymes from leaves of barley infected with brown rust (Puccinia-Hordei Otth). *New Phytol.* 120, 475–480. doi: 10.1111/j.1469-8137.1992.tb01795.x

Thibaud, M. C., Gineste, S., Nussaume, L., and Robaglia, C. (2004). Sucrose increases pathogenesis-related PR-2 gene expression in Arabidopsis thaliana through an SA-dependent but NPR1-independent signaling pathway. *Plant Physiol. Biochem.* 42, 81–88. doi: 10.1016/j.plaphy.2003.10.012

Thines, E., Weber, R. W., and Talbot, N. J. (2000). MAP kinase and protein kinase A-dependent mobilization of triacylglycerol and glycogen during appressorium turgor generation by Magnaporthe grisea. *Plant Cell* 12, 1703–1718. doi: 10.1105/tpc.12.9.1703

Tognetti, J. A., Pontis, H. G., and Martinez-Noel, G. M. (2013). Sucrose signaling in plants: A world yet to be explored. *Plant Signal Behav.* 8:e23316. doi: 10.4161/psb.23316

Van Loon, L. C., Rep, M., and Pieterse, C. M. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162. doi: 10.1146/annurev.phyto.44.070505.143425

Vargas, W. A., and Salerno, G. L. (2010). The Cinderella story of sucrose hydrolysis: Alkaline/neutral invertases, from cyanobacteria to unforeseen roles in plant cytosol and organelles. *Plant Sci.* 178, 1–8. doi: 10.1016/j.plantsci.2009.09.015

Vaughn, M. W., Harrington, G. N., and Bush, D. R. (2002). Sucrose-mediated transcriptional regulation of sucrose symporter activity in the phloem. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10876–10880. doi: 10.1073/pnas.172198599

Voegele, R. T., Wirsel, S., Moll, U., Lechner, M., and Mendgen, K. (2006). Cloning and characterization of a novel invertase from the obligate biotroph Uromyces fabae and analysis of expression patterns of host and pathogen invertases in the course of infection. *Mol. Plant Microbe Interact.* 19, 625–634. doi: 10.1094/MPMI-19-0625

Wachter, R., Langhans, M., Aloni, R., Gotz, S., Weilmunster, A., Koops, A., et al. (2003). Vascularization, high-volume solution flow, and localized roles for enzymes of sucrose metabolism during tumorigenesis by Agrobacterium tumefaciens. *Plant Physiol.* 133, 1024–1037. doi: 10.1104/pp.103.028142

Wang, S. J., Yeh, K. W., and Tsai, C. Y. (2001). Regulation of starch granule-bound starch synthase I gene expression by circadian clock and sucrose in the source tissue of sweet potato. *Plant Sci.* 161, 635–644. doi: 10.1016/S0168-9452(01)00449-6

Wang, W., Barnaby, J. Y., Tada, Y., Li, H., Tor, M., Caldelari, D., et al. (2011). Timing of plant immune responses by a central circadian regulator. *Nature* 470, 110–114. doi: 10.1038/nature09766

Welham, T., Pike, J., Horst, I., Flemetakis, E., Katinakis, P., Kaneko, T., et al. (2009). A cytosolic invertase is required for normal growth and cell development in the model legume, lotus japonicus. *J. Exp. Bot.* 60, 3353–3365. doi: 10.1093/jxb/erp169

Wind, J., Smeekens, S., and Hanson, J. (2010). Sucrose: metabolite and signaling molecule. *Phytochemistry* 71, 1610–1614. doi: 10.1016/j.phytochem.2010.07.007

Wright, D. P., Baldwin, B. C., Shephard, M. C., and Scholes, J. D. (1995). Source-sink relationships in wheat leaves infected with powdery mildew.1. Alterations

in carbohydrate-metabolism. *Physiol. Mol. Plant Pathol.* 47, 237–253. doi: 10.1006/pmpp.1995.1055

Xiang, L., Le Roy, K., Bolouri-Moghaddam, M. R., Vanhaecke, M., Lammens, W., Rolland, F., et al. (2011). Exploring the neutral invertase-oxidative stress defence connection in Arabidopsis thaliana. *J. Exp. Bot.* 62, 3849–3862. doi: 10.1093/jxb/err069

Zhang, C., Xie, Q., Anderson, R. G., Ng, G., Seitz, N. C., Peterson, T., et al. (2013). Crosstalk between the circadian clock and innate immunity in Arabidopsis. *PLoS Pathog.* 9:e1003370. doi: 10.1371/journal.ppat.1003370

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 February 2014; accepted: 04 June 2014; published online: 23 June 2014.
Citation: Tauzin AS and Giardina T (2014) Sucrose and invertases, a part of the plant defense response to the biotic stresses. Front. Plant Sci. ***5****:293. doi: 10.3389/fpls.2014.00293*
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

PERSPECTIVE ARTICLE
published: 28 April 2014
doi: 10.3389/fpls.2014.00168

Callose-mediated resistance to pathogenic intruders in plant defense-related papillae

Christian A. Voigt*

Phytopathology and Biochemistry, Biocenter Klein Flottbek, University of Hamburg, Hamburg, Germany

Edited by:
Vincenzo Lionetti, Sapienza – Università di Roma, Italy

Reviewed by:
Hans Thordal-Christensen, University of Copenhagen, Denmark
Laurence Veronique Bindschedler, Royal Holloway University of London, UK

***Correspondence:**
Christian A. Voigt, Phytopathology and Biochemistry, Biocenter Klein Flottbek, University of Hamburg, Ohnhorststrasse 18, 22609 Hamburg, Germany
e-mail: christian.voigt@uni-hamburg.de

Plants are exposed to a wide range of potential pathogens, which derive from diverse phyla. Therefore, plants have developed successful defense mechanisms during co-evolution with different pathogens. Besides many specialized defense mechanisms, the plant cell wall represents a first line of defense. It is actively reinforced through the deposition of cell wall appositions, so-called papillae, at sites of interaction with intruding microbial pathogens. The papilla is a complex structure that is formed between the plasma membrane and the inside of the plant cell wall. Even though the specific biochemical composition of papillae can vary between different plant species, some classes of compounds are commonly found which include phenolics, reactive oxygen species, cell wall proteins, and cell wall polymers. Among these polymers, the (1,3)-β-glucan callose is one of the most abundant and ubiquitous components. Whereas the function of most compounds could be directly linked with cell wall reinforcement or an anti-microbial effect, the role of callose has remained unclear. An evaluation of recent studies revealed that the timing of the different papilla-forming transport processes is a key factor for successful plant defense.

Keywords: callose, defense response, innate immunity, penetration resistance, plant-pathogen interaction

INTRODUCTION

During plant-pathogen co-evolution, plants have evolved a range of defense mechanisms to prevent ingress and colonization by potential pathogens, which derive from diverse phyla and include fungi, oomycetes, animals, bacteria, and viruses. Successful plant defense depends on an early and rapid perception of the invading pathogen and subsequent induction and mobilization of biochemical and structural defense-related mechanisms. In most cases, plant defense to pathogens is successful and infection is the exception, which reflects the general effectiveness of the plant's immune response (Deverall, 1977; Smith, 1978; Bailey, 1983; Thordal-Christensen, 2003).

The perception of pathogens and the induced plant defense response generally follows two branches of the active immune system. R proteins, which are encoded by plant disease resistance (*R*) genes, control plant defense (Flor, 1971) by recognizing the presence of corresponding avirulence (avr) proteins or effectors deriving from the pathogen. This can occur through direct binding of the avr protein or effector, the binding of an effector-modified target, or recognition of an effector/target complex (Dodds and Rathjen, 2010). In extension to these binding possibilities, a current model suggests that R proteins can guard key cellular hubs, which might be common targets of effectors from different pathogen origin (Mukhtar et al., 2011). Even though most of the R proteins identified contain a nucleotide binding site (NBS) and leucine-rich repeats (LRRs), and are localized intracellularly, a growing number of R proteins has been discovered that contain membrane anchorage motifs, e.g., a myristoylation motif, and an extracellular LRR but lack a NBS (Dangl and Jones, 2001; Martin et al., 2003; Glowacki et al., 2011). Plant NBS-LRR- or atypical R protein-mediated pathogen resistance is only effective against obligate biotrophic or hemi-biotrophic pathogens that require living host tissue for propagation. However, plant NBS-LRR- or atypical R protein-mediated pathogen resistance is not effective against necrotrophic pathogens that macerate and degrade host tissue during colonization (Glazebrook, 2005). In the second arm of the active immune system, transmembrane pattern recognition receptors (PRR) that perceive microbe- or pathogen molecular patterns (MAMPs; Ausubel, 2005) respond to intruding pathogens (Jones and Takemoto, 2004; Nürnberger et al., 2004). MAMPs are characterized by their general occurrence in all members of a pathogen class and their often requirement for pathogen viability. Prominent examples for MAMPs are flagellin from bacterial pathogens and chitin from fungal pathogens, for which the respective PRR have been identified and characterized (Chinchilla et al., 2006; Kaku et al., 2006; Chinchilla et al., 2007; Miya et al., 2007). The widely accepted four phased Zig-Zag model from Jones and Dangl (2006) represents how these two branches of the plant immune system interact.

The plant immune system triggers a variety of defense mechanisms and include a hypersensitive response (HR) for rapid collapse of attacked host cells (Coll et al., 2011), production of anti-microbial phytoalexins (Bednarek and Osbourn, 2009), biosynthesis of enzymes, which can decompose pathogen cell walls (Mauch et al., 1988), and plant cell wall modifications; notably the deposition of papillae, which are enriched with the (1,3)-β-glucan cell wall polymer callose (Aist, 1976). These cell wall thickenings are formed at sites of microbial attack and are thought to act as a physical barrier to slow pathogen invasion (Stone and Clarke, 1992). Compared with many plant defense responses that can be specific to a phylum or even a species, the formation of

callose-rich papillae can be regarded as a ubiquitous response because it appears to be induced in essentially all plants following pathogen challenge.

THE ROLE OF CALLOSE IN PATHOGEN-INDUCED PAPILLAE

The formation of papillae is one of the earliest observed plant defense responses that has been analyzed on a cellular level for over 150 years. deBary (1863) discovered papillae at sites of fungal penetration, and Mangin (1895) reported that callose commonly occurs in papillae. Since then, chemical analyses have identified additional chemical components, which comprise phenolic compounds and lignin, an additional cell wall polymer to callose, reactive oxygen species (ROS), and cell wall proteins like peroxidases and antimicrobial thionins (Aist and Williams, 1971; Mercer et al., 1974; McLusky et al., 1999; Mims et al., 2000). Whereas the role of callose in papillae remained unclear, a function in defense could be attributed to most of the other components. For instance, hydrogen peroxide is a ROS that accumulates in forming papillae and can be used by peroxidases to promote cross-linking of proteins and phenolics to reinforce cell wall appositions (Thordal-Christensen et al., 1997; Brown et al., 1998).

In general, papillae formation is an early defense response and can contribute to the plant's innate immunity (Jones and Dangl, 2006; Schwessinger and Ronald, 2012). By slowing pathogen invasion in the attacked tissue, papillae formation can gain time for an induction of additional defense responses that may require gene activation and expression (Lamb and Dixon, 1997; Brown et al., 1998; Boller and Felix, 2009). However, the extent to which papillae and the deposited callose would contribute to the plant's innate immunity and penetration resistance has been subject to an ongoing discussion.

Callose-rich papillae were not only found in cases of successful resistance but also at sites of pathogen penetration (Aist, 1976). In this regard, the proposed function of callose in strengthening cell wall appositions and contributing to penetration resistance was further challenged by studies using *Arabidopsis* (*Arabidopsis thaliana*) mutants. Disruption mutants that lack the stress-induced callose synthase PMR4 [POWDERY MILDEW RESISTANT 4; also known as GSL5 (GLUCAN SYNTHASE-LIKE 5)] and do not deposit callose at sites of attempted fungal penetration, showed an unexpected, increased resistance to powdery mildew species (Jacobs et al., 2003; Nishimura et al., 2003). This result revealed that in *Arabidopsis* wild-type leaves, callose levels at penetration sites do not contribute to penetration resistance to adapted powdery mildews. However, callose deposition is required to maintain the high penetration resistance to the non-adapted powdery mildew *Blumeria graminis* f.sp. *hordei*, which was challenged in *pmr4* mutants (Jacobs et al., 2003; Ellinger et al., 2013). Additional double-mutant and microarray analyses revealed that the hyperactivated salicylic acid (SA) pathway caused the high resistance to adapted powdery mildews in *pmr4* mutants (Nishimura et al., 2003). The SA-dependent resistance of *pmr4* mutants, however, might not be directly related to missing callose because the penetration success of an adapted powdery mildew was not different between wild-type and *pmr4* mutant at early stages of infection (Consonni et al., 2010; Ellinger et al., 2013).

Contrary results about an active role of callose in forming papillae derived from studies using *mlo* (*MILDEW RESISTANCE LOCUS O*) disruption mutants. In *Arabidopsis*, the observed *mlo2*-conditioned penetration resistance to powdery mildew did not require PMR4-dependent callose formation (Consonni et al., 2010) whereas *mlo*-resistant barley (*Hordeum vulgare*) coleptiles seemed to be dependent on papillae containing callose to maintain their penetration resistance to powdery mildew (Bayles et al., 1990). However, it has to be considered that the results in barley were based on a treatment with a callose synthase inhibitor that is not specific to stress-induced callose biosynthesis. Therefore, inhibition of additional callose synthases might have contributed to increased penetration susceptibility in this experiment. In our recent study, we could directly confirm that callose deposition in papillae can have an active role in penetration resistance also in *Arabidopsis*. The overexpression of *PMR4* caused an elevated early callose deposition at sites of attempted fungal penetration, which provided complete penetration resistance to the adapted powdery mildew *Golovinomyces cichoracearum* and the non-adapted powdery mildew *B. graminis* (Ellinger et al., 2013). This example reveals that timing and rapid transportation in papillae formation is important to slow or even stop pathogen invasion.

The extent to which overexpression of *PMR4* could be applied in crops to induce callose-mediated pathogen resistance is currently under investigation. First results from increased penetration resistance to the virulent powdery mildew *B. graminis* in barley leaves after transient *PMR4* overexpression (Blümke et al., 2013) prompted us to generate stable *PMR4* expression lines in barley, wheat (*Triticum aestivum*), and the model grass *Brachypodium distachyon*. To test whether induced penetration resistance would be dependent on overexpression of the callose synthase gene *PMR4* from *Arabidopsis*, callose synthase genes from the respective plant of interest could be used for overexpression. In this regard, new breeding strategies, like non-GMO-considered TAL effector nuclease mutagenesis (Wendt et al., 2013) would open new possibilities in applying this type of induced resistance in crops, e.g., by site-directed editing of the promoter region for constitutive expression of the gene of interest.

REGULATION OF TRANSPORT PROCESSES AT THE FORMING PAPILLAE

The spatial confinement of papillae to the paramural space between the cell wall and the plasma membrane at sites of attempted pathogen penetration suggests a site-directed transport of papilla components and cell wall-synthesizing enzymes, which would imply an induction and regulation of cell polarization processes (Schmelzer, 2002; Koh et al., 2005). The rearrangement of the cytoskeleton is an important factor in these processes. Actin filaments might be especially involved in the delivery of vesicles and transportation of organelles, like the Golgi and the nucleus, to the infection site and the forming papilla. This was supported in experiments where actin formation and rearrangements were inhibited, which resulted in increased fungal penetration (Kobayashi et al., 1997; Yun et al., 2003). Also *mlo*-mediated penetration resistance to powdery mildew was shown to be dependent on active actin reorganization (Opalski et al., 2005; Miklis et al., 2007). However,

the MLO protein itself accumulated at the forming papillae at penetration sites in absence of an intact actin cytoskeleton (Bhat et al., 2005). This suggests that also actin-independent mechanisms for protein recruitment to infection sites may exist. MLO was also shown to negatively regulate penetration resistance to powdery mildew because in different plant species, like barley, tomato (*Solanum lycopersicum*), and *Arabidopsis*, mutation or disruption of the *MLO* locus conferred increased resistance (Jørgensen, 1992; Piffanelli et al., 2004; Consonni et al., 2006; Bai et al., 2008; Consonni et al., 2010). A putative interaction of MLO and ROR2 (REQUIRED FOR *mlo* RESISTANCE 2) in barley and PEN1 (PENETRATION 1) in *Arabidopsis* (Schulze-Lefert, 2004; Bhat et al., 2005; Panstruga, 2005) could link MLO function with the regulation of transport processes to papillae. However, genetic data suggest that MLO and ROR2/PEN1 function independently. Therefore, observed effects are likely additive and would not support a direct functional link on a molecular level.

PEN1 and ROR2 are functionally homologous members of the syntaxin family (Collins et al., 2003). Similar to target SNAP (SOLUBLE NSF ATTACHEMENT PROTEIN) receptors (tSNARE), syntaxins form ternary SNARE complexes with corresponding VAMPs (VESICLE ASSOCIATED MEMBRANE PROTEIN), which reflects their direct involvement in vesicle fusion processes. PEN1 and ROR2 accumulation at the plasma membrane at sites of attempted fungal penetration allowed a further specification of their function in targeting vesicle trafficking to the forming papilla (Assaad et al., 2004; Bhat et al., 2005; Underwood and Somerville, 2008). This possible function of PEN1 was supported by findings in *Arabidopsis pen1* disruption mutants where papillae formation was reduced at early time-points of powdery mildew infection due to a delay in papilla deposition (Assaad et al., 2004). However, *pen1* disruption did not change the general morphology of papillae, which might indicate an involvement of one or more additional syntaxins that could substitute PEN1 during papilla formation. In contrast to PEN1, PEN2 was found to localize to peroxisomes. These organelles accumulate at sites of attempted fungal penetration where they are thought to deliver compounds with a potential antifungal activity to papillae. Because *PEN2* encodes a glycosyl hydrolase, it might directly participate in compound formation (Lipka et al., 2005; Bednarek et al., 2009). Similar to PEN1, the ATP-binding cassette (ABC) transporter PEN3 localized to the plasma membrane in unchallenged *Arabidopsis* leaves and was transported to the site of papilla formation after pathogen attack where it strongly accumulated (Stein et al., 2006; Underwood and Somerville, 2008). Interestingly, *pen3* disruption mutants revealed a higher resistance to biotrophic and hemibiotrophic pathogens, which was associated with an upregulation of SA biosynthesis or signaling and induced HR-like cell death (Kobae et al., 2006; Stein et al., 2006). Based on the transporter function and the observed phenotypes of *PEN3* mutants, it has been proposed that PEN3 might participate in the export of anti-microbial compounds, which could derive from PEN2-dependent processing in peroxisomes. For its recruitment and focal accumulation at sites of attempted penetration, PEN3 required functional actin filaments but not microtubules, secretory trafficking or protein biosynthesis. Hence, an unknown trafficking pathway might be involved in translocation of existing PEN3 (Underwood and Somerville, 2013).

Regarding one of the most prominent components of the forming papilla, the localized deposition of the (1,3)-β-glucan callose in response to pathogen attack suggests a precise timing of preceding transport processes (Zimmerli et al., 2004; Koh et al., 2005; Nielsen et al., 2012; Ellinger et al., 2013). Our recent results of the overexpression of the GFP-tagged callose synthase PMR4 in *Arabidopsis* suggested that this enzyme was released from vesicle-like bodies and reintegrated into the plasma membrane at sites of attempted penetration where callose deposition started (Ellinger et al., 2013). Therefore, recruitment from the plasma membrane and transport in vesicle-like bodies to the site of attempted penetration could be anticipated after fungal infection. This would also explain why application of brefeldin A, which is a known inhibitor of vesicle transport (Sciaky et al., 1997), prevented callose accumulation at forming papillae (Nielsen et al., 2012). However, a direct proof of PMR4 transportation to sites of attempted pathogen penetration in vesicles is still missing.

In the current discussion about vesicles that are required for transportation and delivery of defense components to the forming papilla, growing evidence of an involvement of multi-vesicular bodies (MVBs) has been provided. MVBs mediate exocytosis that would facilitate a delivery of vesicles and their content to the cell exterior, namely the forming papilla in the paramural space between the plasma membrane and the cell wall. In barley, MVBs contained the ADP-ribosylation factor (ARF) GTPase ARFA1b/1c that was required for callose deposition in papillae and penetration resistance to the powdery mildew *B. graminis*; but absence of this GTPase did not prevent basic papillae formation (Böhlenius et al., 2010). The importance of a timely and coordinated delivery of components required for proper papillae formation was further supported in experiments with *Arabidopsis* where mutation of the ARF-GTP exchange factor GNOM resulted in a 30 min delay of callose deposition in papillae (Nielsen et al., 2012).

A NEW DIRECTION OF PAPILLA EXPENSION

The currently discussed and presented models of papillae formation favor a transportation and delivery of papilla components by vesicles, which are controlled by a complex, underlying network of regulatory mechanisms. This would ensure a rapid and coordinated assembly of this pathogen-induced cell wall structure to support penetration resistance.

These models of delivery and transport processes would be sufficient to explain papillae expansion pointing to the cytosol (Nishimura et al., 2003) and a lateral expansion in the paramural space, which was strongly induced in *Arabidopsis PMR4* overexpression lines where an additional field of callose surrounded the dense core region of the papilla (Ellinger et al., 2013; Naumann et al., 2013). Material or cell wall enzymes, like callose synthases that would be required for a growth of the papilla in these directions, could be delivered along the plasma membrane. However, we recently detected an expansion of the papilla into the pre-existing cellulosic cell wall. Super-resolution

microscopy at sites of papillae formation, where we stained callose with aniline blue fluorochrome and the overlying cellulosic cell wall with pontamine fast scarlet 4B, revealed a migration of callose fibrils into the cell wall (Eggert et al., 2014). Only single callose fibrils, which originated from the dense callosic core of the papilla, migrated into and penetrated through the cellulosic cell wall in wild-type *Arabidopsis* leaves. In contrast, a dense network of callose/cellulose fibrils was established along the papilla core region and the lateral field of callose in epidermal leaf cells of *PMR4* overexpression lines at sites of attempted powdery mildew penetration. In addition to this polymer network, a callose layer was formed on top of the cellulosic cell wall (**Figure 1**). We showed that the complex of the callose/cellulose network and the additional callose layer provided enhanced resistance to cell wall degrading enzymes (Eggert et al., 2014), which helped to explain the observed complete penetration resistance to powdery mildew in *PMR4* overexpression plants (Ellinger et al., 2013).

These findings raised the question whether this new direction of papilla expansion would be a regulated process or the consequence of an ongoing callose production at the plasma membrane of the forming papilla. Because the expansion of the callosic papilla into the cellulosic cell wall occurred at sites without contact to the plasma membrane, the previously discussed transport and delivery mechanisms would not apply. Our results from super-resolution microscopy suggest a permeation of callose fibrils through internal cell wall nanopores (Carles and Scallan, 1973; Hubbe et al., 2007). This could be facilitated if callose would have a gel-like condition at these migration sites. In general, a gel-forming property of callose has been described as being pH-dependent (Harada et al., 1968; Saito et al., 1979). Hence, either conditions at the interphase of the callose deposition and the cellulosic cell wall would favor a gel-formation of callose without active regulation or cellular processes might actively regulate pH condition at the interphase. The apoplastic alkalinization that has been discussed as a general stress factor caused by abiotic and biotic stress, would not be sufficient to induce a gel-formation of callose. Even though the apoplastic pH peaked in short term responses to powdery mildew in barley, the apoplastic pH remained acidic (Felle et al., 2004) whereas alkaline condition would be required for gel-formation of callose (Saito et al., 1979). An active, local regulation of the pH might be possible through vesicle-like bodies and MVBs that were detected within

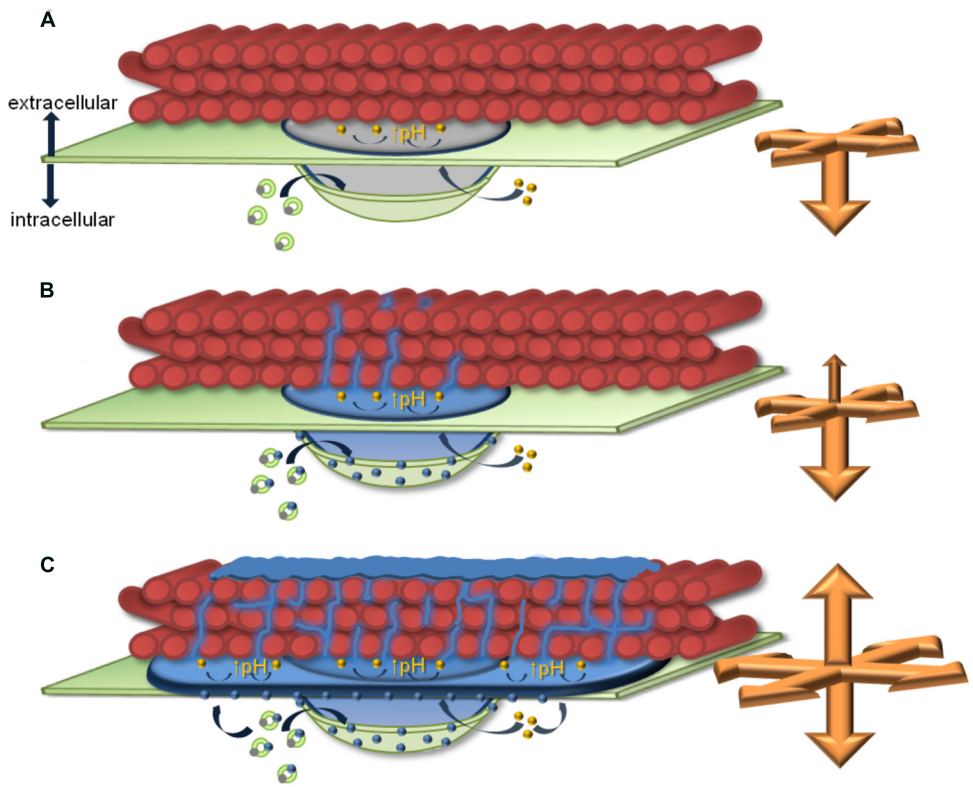


FIGURE 1 | Model of callosic papilla expansion at infection sites. The presented model highlights similarities and differences of callosic papilla expansion and callose/cellulose polymer network formation in *Arabidopsis* epidermal leaf cells at sites of attempted powder-mildew infection in **(A)** the *pmr4* disruption mutant without pathogen-induced callose deposition in the papilla, **(B)** wild-type, and **(C)** the penetration-resistant *PMR4* overexpression line. Green circles represent possible multi-vesicular bodies (MVBs) involved in the delivery of non-callosic papilla matrix and/or papilla-forming enzymes (gray dots) and the callose synthase PMR4 (blue dots) to the forming papilla. Yellow dots inside the papilla matrix indicate a putative involvement of vesicles/vesicle-like bodies in regulating the pH at the interphase of the papilla matrix and the cellulosic cell wall to induce gel-formation of callose (↑pH). Orange arrows indicate the direction and strength of papilla expansion. Green: plasma membrane, red: cellulose fibrils of the cell wall, blue: callosic papilla matrix and callose fibrils, gray: non-callosic papilla matrix.

the papilla structure (An et al., 2006) and could have access to the callose/cellulose interphase for regulatory activities. A further application of super-resolution microscopy combined with specific and efficient labeling techniques could support the analysis of papilla expansion in all directions.

REFERENCES

Aist, J. R. (1976). "Papillae and related wound plugs of plant cells," in *Annual Review of Phytopathology*, eds K. F. Baker, G. A. Zentmeyer, and E. B. Cowling (Palo Alto: Annual Reviews mInc.), 145–163.

Aist, J. R., and Williams, P. H. (1971). The cytology and kinetics of cabbage root hair penetration by *Plasmodiophora brassicae*. *Can. J. Bot.* 49, 2023–2034. doi: 10.1139/b71-284

An, Q., Hückelhoven, R., Kogel, K. H., and Van Bel., A. J. (2006). Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. *Cell. Microbiol.* 8, 1009–1019. doi: 10.1111/j.1462-5822.2006.00683.x

Assaad, F. F., Qiu, J. L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., et al. (2004). The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. *Mol. Biol. Cell* 15, 5118–5129. doi: 10.1091/mbc.E04-02-0140

Ausubel, F. M. (2005). Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* 6, 973–979. doi: 10.1038/ni1253

Bai, Y., Pavan, S., Zheng, Z., Zappel, N. F., Reinstadler, A., Lotti, C., et al. (2008). Naturally occurring broad-spectrum powdery mildew resistance in a Central American tomato accession is caused by loss of mlo function. *Mol. Plant Microbe Interact.* 21, 30–39. doi: 10.1094/MPMI-21-1-0030

Bailey, J. A. (1983). "Biological perspectives of host-pathogen interactions," in *The Dynamics of Host Defence*, eds J. A. Bailey and B. J. Deverall (Sydney: Academic Press), 1–32.

Bayles, C. J., Ghemawat, M. S., and Aist, J. R. (1990). Inhibition by 2-deoxy-D-glucose of callose formation, papilla deposition, and resistance to powdery mildew in an ml-o barley mutant. *Physiol. Mol. Plant Pathol.* 36, 63–72. doi: 10.1016/0885-5765(90)90092-C

Bednarek, P., and Osbourn, A. (2009). Plant-microbe interactions: chemical diversity in plant defense. *Science* 324, 746–748. doi: 10.1126/science.1171661

Bednarek, P., Piślewska-Bednarek, M., Svatoš, A., Schneider, B., Doubsky, J., Mansurova, M., et al. (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323, 101–106. doi: 10.1126/science.1163732

Bhat, R. A., Miklis, M., Schmelzer, E., Schulze-Lefert, P., and Panstruga, R. (2005). Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3135–3140. doi: 10.1073/pnas.0500012102

Blümke, A., Somerville, S. C., and Voigt, C. A. (2013). Transient expression of the *Arabidopsis thaliana* callose synthase PMR4 increases penetration resistance to powdery mildew in barley. *Adv. Biosci. Biotechnol.* 4, 810–813. doi: 10.4236/abb.2013.48106

Böhlenius, H., Mørch, S. M., Godfrey, D., Nielsen, M. E., and Thordal-Christensen, H. (2010). The multivesicular body-localized GTPase ARFA1b/1c is important for callose deposition and ROR2 syntaxin-dependent preinvasive basal defense in barley. *Plant Cell* 22, 3831–3844. doi: 10.1105/tpc.110.078063

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Brown, I., Trethowan, J., Kerry, M., Mansfield, J., and Bolwell, G. P. (1998). Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of Xanthomonas campestris and French bean mesophyll cells. *Plant J.* 15, 333–343. doi: 10.1046/j.1365-313X.1998.00215.x

Carles, J. E., and Scallan, A. M. (1973). The determination of the amount of bound water within cellulosic gels by NMR spectroscopy. *J. Appl. Polym. Sci.* 17, 1855–1865. doi: 10.1002/app.1973.070170618

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* 18, 465–476. doi: 10.1105/tpc.105.036574

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J. D., et al. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448, 497–500. doi: 10.1038/nature05999

Coll, N. S., Epple, P., and Dangl, J. L. (2011). Programmed cell death in the plant immune system. *Cell Death Differ.* 18, 1247–1256. doi: 10.1038/cdd.2011.37

Collins, N. C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J. L., et al. (2003). SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* 425, 973–977. doi: 10.1038/nature02076

Consonni, C., Bednarek, P., Humphry, M., Francocci, F., Ferrari, S., Harzen, A., et al. (2010). Tryptophan-derived metabolites are required for antifungal defense in the *Arabidopsis* mlo2 mutant. *Plant Physiol.* 152, 1544–1561. doi: 10.1104/pp.109.147660

Consonni, C., Humphry, M. E., Hartmann, H. A., Livaja, M., Durner, J., Westphal, L., et al. (2006). Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. *Nat. Genet.* 38, 716–720. doi: 10.1038/ng1806

Dangl, J. L., and Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411, 826–833. doi: 10.1038/35081161

deBary, A. (1863). Recherches sur le développement de quelques champignons parasites. *Ann. Sci. Nat. Bot. Biol. Vég.* 20, 5–148.

Deverall, B. J. (1977). *Defence Mechanisms of Plants.* Cambridge: Cambridge University Press. doi: 10.1017/CBO9780511735707

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812

Eggert, D., Naumann, M., Reimer, R., and Voigt, C. A. (2014). Nanoscale glucan polymer network causes pathogen resistance. *Sci. Rep.* 4, 4159. doi: 10.1038/srep04159

Ellinger, D., Naumann, M., Falter, C., Zwikowics, C., Jamrow, T., Manisseri, C., et al. (2013). Elevated early callose deposition results in complete penetration resistance to powdery mildew in *Arabidopsis*. *Plant Physiol.* 161, 1433–1444. doi: 10.1104/pp.112.211011

Felle, H. H., Herrmann, A., Hanstein, S., Hückelhoven, R., and Kogel, K.-H. (2004). Apoplastic pH signaling in barley leaves attacked by the powdery mildew fungus Blumeria graminis f. sp. hordei. *Mol. Plant Microbe. Interact.* 17, 118–123. doi: 10.1094/MPMI.2004.17.1.118

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9, 275–296. doi: 10.1146/annurev.py.09.090171.001423

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Glowacki, S., Macioszek, V. K., and Kononowicz, A. K. (2011). R proteins as fundamentals of plant innate immunity. *Cell. Mol. Biol. Lett.* 16, 1–24. doi: 10.2478/s11658-010-0024-2

Harada, T., Misaki, A., and Saito, H. (1968). Curdlan: a bacterial gel-forming β-1,3-glucan. *Arch. Biochem. Biophys.* 124, 292–298. doi: 10.1016/0003-9861(68)90330-5

Hubbe, M. A., Rojas, O. J., Lucia, L. A., and Jung, T. M. (2007). Consequences of the nanoporosity of cellulosic fibers on their streaming potential and their interactions with cationic polyelectrolytes. *Cellulose* 14, 655–671. doi: 10.1007/s10570-006-9098-4

Jacobs, A. K., Lipka, V., Burton, R. A., Panstruga, R., Strizhov, N., Schulze-Lefert, P., et al. (2003). An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* 15, 2503–2513. doi: 10.1105/tpc.016097

Jones, D. A., and Takemoto, D. (2004). Plant innate immunity - direct and indirect recognition of general and specific pathogen-associated molecules. *Curr. Opin. Immunol.* 16, 48–62. doi: 10.1016/j.coi.2003.11.016

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jørgensen, J. H. (1992). Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. *Euphytica* 63, 141–152. doi: 10.1007/BF00023919

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., et al. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11086–11091. doi: 10.1073/pnas.0508882103

Kobae, Y., Sekino, T., Yoshioka, H., Nakagawa, T., Martinoia, E., and Maeshima, M. (2006). Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis*

thaliana, causes hypersensitive cell death upon pathogen infection. *Plant Cell Physiol.* 47, 309–318. doi: 10.1093/pcp/pcj001

Kobayashi, Y., Yamada, M., Kobayashi, I., and Kunoh, H. (1997). Actin microfilaments are required for the expression of nonhost resistance in higher plants. *Plant Cell Physiol.* 38, 725–733. doi: 10.1093/oxfordjournals.pcp.a029226

Koh, S., Andre, A., Edwards, H., Ehrhardt, D., and Somerville, S. (2005). *Arabidopsis thaliana* subcellular responses to compatible *Erysiphe cichoracearum* infections. *Plant J.* 44, 516–529. doi: 10.1111/j.1365-313X.2005.02545.x

Lamb, C., and Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 251–275. doi: 10.1146/annurev.arplant.48.1.251

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., et al. (2005). Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. *Science* 310, 1180–1183. doi: 10.1126/science.1119409

Mangin, L. (1895). Recherches sur les Péronosporées. *Bull. Soc. d'Hist. Nat. d'Autun* 8, 55–108.

Martin, G. B., Bogdanove, A. J., and Sessa, G. (2003). Understanding the functions of plant disease resistance proteins. *Annu. Rev. Plant Biol.* 54, 23–61. doi: 10.1146/annurev.arplant.54.031902.135035

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal Hydrolases in Pea Tissue : II. Inhibition of Fungal Growth by Combinations of Chitinase and beta-1,3-Glucanase. *Plant Physiol.* 88, 936–942. doi: 10.1104/pp.88.3.936

McLusky, S. R., Bennett, M. H., Beale, M. H., Lewis, M. J., Gaskin, P., and Mansfield, J. W. (1999). Cell wall alterations and localized accumulation of feruloyl-3′-methoxytyramine in onion epidermis at sites of attempted penetration by Botryis allii are associated with actin polarization, peroxidase activity and suppression of flavenoid biosynthesis. *Plant J.* 17, 523–534. doi: 10.1046/j.1365-313X.1999.00403.x

Mercer, P. C., Wood, R. K. S., and Greenwood, A. D. (1974). Resistance to anthracnose of French bean. *Physiol. Plant Pathol.* 4, 291–306. doi: 10.1016/0048-4059(74)90016-2

Miklis, M., Consonni, C., Bhat, R. A., Lipka, V., Schulze-Lefert, P., and Panstruga, R. (2007). Barley MLO modulates actin-dependent and actin-independent antifungal defense pathways at the cell periphery. *Plant Physiol.* 144, 1132–1143. doi: 10.1104/pp.107.098897

Mims, C. W., Sewall, T. C., and Richardson, E. A. (2000). Ultrastructure of the host-pathogen relationship in Entomosporium leaf spot disease of Photinia. *Int. J. Plant Sci.* 161, 291–295. doi: 10.1086/314246

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., et al. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19613–19618. doi: 10.1073/pnas.0705147104

Mukhtar, M. S., Carvunis, A. R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., et al. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333, 596–601. doi: 10.1126/science.1203659

Naumann, M., Somerville, S., and Voigt, C. (2013). Differences in early callose deposition during adapted and non-adapted powdery mildew infection of resistant *Arabidopsis* lines. *Plant Signal. Behav.* 8, e24408 doi: 10.4161/psb.24408

Nielsen, M. E., Feechan, A., Böhlenius, H., Ueda, T., and Thordal-Christensen, H. (2012). *Arabidopsis* ARF-GTP exchange factor, GNOM, mediates transport required for innate immunity and focal accumulation of syntaxin PEN1. *Proc. Natl. Acad. Sci. U.S.A.* 109, 11443–11448. doi: 10.1073/pnas.1117596109

Nishimura, M. T., Stein, M., Hou, B. H., Vogel, J. P., Edwards, H., and Somerville, S. C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* 301, 969–972. doi: 10.1126/science.1086716

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* 198, 249–266. doi: 10.1111/j.0105-2896.2004.0119.x

Opalski, K. S., Schultheiss, H., Kogel, K. H., and Hückelhoven, R. (2005). The receptor-like MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley attacked by the biotrophic powdery mildew fungus Blumeria graminis f.sp. hordei. *Plant J.* 41, 291–303. doi: 10.1111/j.1365-313X.2004.02292.x

Panstruga, R. (2005). Serpentine plant MLO proteins as entry portals for powdery mildew fungi. *Biochem. Soc. Trans.* 33, 389–392. doi: 10.1042/BST0330389

Piffanelli, P., Ramsay, L., Waugh, R., Benabdelmouna, A., D'Hont, A., Hollricher, K., et al. (2004). A barley cultivation-associated polymorphism conveys resistance to powdery mildew. *Nature* 430, 887–891. doi: 10.1038/nature02781

Saito, H., Ohki, T., and Sasaki, T. (1979). A 13C-nuclear magnetic resonance study of polysaccharide gels. Molecular architecture in the gels consisting of fungal, branched (1→3)-β-D-glucans (lentinan and schizophyllan) as manifested by conformational changes induced by sodium hydroxide. *Carbohydrate Res.* 74, 227–240. doi: 10.1016/S0008-6215(00)84779-5

Schmelzer, E. (2002). Cell polarization, a crucial process in fungal defence. *Trends Plant Sci.* 7, 411–415. doi: 10.1016/S1360-1385(02)02307-5

Schulze-Lefert, P. (2004). Knocking on the heaven's wall: pathogenesis of and resistance to biotrophic fungi at the cell wall. *Curr. Opin. Plant Biol.* 7, 377–383. doi: 10.1016/j.pbi.2004.05.004

Schwessinger, B., and Ronald, P. C. (2012). Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* 63, 451–482. doi: 10.1146/annurev-arplant-042811-105518

Sciaky, N., Presley, J., Smith, C., Zaal, K. J., Cole, N., Moreira, J. E., et al. (1997). Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *J. Cell Biol.* 139, 1137–1155. doi: 10.1083/jcb.139.5.1137

Smith, H. (1978). Recognition and defence in plants. *Nature* 273, 266–268. doi: 10.1038/273266a0

Stein, M., Dittgen, J., Sanchez-Rodriguez, C., Hou, B. H., Molina, A., Schulze-Lefert, P., et al. (2006). *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. *Plant Cell* 18, 731–746. doi: 10.1105/tpc.105.038372

Stone, B. A., and Clarke, A. E. (1992). *Chemistry and Biology of (1→3)-β-glucans*. Bundoora: La Trobe University Press.

Thordal-Christensen, H. (2003). Fresh insights into processes of nonhost resistance. *Curr. Opin. Plant Biol* 6, 351–357. doi: 10.1016/S1369-5266(03)00063-3

Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D. B. (1997). Subcellular localization of H2O2 in plants. *H*2O2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. *Plant J.* 11, 1187–1194. doi: 10.1046/j.1365-313X.1997.11061187.x

Underwood, W., and Somerville, S. C. (2008). Focal accumulation of defences at sites of fungal pathogen attack. *J. Exp. Bot.* 59, 3501–3508. doi: 10.1093/jxb/ern205

Underwood, W., and Somerville, S. C. (2013). Perception of conserved pathogen elicitors at the plasma membrane leads to relocalization of the *Arabidopsis* PEN3 transporter. *Proc. Natl. Acad. Sci. U.S.A.* 110, 12492–12497. doi: 10.1073/pnas.1218701110

Wendt, T., Holm, P. B., Starker, C. G., Christian, M., Voytas, D. F., Brinch-Pedersen, H., et al. (2013). TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. *Plant Mol. Biol.* 83, 279–285. doi: 10.1007/s11103-013-0078-4

Yun, B. W., Atkinson, H. A., Gaborit, C., Greenland, A., Read, N. D., Pallas, J. A., et al. (2003). Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in *Arabidopsis* against wheat powdery mildew. *Plant J.* 34, 768–777. doi: 10.1046/j.1365-313X.2003.01773.x

Zimmerli, L., Stein, M., Lipka, V., Schulze-Lefert, P., and Somerville, S. (2004). Host and non-host pathogens elicit different jasmonate/ethylene responses in *Arabidopsis*. *Plant J.* 40, 633–646. doi: 10.1111/j.1365-313X.2004.02236.x

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 February 2014; accepted: 08 April 2014; published online: 28 April 2014.
Citation: Voigt CA (2014) Callose-mediated resistance to pathogenic intruders in plant defense-related papillae. Front. Plant Sci. ***5****:168. doi: 10.3389/fpls.2014.00168*
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 05 August 2014
doi: 10.3389/fpls.2014.00358

The role of the secondary cell wall in plant resistance to pathogens

Eva Miedes[1,2], Ruben Vanholme[3,4], Wout Boerjan[3,4] and Antonio Molina[1,2]*

[1] Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica Madrid, Madrid, Spain
[2] Departamento Biotecnología, Escuela Técnica Superior Ingenieros Agrónomos, Universidad Politécnica Madrid, Madrid, Spain
[3] Department of Plant Systems Biology, VIB (Flanders Institute for Biotechnology), Gent, Belgium
[4] Department of Plant Biotechnology and Bioinformatics, Ghent University, Gent, Belgium

Edited by:
Vincenzo Lionetti, Sapienza Università di Roma, Italy

Reviewed by:
William Underwood, University of California, Berkeley, USA
Lina Gallego-Giraldo, University of North Texas, USA

***Correspondence:**
Antonio Molina, Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica Madrid, Campus Montegancedo, M40 (Km. 38), Pozuelo de Alarcón, Madrid 28223, Spain
e-mail: antonio.molina@upm.es

Plant resistance to pathogens relies on a complex network of constitutive and inducible defensive barriers. The plant cell wall is one of the barriers that pathogens need to overcome to successfully colonize plant tissues. The traditional view of the plant cell wall as a passive barrier has evolved to a concept that considers the wall as a dynamic structure that regulates both constitutive and inducible defense mechanisms, and as a source of signaling molecules that trigger immune responses. The secondary cell walls of plants also represent a carbon-neutral feedstock (lignocellulosic biomass) for the production of biofuels and biomaterials. Therefore, engineering plants with improved secondary cell wall characteristics is an interesting strategy to ease the processing of lignocellulosic biomass in the biorefinery. However, modification of the integrity of the cell wall by impairment of proteins required for its biosynthesis or remodeling may impact the plants resistance to pathogens. This review summarizes our understanding of the role of the plant cell wall in pathogen resistance with a focus on the contribution of lignin to this biological process.

Keywords: cell wall, plant pathogen, plant immunity, xylan, cellulose, lignin

INTRODUCTION

In their natural environments, plants are under continuous threat of biotic stresses caused by pathogenic bacteria, fungi, viruses, and oomycetes, that compromise plant survival and reproduction (Panstruga et al., 2009). Given that green plants are the ultimate source of energy for most other organisms, it is not surprising that plants have evolved a plethora of resistance mechanisms which are either constitutively present or induced after pathogen attack (Glazebrook, 2005; Panstruga et al., 2009). An important defense element common to all plants is the cell wall.

All the plant cells that are in developmental expansion have a constantly remodeled primary cell wall that mainly consists of carbohydrate-based polymers (classified as cellulose, hemicelluloses and pectins) and hydroxyproline-rich *O*-glycoproteins, such as extensins and arabinogalactan proteins (AGPs; Carpita and McCann, 2000). In addition, those cells that have completed their cellular expansion and need to reinforce their structure for functional reasons (e.g., to form vessel or fiber cells) generate a secondary cell wall that is mainly composed of cellulose, hemicelluloses (mostly xylans) and lignin (Cosgrove, 2005; Sarkar et al., 2009). Besides having multiple essential functions during plant development, plant cell walls also play important roles in preventing pathogen invasion. First, cell walls act as a passive barrier; local or extensive breakdown of the wall matrix is typically required for the progression of pathogen infection (Cantu et al., 2008; Hematy et al., 2009). Second, the cell wall is a reservoir of antimicrobial compounds, which are released during cell wall degradation (García-Olmedo et al., 2001; Schulze-Lefert, 2004; Vorwerk et al., 2004). Moreover, plants have a dedicated cell wall integrity (CWI) maintenance mechanism similar to that existing in fungi, which initiates responses to regulate CWI during plant development and in response to external stimuli (Wolf et al., 2012; Engelsdorf and Hamann, 2014). Impairment of CWI by pathogen attack or wounding results in the release of plant signaling molecules, the so-called Damage-Associated Molecular Patterns (DAMPs; Vorwerk et al., 2004; Cantu et al., 2008). DAMPs can modulate plant innate immune responses upon recognition by plant Pattern Recognition Receptors (PRRs), through molecular mechanisms that are similar to those regulating the activation of immune responses by Pathogen-Associated Molecular Patterns (PAMPs) derived from microbial pathogens (reviewed by Dodds and Rathjen, 2010; Macho and Zipfel, 2014; Malinovsky et al., 2014). The recognition of DAMPs and PAMPs by PRRs activates protein kinase cascades, which regulate downstream immune responses that can lead, among others, to cell-wall reinforcement (Ringli, 2010; Ferrari et al., 2013; Engelsdorf and Hamann, 2014; Malinovsky et al., 2014). Notably, in addition to its role in protecting plants against infection, the plant cell wall can also act as a source of nutrients from the pathogen point of view, thereby promoting pathogen growth and development (Cantu et al., 2008; Hematy et al., 2009).

Cell walls are also considered as a valuable feedstock for the production of second generation biofuels and bio-based chemicals. These so-called lignocellulosic feedstocks can either derive from agricultural and industrial practices, such as maize stover, straw and sugarcane bagasse, or from dedicated crops, such as fast growing grasses and trees, grown for the purpose of generating large volumes of lignocellulosic biomass. In both cases, engineering cell wall composition is a promising strategy to ease lignocellulosic biomass conversion toward fuels and chemicals

in industrial processes (Simmons et al., 2010; Ong et al., 2014). Importantly, the changes in cell-wall composition needed for industrial biomass processing should not conflict with the principal biological roles of the cell wall as a supportive and protective structure. Indeed, some cell wall modifications result in negative repercussions on biomass yield (Bonawitz and Chapple, 2013). A better understanding of the processes underlying the yield-penalty in plants with modified cell walls has led to successful engineering strategies to recover the biomass yield, while maintaining the anticipated cell wall modifications (Petersen et al., 2012; Yang et al., 2013; Bonawitz et al., 2014). Likewise, cell wall modifications should not have negative repercussions on crop susceptibility toward pathogens. Clearly, a better understanding of the processes underlying the interactions between pathogens and the cell wall will support the development of plants with optimized lignocellulosic characteristics, without negatively affecting disease resistance.

A relatively large number of studies have described the influence of plant cell-wall modifications on pathogen infection (Cantu et al., 2008; Bellincampi et al., 2014; Malinovsky et al., 2014). Contra-intuitively, "weakening" the cell wall by knocking out essential genes involved in cell-wall biosynthesis sometimes leads to enhanced resistance toward specific pathogens. In this review, we summarize the consequences of secondary cell-wall modifications on pathogenic infection and link them with our current knowledge on the role of the cell wall in plant resistance to pathogens. Because lignin is both stress-induced and developmentally deposited in the secondary thickened cell wall and because it is a major target for lignocellulosic biomass engineering, we have put special emphasis on the effect of altering lignin amount and composition on pathogen infection and spread.

THE EFFECT OF ALTERING CELL WALL POLYSACCHARIDES ON RESISTANCE TO PATHOGENS

The contribution of the secondary cell wall to plant immunity has been mainly demonstrated through the characterization of plant mutants impaired in secondary wall composition (Cantu et al., 2008; Underwood, 2012). For instance, the resistance of a set of *Arabidopsis thaliana* (*Arabidopsis* herein) mutants defective in cellulose synthase (CESA) subunits required for secondary cell wall formation (i.e., CESA4, CESA7, and CESA8) toward a series of pathogens has been tested. These mutants make less cellulose, which results in collapsed xylem vessels, and therefore they are called *ir*regular *x*ylem mutants (*irx5*, *irx3*, and *irx1*, respectively). These mutants showed enhanced resistance to different pathogens, including the necrotrophic fungi *Plectosphaerella cucumerina* and *Botrytis cinerea*, the vascular bacterium *Ralstonia solanacearum*, and the biotrophic bacterium *Pseudomonas syringae* (**Table 1**; Hernández-Blanco et al., 2007). In line with these results, an *Arabidopsis* mutant defective in the MYB46 transcription factor that directly regulates the expression of genes required for secondary cell wall formation, including lignin and cellulose biosynthesis (among which *CESA4, CESA7*, and *CESA8*), also showed enhanced resistance to necrotrophic fungi (Ramírez et al., 2011). The disease resistance phenotype of *irx1*, *irx3*, *irx5*, and *myb46* mutants was in part explained by the constitutive activation of plant immune responses rather than by alterations of the passive wall barrier. In these mutants, the abscisic acid signaling pathway was constitutively active and antimicrobial peptides and tryptophan-derived metabolites accumulated to a higher extent than in wild-type plants (Hernández-Blanco et al., 2007; Sánchez-Vallet et al., 2010). Plant resistance to pathogens is also altered in *Arabidopsis* mutants affected in CESAs subunits required for cellulose biosynthesis of the primary cell wall, such as the CESA3 defective *isoxaben resistant* (*ixr1*)/*constitutive expression of VSP* (*cev1*) mutants (Ellis et al., 2002). The *ixr1/cev1* mutant alleles are more resistant than wild-type plants to *B. cinerea*, *P. syringae*, and *Erysiphe cichoracearum* (Ellis et al., 2002), whereas their resistance to *R. solanacearum* and *P. cucumerina* does not differ from that of wild-type plants, which contrasts with the resistance phenotype of the secondary cell wall cellulose mutants, *irx1, irx3* and *irx5* (Hernández-Blanco et al., 2007). In *ixr1/cev1* plants, the ethylene and jasmonic acid, but not the abscisic acid signaling pathway, are constitutively activated. These results with *Arabidopsis cesA* mutants illustrate that specific immune responses can be activated by alteration of the CWI of either the primary or the secondary wall (Ellis et al., 2002; Hernández-Blanco et al., 2007).

A severe reduction in secondary wall thickness of fibers, but not that of xylem vessels, as it occurs in the *Arabidopsis WALLS ARE THIN 1* (*wat1*) mutant, also increased resistance to vascular plant pathogens, such as the bacteria *R. solanacearum* and *Xanthomonas campestris* pv. *campestris*, the fungi *Verticillium dahliae* and *Verticillium alboatrum*, and the necrotrophic fungus *P. cucumerina* (Denancé et al., 2013). *WAT1* encodes a tonoplast localized indole acetic acid (auxin) transporter (Pesquet et al., 2005; Ranocha et al., 2010, 2013). Auxin content was found to be lower in roots, but not in leaves of the *wat1* mutant than in those of wild-type plants. In contrast, salicylic acid content was higher in the roots of the *wat1* mutant than in those of wild-type plants. Introduction in *wat1* plants of *NahG*, the bacterial gene coding for a salicylic acid-degrading hydroxylase, restored full susceptibility to the bacteria (Denancé et al., 2013). These data and those obtained by comparative transcriptomic analyses of *wat1* and wild-type plants suggest that *wat1*-mediated resistance is again not caused by altering the strength of the wall as a passive barrier, but that it is dependent on the activation of immune responses, mainly localized in the vascular system, which are partially dependent on the salicylic acid pathway. This defense response has been described as "vascular immunity" (Denancé et al., 2013).

Alteration of glucoronoxylans and xyloglucans or modifications in the content of wall xylose, which is the major sugar component of these polysaccharides, also impacts resistance to pathogens in *Arabidopsis*. For example, plants with enhanced levels of wall-bound xylose, as it occurs in the *de-etiolated3* (*det3*) and *irx6* mutants (Brown et al., 2005; Rogers et al., 2005) or with alterations in the structure of xyloglucan, as in the *xyl1-2* mutant (Sampedro et al., 2010), show an enhanced resistance to the necrotrophic fungus *P. cucumerina* (Delgado-Cerezo et al., 2012; **Table 1**). In contrast, impairment of the *ERECTA* (*ER*) gene encoding a PRR resulted in a reduced content of xylose besides other cell wall alterations in *Arabidopsis* (Sánchez-Rodríguez et al.,

Table 1 | Resistance phenotype of plants with alterations in secondary cell wall structure/composition.

Gene name (mutant/transgenic)	Plant species	Pathogen tested	Phenotype[1]	Reference
CESA4, CESA7, CESA8 (irx5, irx3, irx1)	*Arabidopsis thaliana*	*Plectosphaerella cucumerina*, *Ralstonia solanacearum*, *Botrytis cinerea*, *Pseudomonas syringae*	R	Hernández-Blanco et al. (2007)
WAT (wat1)	*Arabidopsis thaliana*	*R. solanacearum*, *P. cucumerina*, *Xanthomonas campestris*, *Verticillium dahlia*, *V. alboatrum*	R	Denancé et al. (2013)
DET3 (det3)	*Arabidopsis thaliana*	*P. cucumerina*	R	Delgado-Cerezo et al. (2012)
XYL1 (xyl1-2)	*Arabidopsis thaliana*	*P. cucumerina*	R	Delgado-Cerezo et al. (2012)
RWA2 (rwa2)	*Arabidopsis thaliana*	*B. cinerea*	R	Manabe et al. (2011)
IRX6 (irx6)	*Arabidopsis thaliana*	*P. cucumerina*	R	Delgado-Cerezo et al. (2012)
MYB46 (myb46)	*Arabidopsis thaliana*	*B. cinerea*	R	Ramírez et al. (2011)
PAL (pal1/2/3/4)	*Arabidopsis thaliana*	*P. syringae*	S	Huang et al. (2010)
COMT (comt1)	*Arabidopsis thaliana*	*X. campestris*, *P. syringae*, *B. cinerea*, *Blumeria graminis*, *Alternaria brassicicola*	S	Quentin et al. (2009)
		Hyaloperonospora arabidopsidis	R[2]	Quentin et al. (2009)
F5H1 (f5h1)	*Arabidopsis thaliana*	*Sclerotinia sclerotiorum*	S	Huang et al. (2009)
F5H1 (fah1-2)	*Arabidopsis thaliana*	*Verticillium longisporum*	S	König et al. (2014)
AXE (35S::AnAXE)[3]	*Arabidopsis thaliana*	*B. cinerea*	R	Pogorelko et al. (2013)
PAL (35S::PvPAL2)[3]	*Nicotiana tabacum*	*Cercospora nicotianae*	R	Shadle et al. (2003)
PAL (35S::ShPAL)[3]	*Nicotiana tabacum*	*Phytophthora parasitica*, *C. nicotianae*	R	Way et al. (2002, 2011)
COMT (comt)	*Nicotiana tabacum*	*Agrobacterium tumefaciens*	R	Maury et al. (2010)
HCT (HCT antisense)	*Medicago sativa*	*Colletotrichum trifolii*	R	Gallego-Giraldo et al. (2011b)
PAL, CCoAOMT, COMT, CAD (RNAi)[4]	*Triticum monococcum*	*Blumeria graminis f. sp. tritici*	S	Bhuiyan et al. (2009)
CAD (RNAi)[4]	*Linum usitatissimum*	*Fusarium oxysporum*	S	Wróbel-Kwiatkowska et al. (2007)
CAD (bmr6)	*Sorghum bicolor*	*Fusarium thapsinum*, *F. proliferatum*, *F. verticillioides*, *Alternaria alternata*	R	Funnell-Harris et al. (2010)
COMT (bmr12)	*Sorghum bicolor*	*F. thapsinum*, *F. proliferatum*, *F. verticillioides*, *A. alternata*	R	Funnell-Harris et al. (2010)
AXE (35S::AnAXE)[3]	*Brachypodium distachyon*	*Bipolaris sorokiniana*	R	Pogorelko et al. (2013)

[1] *R; enhanced resistance compared with wild-type plants; S, enhanced susceptibility compared with wild-type plants.*
[2] *Enhanced resistance to downy mildew was not correlated with increased plant defense responses in comt1 mutant, but coincided with a higher frequency of oomycete sexual reproduction within mutant tissues.*
[3] *Genes from Aspergillus nidulans (An), Phaseolus vulgaris (Pv) and Stylosanthes humilis (Sh).*
[4] *RNA interference constructs were made by a combined ligation/recombination (LR) method using plasmid pIPKTA30N as the final GATEWAY destination vector (Bhuiyan et al., 2009) and a self-complementary hairpin RNA (hpRNA) of CAD gene, under the control of 35S CaMV promoter, was used to silence CAD expression (Wróbel-Kwiatkowska et al., 2007).*

2009). The *er* mutant was found to be more susceptible than wild-type plants to several pathogens, such as the necrotrophic fungus *P. cucumerina*, the vascular bacterium *R. solanacearum* and the vascular oomycete *Pythium irregulare* (**Table 1**; Godiard et al., 2003; Llorente et al., 2005; Adie et al., 2007). The enhanced susceptibility to *P. cucumerina* and the cell wall features of the *er* mutant, including its reduced xylose content, were restored to wild-type levels by mutations in *SUPPRESSOR OF ERECTA 1* and 2 (*SER1* and *SER2)*, further suggesting a link between cell wall xylose content and resistance to pathogens (Sánchez-Rodríguez et al., 2009). Although several defense genes are constitutively upregulated in the *ser1* and *ser2* mutants, the precise molecular basis of their resistance has not yet been fully elucidated and the *SER* genes have not been characterized yet (Sánchez-Rodríguez et al., 2009). *Arabidopsis* mutants in the Gβ and Gγ1/γ2 subunits of the heterotrimeric G protein (i.e., *agb1* single and *agg1 agg2* double mutants, respectively) also have a reduced content of xylose in their cell walls and are hypersusceptible to the necrotrophic fungi *P. cucumerina* and *Alternaria brassicicola*, the biotrophic bacterium *P. syringae* and the vascular fungus *Fusarium oxysporum* (**Table 1**; Llorente et al., 2005; Trusov et al., 2010; Klopffleisch et al., 2011; Delgado-Cerezo et al., 2012; Liu et al., 2013; Lorek et al., 2013; Torres et al., 2013). Interestingly, the reduced resistance of *agb1* single and *agg1 agg2* double mutants was found to be independent of defense pathways required for resistance to these pathogens, such as those regulated by abscisic acid, salicylic acid, jasmonic acid and ethylene, and those that regulate the biosynthesis of tryptophan-derived metabolites (Delgado-Cerezo et al., 2012; Lorek et al., 2013; Torres et al., 2013). It has been suggested that the reduced resistance in the *agb1* and *agg1 agg2* mutants is rather the direct consequence of a weakened cell-wall and a defective production of reactive oxygen species (ROS) upon pathogen infection (Delgado-Cerezo et al., 2012; Jiang et al., 2012; Liu et al., 2013; Lorek et al., 2013). Together, these data suggest that shifts in the xylose content of the cell wall, e.g., by altering the glucoronoxylan and xyloglucan content, are responsible, at least in part, for the altered susceptibility of some *Arabidopsis* secondary cell wall mutants to pathogens.

Cell wall polysaccharides such as xylan, (gluco)mannan and xyloglucan can be acetylated. Four *Reduced Wall Acetylation* genes (*RWA1–RWA4*) are involved in the acetylation of xylan during secondary wall biosynthesis. The expression of these genes is regulated by SND1, a transcriptional master switch of secondary wall biosynthesis (Lee et al., 2011). Remarkably, the *Arabidopsis rwa2* mutant, that has ∼20% lower levels of polysaccharide *O*-acetylation but no obvious alteration in growth and development, is more resistant than wild-type plants to the necrotrophic fungus *B. cinerea* (Manabe et al., 2011). The relevance of the degree of xylan acetylation in plant resistance to pathogens is further supported by the enhanced resistance to the necrotrophic fungi *B. cinera* and *Bipolaris sorkiniana* of transgenic *Arabidopsis* and *Brachypodium distachyon*, respectively, that have a reduced xylan acetylation due to overexpression of a xylan acetylesterase from *Aspergillus nidulans* (*AnAXE*; **Table 1**; Pogorelko et al., 2013). These data indicate that the degree of acetylation of specific secondary cell wall polymers might be a determinant of susceptibility to particular pathogens. In addition to RWA proteins, members of the trichome birefringence (TBR) and TBR-like (TBL) protein families are also involved in the *O*-acetylation of wall polysaccharides (Gille et al., 2011). The *Arabidopsis powdery mildew resistant5* (*pmr5*) mutant, impaired in a TBL member, has a decrease in cell-wall esterification as demonstrated by Fourier transform infrared (FTIR) analysis, but it has yet to be demonstrated whether *pmr5* cell walls have an altered polysaccharide *O*-acetylation (Vogel et al., 2004; Gille and Pauly, 2012). The *pmr5* mutant is more resistant than wild-type plants to powdery mildew fungi (i.e., *E. cichoracearum* and *E. orontii)*, whereas its resistance to the bacterium *P. syringae* or the oomycete *Peronospora parasitica* (re-named *Hyaloperonospora arabidopsidis)* was similar to that of wild-type plants (Vogel et al., 2004). Taken together, these data indicate that a decrease in cell wall acetylation in *Arabidopsis* resulted in an enhanced resistance to several fungi, but the molecular mechanisms explaining this resistance phenotype have yet to be elucidated.

Primary cell wall remodeling can also impact pathogen resistance, as exemplified by the enhanced resistance to some pathogens of mutants defective in the CESA subunits required for primary cell wall cellulose biosynthesis (i.e., *ixr1/cev1*; Ellis et al., 2002; Hernández-Blanco et al., 2007). Similarly, modification of the biosynthesis and/or structure (e.g., degree of methylesterification or acetylation) of wall pectins can affect pathogen resistance (Vogel et al., 2002, 2004; Lionetti et al., 2007; Raiola et al., 2011; Volpi et al., 2011; Bethke et al., 2014). The complex contribution of pectin amount/structure to the regulation of plant innate immunity has been nicely reviewed in several recent publications that also describe the different virulence mechanism used by pathogens to modify or degrade pectins in order to favor plant colonization (Ferrari et al., 2012; Lionetti et al., 2012; Bellincampi et al., 2014).

PHENYLPROPANOID AND LIGNIN BIOSYNTHESIS

Lignin is an aromatic polymer that is mainly deposited in secondary thickened cell walls where it provides strength and imperviousness. In monocot and dicot plants, lignin is mainly made from the monolignols coniferyl and sinapyl alcohol that give rise to the guaiacyl (G) and syringyl (S) units in the lignin polymer, respectively. *p*-Coumaryl alcohol, that gives rise to the *p*-hydroxyphenyl (H) units in the lignin polymer, is a minor monolignol that is slightly more abundant in monocot than in dicot cell walls. Lignin of gymnosperms is typically composed of G units and low levels of H units, but lacks S units. In several plant species, the traditional monomers are incorporated into the lignin in acylated forms. For instance, kenaf lignin is rich in sinapyl acetate-derived units, lignin of grasses has a high content of sinapyl *p*-coumarate-derived lignin units and poplar lignin incorporates sinapyl *p*-hydroxybenzoate (Morreel et al., 2004; Del Río et al., 2007; Lu and Ralph, 2008; Hatfield et al., 2009). In addition, plants do accept a range of other phenolics as lignin monomers. For example, lignin in wheat straw has relatively high levels of the flavonoid tricin (Del Río et al., 2012), whereas phenylpropanoid aldehydes and acids are found in the lignin of a range of wild-type and genetically engineered plants (Kim et al., 2000; Dauwe et al., 2007; Vanholme et al., 2012a; Van Acker et al., 2013, 2014).

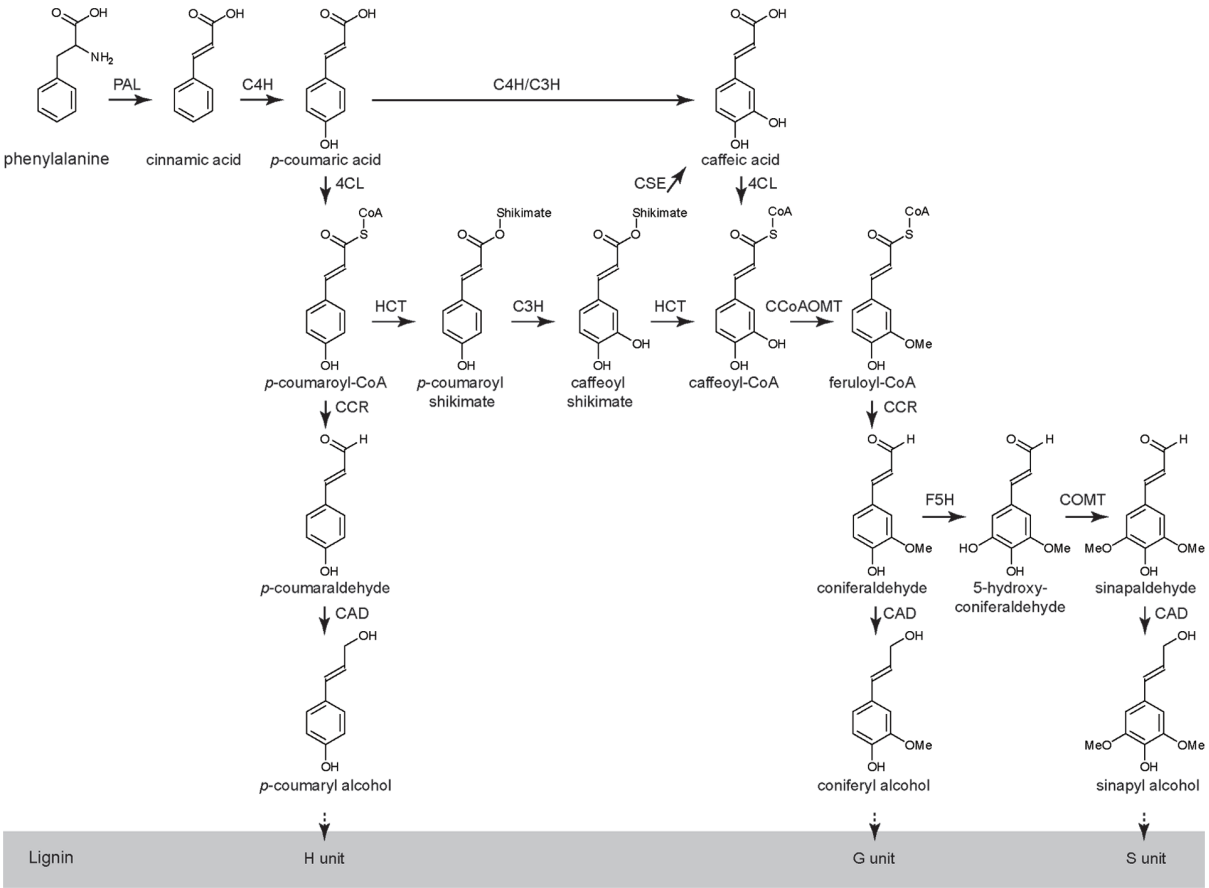


FIGURE 1 | The phenylpropanoid and monolignol biosynthetic pathway in *Arabidopsis* (adapted from Vanholme et al., 2010, 2013).

The lignin biosynthetic pathway is relatively well described in many species including *Arabidopsis*, tobacco, alfalfa and poplar, and is generally divided in two branches: (i) the general phenylpropanoid pathway from Phe to feruloyl-CoA and (ii) the monolignol-specific pathway from feruloyl-CoA to the monolignols (**Figure 1**). At least eleven enzymes are involved in the biosynthesis of the monolignols from Phe: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), *p*-coumarate 3-hydroxylase (C3H), caffeoyl shikimate esterase (CSE), caffeoyl-CoA *O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid *O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD; **Figure 1**; Boerjan et al., 2003; Bonawitz and Chapple, 2010; Vanholme et al., 2010, 2013). After their biosynthesis, the monolignols are transported to the cell wall where they are oxidized by laccases and/or peroxidases to monolignol radicals. Subsequently, these monolignol radicals couple in a combinatorial fashion with the formation of various types of chemical bonds of which the ether (8-O-4), resinol (8–8), and coumaran (8–5) bonds are the most prominent ones (Boerjan et al., 2003; Ralph et al., 2004; Vanholme et al., 2012a).

The biosynthesis and deposition of lignin in secondary cell walls is developmentally programmed and it is generally accepted that lignin provides a physical barrier against initial pathogen colonization (Buendgen et al., 1990; Bonello et al., 2003). In addition, lignin or lignin-like phenolic polymers are synthesized and rapidly deposited in cell walls in response to biotic and abiotic stresses and to cell wall structure perturbations (Caño-Delgado et al., 2003; Tronchet et al., 2010; Sattler and Funnell-Harris, 2013). The deposition of lignin in infected cells may prevent the spread of toxins and enzymes of the pathogen into the host and at the same time also the transfer of water and nutrients from the host cells to the pathogen (Smith et al., 2007). Both biotic and abiotic stresses have been shown to induce the expression of genes of the phenylpropanoid pathway in different tissues and cell cultures of several plants species, resulting in the enhanced accumulation of the corresponding enzymes, increased enzymatic activities and wall lignification (Kliebenstein et al., 2002; Bhuiyan et al., 2007; Zhao et al., 2009). For example, in Chinese cabbage (*Brassica rapa L.* ssp. *pekinensis)* infected with the necrotrophic bacterium *Erwinia carotovora* subsp. *carotovora*, transcriptomic analyses revealed that 12 genes putatively encoding enzymes involved in lignin biosynthesis were up-regulated (Zhang et al., 2007), and in *Camelina sativa* the expression of *CsCCR2* was induced after

inoculation with the necrotrophic fungus *Sclerotinia sclerotiorum* (Eynck et al., 2012). In cell suspension cultures of *Linum usitatissimum* treated with different fungal PAMPs, the expression of genes encoding PAL, CCR, and CAD was elevated, PAL activity was enhanced and monolignol-derived compounds accumulated (Hano et al., 2006). Similarly, in suspension cultured cells of a blast-resistant rice genotype (*Oryza sativa* L. cv. Gigante Vercelli) treated with cell wall hydrolysates from the fungal pathogen *Magnaporthe oryzae*, up-regulation of *PAL* genes was observed (Giberti et al., 2012). Also in wheat lignification acts as a defense response against pathogens: for example, S-rich lignin was found to accumulate during the hypersensitive reaction of wheat to *Puccinia graminis* infection (Menden et al., 2007), and S lignin was made in wheat sheath epidermal cells infected with *F. proliferatum* (Bishop et al., 2002). In contrast, no changes in the lignin content occurred in wheat leaves infected with Wheat Streak Mosaic Virus (Kofalvi and Nassuth, 1995).

In addition to its function in the biosynthesis of lignin, the phenylpropanoid pathway is required for the synthesis of numerous other phenolic compounds, such as stilbenes, coumarins, (neo-)lignans, phenylpropanoid conjugates, and flavonoids (Lo and Nicholson, 1998; Yu et al., 2000, 2005; Dixon et al., 2002; Naoumkina et al., 2010). Many of these compounds are considered to be phytoalexins, i.e., antimicrobial compounds implicated in plant defense (Daayf et al., 2012; König et al., 2014). Therefore, impairing steps of the phenylpropanoid pathway can result in either accumulation or reduced abundance of these compounds, often resulting in pleiotropic effects on plant resistance (Weiergang et al., 1996; Ruuhola and Julkunen-Tiitto, 2003; Dicko et al., 2005; Pan et al., 2006; Lozovaya et al., 2007). Phenolic compounds are also important in plant-pathogen recognition. For example, the expression of *Agrobacterium tumefaciens Virulence* (*Vir*) genes, needed for infection, is induced by phenolic compounds and infection of the host plant cannot take place in the absence of these compounds (Lee et al., 1992, 1995; Maury et al., 2010).

EFFECT OF PHENOLIC CONTENT AND LIGNIN MODIFICATIONS ON PATHOGEN RESISTANCE

Evidence for a role for lignin and soluble phenolics in plant defense has been obtained from the analysis of the pathogen resistance of transgenic plants and mutants with contrasting lignin amount or composition. For example, in tomato, the total content of soluble phenolics and lignin were significantly higher in varieties that were resistant to the vascular bacterium *R. solanacearum* than in susceptible ones, and this enhanced resistance was associated to a greater accumulation of lignin in roots upon bacterial infection, a process that was triggered by salicylic acid (Mandal et al., 2011, 2013). In tobacco, plants down-regulated for *PAL* had reduced levels of chlorogenic acid and exhibited more rapid and extensive lesion development than wild-type plants upon infection with the fungal pathogen *Cercospora nicotianae* (Maher et al., 1994). The increased disease susceptibility in this *PAL*-suppressed line was suggested not to result from the inhibition of the pathogen-induced response, but rather from the decrease in the developmental accumulation of chlorogenic acid (Maher et al., 1994). However, the lignin content in this *PAL*-suppressed line was not determined and therefore it cannot be excluded that the enhanced susceptibility was caused by reduced lignin content or a weaker cell wall (Maher et al., 1994). In accordance with the latter studies, transgenic tobacco plants constitutively overexpressing *PAL* genes showed a higher tolerance toward *C. nicotianae* and *Phytophthora parasitica* pv. *nicotianae* (Way et al., 2002, 2011; Shadle et al., 2003). Notably, *COMT* and *CCoAOMT* antisense tobacco lines were more resistant to *Agrobacterium tumefaciens* infection and showed a reduced tumor area and mass relative to wild-type plants (Maury et al., 2010). The phenolic compounds secreted by these antisense plants upon wounding did not induce the expression of the bacterial *Vir* genes equally well as those secreted from wounded wild-type plants (Maury et al., 2010). In other words, the *Agrobacterium* did not recognize its host because of the difference in soluble phenolics.

In cotton (*Gossypium hirsutum*), quantitative analysis of resistance to the wilt fungus *V. dahliae* revealed an association between increased lignification in the stems upon infection and resistance against wilt (Xu et al., 2011). In line with these data, overexpression of the cotton *DIRIGENT1* gene, which enhances lignification, blocks the spread of *V. dahliae* (Shi et al., 2012). In alfalfa (*Medicago sativa*), down-regulation of the *HCT* gene leads to plants with reduced lignin levels, constitutive defense responses and enhanced tolerance to the fungal pathogen *Colletotrichum trifolii*. This activation of defense responses was hypothesized to be triggered by bioactive cell wall fragments released from the secondary cell wall (Gallego-Giraldo et al., 2011b). In melon (*Cucumis melo*), lignin accumulation upon infection was found to increase faster and to a higher level in lines resistant to the powdery mildew fungus *Podosphaera fusca* than in susceptible lines, and this differential accumulation correlated with enhanced PAL levels (Romero et al., 2008). Lignin composition seems to play an important role in pathogen resistance in flax, as RNAi-mediated suppression of a *CAD* gene increased flax susceptibility to the vascular fungus *F. oxysporum* (Wróbel-Kwiatkowska et al., 2007).

Also in grasses the effect of modifying lignin biosynthesis on plant susceptibility has been investigated. For instance, in wheat *(Triticum monococcum)*, silencing the monolignol biosynthesis genes *TmPAL*, *TmCOMT*, *TmCCoAOMT*, and *TmCAD* led to super-susceptibility of leaf tissues to the fungus *B. graminis* f. sp. *tritici*, the causal agent of powdery mildew disease (Bhuiyan et al., 2009). The increased accumulation of mono- and diferulates in the cell walls of oat and wheat upon infection with *P. coronate* sp. *avenae* and *Agrobacterium* sp., respectively, has been associated with resistance toward these pathogens (Ikegawa et al., 1996; Parrott et al., 2002). In transgenic rice overexpressing the *NPR1 HOMOLOG 1* (*NH1*), a suppressor mutant screening was performed and a mutation in the *SUPPRESSSOR OF NH1-MEDIATED LESION FORMATION AND RESISTANCE* (*SNL6*) gene, which encodes a CCR-like protein, was selected. *snl6* mutants had a lower lignin content and a reduced resistance to the bacterium *X. oryzae* pv. *oryzae* (Bart et al., 2010). Mutations in *BROWN MIDRIB 6* (*BMR6*) and *BMR12* in sorghum (*Sorghum bicolor* L.) allowed the development of forage and grain lines with a reduced lignin content and modified lignin composition (Oliver et al., 2005). The *bmr6* and *bmr12* mutants, that are defective in CAD and COMT proteins, respectively, restricted the growth of

different *Fusarium* spp. (*F. thapsinum*, *F. proliferatum*, and *F. verticillioides*), but not that of *Gibberella fujikuroi* (Bout and Vermerris, 2003; Sattler et al., 2009; Funnell-Harris et al., 2010). It is unknown whether the alteration of lignin composition or the accumulation of phenolic compounds is causative to the enhanced resistance of these sorghum mutants to *Fusarium* sp.

In trees, the contribution of lignin amount or composition on susceptibility to pathogens has been also investigated. For example, in eucalyptus, the deposition of lignin in necrophylatic periderm in the early stages of infection by *Mycosphaerella* explains the greater resistance of *Eucalyptus nitens* as compared with *E. globulus* (Smith et al., 2007). Comparative metabolite profiling of xylem tissue of *Ulmus minor* and *Ulmus minor* × *Ulmus pumila* after inoculation with *Ophiostoma novo-ulmi* showed that the hybrid has a faster defense response, which is characterized by an increase in the amount of lignin (Martin et al., 2007). Similarly, the infection of *Pinus nigra* by *Sphaeropsis sapinea* induces an increase in the deposition of lignin that was associated to resistance (Bonello and Blodgett, 2003). Interestingly, in hybrid poplar (*Populus tremula* × *Populus alba*) no increased disease incidence was observed in field-grown antisense *COMT* and *CAD* lines relative to that observed in wild-type trees, nicely showing that altered lignin biosynthesis does not necessarily negatively impact resistance to pathogens (Pilate et al., 2002; Halpin et al., 2007).

In summary, a general positive correlation between lignin amount and pathogen resistance has been observed, in particular when the plant-pathogen interaction concerns vascular pathogens, such as *Fusarium* sp., *Xanthomonas* sp. or *Verticilium* sp., that generally spread through the secondary-thickened xylem. In the majority of the examples analyzed, the impact of lignin modification on the regulation of other defense responses has not been studied, and it is not yet possible to conclude whether the role of lignin in resistance is merely passive or active by regulating specific immune responses.

CONTRIBUTION OF LIGNIN TO PATHOGEN RESISTANCE IN *Arabidopsis*

Several lines of evidence support a role for lignin in immunity of *Arabidopsis* to pathogens. The expression of some lignin-biosynthesis genes was induced, and the amount of lignin increased, by treating *Arabidopsis* with hormones (i.e., salicylic acid, abscisic acid or jasmonic acid) that regulate plant defense (Mohr and Cahill, 2007; Chen et al., 2009; Gallego-Giraldo et al., 2011a). Similarly, infection of *Arabidopsis* with particular pathogens, such as the bacteria *P. syringae* pv. *tomato* and *X. campestris,* resulted in increased expression of lignin-biosynthesis genes and in higher lignin levels (Mohr and Cahill, 2007; Quentin et al., 2009). Experiments also hinted to specific stress-related roles for the different gene family members involved in certain enzymatic conversions. For example, the *Arabidopsis CCR2* gene has been suggested to participate in the hypersensitive response to *X. campestris* as its expression was up-regulated after inoculation with this bacterium, in contrast to *CCR1* which was preferentially expressed during development (Lauvergeat et al., 2001).

The analysis of *Arabidopsis* mutants defective in lignin biosynthesis and of transgenic plants overexpressing lignin biosynthesis genes has contributed to unravel the role of lignin in plant immunity. For example, two *pal1/2/3/4* quadruple mutants with 20% and 25% residual lignin levels and 25% residual salicylic acid levels, showed a stunted growth and were hypersusceptible to *P. syringae*. In addition, the total salicylic acid levels in the quadruple mutants after infection were about 50% of those in wild type, suggesting that pathogen-induced, salicylic acid-mediated resistance might be partially impaired in this mutant (Huang et al., 2010). The *Arabidopsis comt* mutant was found to be slightly more susceptible than wild-type plants to *P. syringae* pv. *tomato DC3000*, but also to *B. cinerea*, *A. brassicicola* and *X. campestris* pv. *campestris*, and *B. graminis* f. sp. *hordei*, that is a barley pathogen that does not colonize *Arabidopsis* plants (Quentin et al., 2009). Unexpectedly, asexual sporulation of the oomycete *H. arabidopsidis*, causing the downy mildew disease, was impaired in the *comt* mutant (Quentin et al., 2009). This phenotype was not correlated with an increased salicylic and jasmonic acid-dependent defense, but with a higher frequency of oomycete sexual reproduction within *comt* mutant tissues (Quentin et al., 2009). It was further proven that *comt* mutants accumulated soluble 5-hydroxyferuloyl-malate and that this compound promoted sexual oomycete reproduction *in vitro* (Quentin et al., 2009). *Arabidopsis f5h1* mutants showed an increased susceptibility to the fungal pathogen *S. sclerotiorum* and to the vascular fungus *Verticillium longisporum* (Huang et al., 2009; König et al., 2014). The *f5h1* mutants have similar amounts of lignin as compared to wild type, but lack S units in the lignin and are sinapate ester deficient (Meyer et al., 1998; Vanholme et al., 2012b; König et al., 2014). Because sinapate esters inhibit fungal growth *in vitro*, their absence in *f5h1* might explain the mutant's increased susceptibility toward fungal pathogens (König et al., 2014). Interestingly, the *UGT72E2* over-expressing *Arabidopsis* line, in which lignin was not altered but the soluble phenylpropanoid coniferin accumulated, was less susceptible to *V. longisporum* (König et al., 2014). All together these results again strongly support that not only the lignin polymer but also the soluble phenolic pool plays a significant role in the defense of plants against pathogens. As described above for crops and other plant species, a general positive correlation between lignin amount and resistance toward pathogens has been observed in *Arabidopsis* too. However, a deeper characterization of the impact of the lignin alterations on *Arabidopsis* immune responses and its relation with the content and profile of phenolics is needed to elucidate the molecular mechanisms explaining the differential responses of the mutants to pathogen infection (**Table 1**).

In *Arabidopsis*, the plant's response to the perturbation of lignin has been studied in a collection of mutants, each mutated in a single gene of this pathway, by combining transcriptomics and metabolomics (Vanholme et al., 2012b; **Figure 1**). These analyses revealed that *c4h*, *4cl1*, *ccoaomt1*, and *ccr1* mutants, that produced less lignin, upregulated the shikimate, methyl-donor, and phenylpropanoid pathways (i.e., the pathways supplying the monolignols), whereas, *f5h1* and *comt* mutants, that provoked lignin compositional shifts, downregulated the very same pathways (Vanholme et al., 2012b). Moreover, some of these mutant alleles revealed subtle differences in the metabolic and gene

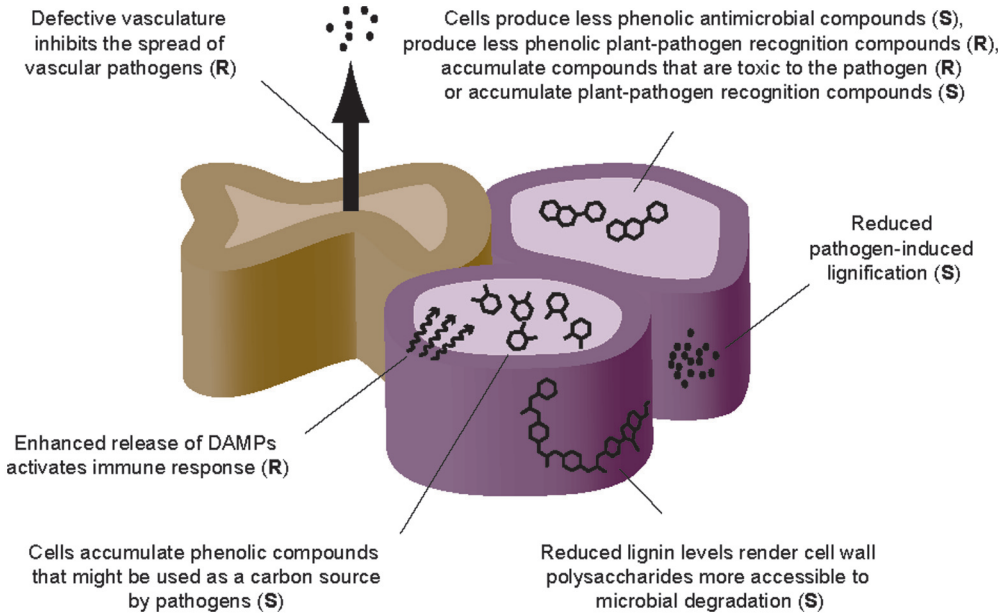


FIGURE 2 | Model illustrating lignin and secondary cell wall modifications resulting in enhanced resistance (R) or enhanced susceptibility (S) to pathogens.

expression profiles that might contribute to differential resistance responses to pathogens (Vanholme et al., 2012b). This collection of mutants represents a unique tool to further characterize the specific contribution of lignin biosynthesis to resistance against different types of pathogens in *Arabidopsis*.

EFFECTS OF MODIFYING LIGNIN AND SECONDARY CELL WALL STRUCTURE ON PLANT PATHOGEN RESISTANCE: HYPOTHESES AND OPEN QUESTIONS

Several hypotheses can be formulated to explain the resistance or the susceptibility observed in mutants and transgenic plants affected in the amount and/or composition of lignin and, more broadly, the secondary cell wall (**Figure 2**): (i) The perturbation of lignin or secondary cell wall structure modifies the physical barrier that pathogens must overcome to invade the plant. This perturbation can lead to enhanced resistance or susceptibility as these pathogens might lack the enzymes required for a proper degradation of this novel physical barrier, or wall degradation by pathogens is now facilitated, respectively; (ii) The reduced amount of lignin or the modification of lignin or secondary cell wall composition impacts the strength of the secondary cell wall resulting in collapsed xylem. A drop in vascular conduction might negatively contribute to plant colonization by vascular pathogens; (iii) The reduction of lignin amount and the modification of the secondary cell wall can loosen the wall, facilitating the constitutive or pathogen-induced release of cell wall DAMPs, which might trigger immune responses resulting in enhanced resistance to pathogens; (iv) The perturbation of the lignin pathway could lead to the accumulation of soluble phenolic compounds that are either toxic to some pathogens (e.g., resulting in a reduced virulence), or serve as a new carbon or nutrient source for pathogens that then will grow better (e.g., resulting in enhanced virulence); (v) Similarly, the perturbation of the lignin pathway could also lead to the accumulation or decrease of soluble phenolics that are plant-pathogen recognition compounds, which would result in an enhanced susceptibility or resistance, respectively. Although these hypotheses might explain some of the published phenotypes, other molecular explanations cannot be excluded and a deeper molecular and biochemical characterization is required for a better understanding of the contribution of the secondary cell wall to pathogen resistance.

PERSPECTIVES

Lignin negatively impacts the conversion of lignocellulosic biomass into fermentable sugars, making it one of the most important limiting factors in the processing of plant biomass to pulp and biofuels (Chen and Dixon, 2007; Dien et al., 2009; Van Acker et al., 2013). Hence, modifications of the plant secondary cell wall can contribute to the improvement of biomass processing in paper mills and bio-refineries. However, one critical question for lignocellulosic feedstock development is whether engineering the secondary cell wall, including its lignin content and composition, will affect plant defense against pathogens. In this review, we have summarized the role of the cell wall in plant resistance toward pathogens. We conclude that plants with altered secondary cell walls may either have an enhanced or a reduced resistance toward pathogens, or no effect at all, depending on the alterations made and the pathogens tested. Because our current knowledge on the role of the cell wall (primary or secondary) in defense against pathogens is still fragmentary, it is difficult to predict how specific alterations of the cell wall will influence a plant's resistance toward pathogens. A deeper investigation of the role of the plant cell wall

in pathogen resistance and the biochemical networks underlying this resistance is required.

ACKNOWLEDGMENTS

We gratefully acknowledge funding through the Spanish Ministry of Economy and Competitiveness (MINECO, grants BIO2012-32910 and PIM2010PKB-00749), the European Commission's Directorate-General for Research within the 7th Framework Program (FP7/2007–2013) under the grant agreement No. 311804 (SUNLIBB) and No. 270089 (MULTIBIOPRO), Stanford University's Global Climate and Energy Project (GCEP, "Lignin management: optimizing yield and composition in lignin−modified plants"), and the Multidisciplinary Research Partnership "Biotechnology for a Sustainable Economy" (01MRB510W) of Ghent University. Ruben Vanholme is indebted to the Research Foundation-Flanders for a postdoctoral fellowship. Eva Miedes was a JdC postdoctoral fellow from the Spanish MINECO.

REFERENCES

Adie, B. A., Perez-Perez, J., Perez-Perez, M. M., Godoy, M., Sanchez-Serrano, J. J., Schmelz, E. A., et al. (2007). ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. *Plant Cell* 19, 1665–1681. doi: 10.1105/tpc.106.048041

Bart, R. S., Chern, M., Vega-Sánchez, M. E., Canlas, P., and Ronald, P. C. (2010). Rice Snl6, a cinnamoyl-CoA reductase-Like gene family member, is required for NH1-mediated immunity to *Xanthomonas oryzae* pv. *oryzae*. *PLoS Genet.* 6:e1001123. doi: 10.1371/journal.pgen.1001123

Bellincampi, D., Cervone, F., and Lionetti, V. (2014). Plant cell wall dynamics and wall-related susceptibility in plant–pathogen interactions. *Front. Plant Sci.* 5:228. doi: 10.3389/fpls.2014.00228

Bethke, G., Grundman, R. E., Sreekanta, S., Truman, W., Katagiri, F., and Glazebrook, J. (2014). *Arabidopsis* PECTIN METHYLESTERASEs contribute to immunity against *Pseudomonas syringae*. *Plant Physiol.* 164, 1093–1107. doi: 10.1104/pp.113.227637

Bhuiyan, N., Liu, W., Liu, G., Selvaraj, G., Wei, Y., and 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 Mol. Biol.* 64, 305–318. doi: 10.1007/s11103-007-9155-x

Bhuiyan, N. H., Selvaraj, G., Wei, Y., and 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. *J. Exp. Bot.* 60, 509–521. doi: 10.1093/jxb/ern290

Bishop, D. L., Chyatterton, N. J., Harrison, P. A., and Hatfield, R. D. (2002). Changes in carbohydrate partitioning and cell wall remodelling with stress-induced pathogenesis in wheat sheaths. *Physiol. Mol. Plant Pathol.* 61, 53–63. doi: 10.1016/S0885-5765(02)90416-9

Boerjan, W., Ralph, J., and Baucher, M. (2003). Lignin biosynthesis. *Annu. Rev. Plant Biol.* 54, 519–546. doi: 10.1146/annurev.arplant.54.031902.134938

Bonawitz, N. D., and Chapple, C. (2010). The genetics of lignin biosynthesis: connecting genotype to phenotype. *Annu. Rev. Genet.* 44, 337–363. doi: 10.1146/annurev-genet-102209-163508

Bonawitz, N. D., and Chapple, C. (2013). Can genetic engineering of lignin deposition be accomplished without an unacceptable yield penalty? *Curr. Opin. Plant Biol.* 24, 336–343. doi: 10.1016/j.copbio.2012.11.004

Bonawitz, N. D., Kim, J. I., Tobimatsu, Y., Ciesielski, P. N., Anderson, N. A., Ximenes, E., et al. (2014). Disruption of mediator rescues the stunted growth of a lignin-deficient *Arabidopsis* mutant. *Nature* 15, 509, 376–380. doi: 10.1038/nature13084

Bonello, P., and Blodgett, J. T. (2003). *Pinus nigra–Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiol. Mol. Plant Physiol.* 63, 249–261. doi: 10.1016/j.pmpp.2004.02.002

Bonello, P., Storer, A. J., Gordon, T. R., Wood, D. L., and Heller, W. (2003). Systemic effects of *Heterobasidion annosum* on ferulic acid glucoside and lignin of presymptomatic ponderosa pine phloem, and potential effects on bark-beetle-associated fungi. *J. Chem. Ecol.* 29, 1167–1182. doi: 10.1023/A:1023833707382

Bout, S., and Vermerris, W. (2003). A candidate-gene approach to clone the sorghum Brown midrib gene encoding caffeic acid O-methyltransferase. *Mol. Genet. Genomics* 269, 205–214.

Brown, D. M., Zeef, L. A. H., Ellis, J., Goodacreb, R., and Turner, S. R. (2005). Identification of novel genes in Arabidopsis involved in secondary cell wall formation using expression profiling and reverse genetics. *Plant Cell* 17, 2281–2295. doi: 10.1105/tpc.105.031542

Buendgen, M. R., Coors, J. G., Grombacher, A. W., and Russell, W. A. (1990). European corn borer resistance and cell wall composition of three maize populations. *Crop Sci.* 30, 505–510. doi: 10.2135/cropsci1990.0011183X003000030005x

Caño-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003). Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *Plant J.* 34, 351–362. doi: 10.1046/j.1365-313X.2003.01729.x

Cantu, D., Vicente, A. R., Labavitch, J. M., Bennett, A. B., and Powell, A. L. (2008). Strangers in the matrix: plant cell walls and pathogen susceptibility. *Trends Plant Sci.* 13, 610–617. doi: 10.1016/j.tplants.2008.09.002

Carpita, N., and McCann, M. (2000). "The cell wall," in *Biochemistry and Molecular Biology of Plants*, eds B. B. Buchanan, G. Wilhelm, and R. L. Jones (Rockville, IL: American Society of Plant Physiologists), 52–108.

Chen, F., and Dixon, R. A. (2007). Lignin modification improves fermentable sugar yields for biofuel production. *Nat. Biotechnol.* 25, 759–761. doi: 10.1038/nbt1316

Chen, Z., Zheng, Z., Huang, J., Lai, Z., and Fan, B. (2009). Biosynthesis of salicylic acid in plants. *Plant Signal. Behav.* 4, 493–496. doi: 10.4161/psb.4.6.8392

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* 6, 850–861. doi: 10.1038/nrm1746

Daayf, F., El Hadrami, A., El-Bebany, A. F., Henriquez, M. A., Yao, Z., Derksen, H., et al. (2012). "Phenolic compounds in plant defense and pathogen counter-defense mechanisms," in *Recent Advances in Polyphenol Research*, eds V. Cheynier, P. Sarni-Manchado, and S. Quideau (Oxford, UK: Wiley-Blackwell). doi: 10.1002/9781118299753.ch8

Dauwe, R., Morreel, K., Goeminne, G., Gielen, B., Rohde, A., Van Beeumen, J., et al. (2007). Molecular phenotyping of lignin-modified tobacco reveals associated changes in cell–wall metabolism, primary metabolism, stress metabolism and photorespiration. *Plant J.* 52, 263–285. doi: 10.1111/j.1365-313X.2007.03233.x

Del Río, J. C., Marques, G., Rencoret, J., Martínez, Á. T., and Gutiérrez, A. (2007). Occurrence of naturally acetylated lignin units. *J. Agric. Food Chem.* 55, 5461–5468. doi: 10.1021/jf0705264

Del Río, J. C., Rencoret, J., Prinsen, P., Martínez, Á. T., Ralph, J., and Gutiérrez, A. (2012). Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods. *J. Agric. Food Chem.* 60, 5922–5935. doi: 10.1021/jf301002n

Delgado-Cerezo, M., Sánchez-Rodríguez, C., Escudero, V., Miedes, E., Fernández, P. V., Jordá, L., et al. (2012). *Arabidopsis* heterotrimeric G-protein regulates cell wall defense and resistance to necrotrophic fungi. *Mol. Plant* 5, 98–114. doi: 10.1093/mp/ssr082

Denancé, N., Ranocha, P., Oria, N., Barlet, X., Rivière, M. P., Yadeta, K. A., et al. (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. *Plant J.* 73, 225–239. doi: 10.1111/tpj.12027

Dicko, M. H., Gruppen, H., Barro, C., Traore, A. S., Van Berkel, W. J. H., and Voragen, A. G. J. (2005). Impact of phenolic compounds and related enzymes in sorghum varieties for resistance and susceptibility to biotic and abiotic stresses. *J. Chem. Ecol.* 31, 2671–2688. doi: 10.1007/s10886-005-7619-5

Dien, B. S., Sarath, G., Pedersen, J. F., Sattler, S. E., Chen, H., Funnell-Harris, D. L., et al. (2009). Improved sugar conversion and ethanol yield for forage sorghum (*Sorghum bicolor* L. Moench) lines with reduced lignin contents. *Bioenergy Res.* 2, 153–164. doi: 10.1007/s12155-009-9041-2

Dixon, R. A., Achnine, L., Kota, P., Liu, C. J., Reddy, M. S., and Wang, L. (2002). The phenylpropanoid pathway and plant defence – a genomics

perspective. *Mol. Plant Pathol.* 3, 371–390. doi: 10.1046/j.1364-3703.2002.00131.x

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J. G. (2002). The *Arabidopsis* mutant cev1 links cell wall signaling to jasmonate and ethylene responses. *Plant Cell* 14, 1557–1566. doi: 10.1105/tpc.002022

Engelsdorf, T., and Hamann, T. (2014). An update on receptor-like kinase involvement in plant cell wall integrity maintenance. *Ann. Bot.* doi: 10.1093/aob/mcu043 [Epub ahead of print].

Eynck, C., Séguin-Swartz, G., Clarke, W. E., and Parkin, I. A. (2012). Monolignol biosynthesis is associated with resistance to *Sclerotinia sclerotiorum* in *Camelina sativa*. *Mol. Plant Pathol.* 13, 887–899. doi: 10.1111/j.1364-3703.2012.00798.x

Ferrari, S., Savatin, D. V., Sicilia, F., Gramegna, G., Cervone F., and DeLorenzo, G. (2013). Oligogalacturonides: plant damage associated molecular patterns and regulators of growth and development. *Front. Plant Sci.* 4:49. doi: 10.3389/fpls.2013.00049

Ferrari, S., Sella, L., Janni, M., Favaron, F., and D'Ovidio, R. (2012). Transgenic expression of polygalacturonase-inhibiting proteins in *Arabidopsis* and wheat increases resistance to the flower pathogen *Fusarium graminearum*. *Plant Biol.* 14, 31–38. doi: 10.1111/j.1438-8677.2011.00449.x

Funnell-Harris, D. L., Pedersen, J. F., and Sattler, S. E. (2010). Alteration in lignin biosynthesis restricts growth of *Fusarium* spp. in brown midrib sorghum. *Phytopathology* 100, 671–681. doi: 10.1094/PHYTO-100-7-0671

Gallego-Giraldo, L., Escamilla-Trevino, L., Jackson, L. A., and Dixon, R. A. (2011a). Salicylic acid mediates the reduced growth of lignin down-regulated plants. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20814–20819. doi: 10.1073/pnas.1117873108

Gallego-Giraldo, L., Jikumaru, Y., Kamiya, Y., Tang, Y., and Dixon, R. A. (2011b). Selective lignin down-regulation leads to constitutive defense response expression in alfalfa (*Medicago sativa* L.). *New Phytol.* 190, 627–639. doi: 10.1111/j.1469-8137.2010.03621.x

García-Olmedo, F., Rodríguez-Palenzuela, P., Molina, A., Alamillo, J. M., López-Solanilla, E., Berrocal-Lobo, M., et al. (2001). Antibiotic activities of peptides, hydrogen peroxide and peroxynitrite in plant defence. *FEBS Lett.* 498, 219–222. doi: 10.1016/S0014-5793(01)02456-5

Giberti, S., Bertea, C. M., Narayana, R., Maffei, M. E., and Forlani, G. (2012). Two phenylalanine ammonia lyase isoforms are involved in the elicitor-induced response of rice to the fungal pathogen *Magnaporthe oryzae*. *J. Plant Physiol.* 169, 249–254. doi: 10.1016/j.jplph.2011.10.008

Gille, S., de Souza, A., Xiong, G., Benz, M., Cheng, K., Schultink, A., et al. (2011). O-acetylation of *Arabidopsis* hemicellulose xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. *Plant Cell* 23, 4041–4053. doi: 10.1105/tpc.111.091728

Gille, S., and Pauly, M. (2012). O-acetylation of plant cell wall polysaccharides. *Front. Plant Sci.* 3:12. doi: 10.3389/fpls.2012.00012

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Godiard, L., Sauviac, L., Torii, K. U., Grenon, O., Mangin, B., and Grimsley, N. H., et al. (2003). ERECTA, an LRR receptor-like kinase protein controlling development pleiotropically affects resistance to bacterial wilt. *Plant J.* 36, 353–365. doi: 10.1046/j.1365-313X.2003.01877.x

Halpin, C., Thain, S. C., Tilston, E. L., Guiney, E., Lapierre, C., and Hopkins, D. W. (2007). Ecological impacts of trees with modified lignin. *Tree Genet. Genomes* 3, 101–110. doi: 10.1007/s11295-006-0060-2

Hano, C., Martin, I., Fliniaux, O., Legrand, B., Gutierrez, L., Arroo, R. R., et al. (2006). Pinoresinol–lariciresinol reductase gene expression and secoisolariciresinol diglucoside accumulation in developing flax (*Linum usitatissimum*) seeds. *Planta* 224, 1291–1301. doi: 10.1007/s00425-006-0308-y

Hatfield, R. D., Marita, J. M., Frost, K., Grabber, J., Ralph, J., Lu, F., et al. (2009). Grass lignin acylation: p-coumaroyl transferase activity and cell wall characteristics of C3 and C4 grasses. *Planta* 229, 1253–1267. doi: 10.1007/s00425-009-0900-z

Hematy, K., Cherk, C., and Somerville, S. (2009). Host–pathogen warfare at the plant cell wall. *Curr. Opin. Plant Biol.* 12, 406–413. doi: 10.1016/j.pbi.2009.06.007

Hernández-Blanco, C., Feng, D. X., Hu, J., Sánchez-Vallet, A., Deslandes, L., Llorente, F., et al. (2007). Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. *Plant Cell* 19, 890–903. doi: 10.1105/tpc.106.048058

Huang, J., Bhinu, V. S., Li, X., Bashi, Z. D., Zhou, R., and Hannoufa, A. (2009). Pleiotropic changes in *Arabidopsis* f5h and sct mutants revealed by large-scale gene expression and metabolite analysis. *Planta* 230, 1057–1069. doi: 10.1007/s00425-009-1007-2

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou Y. H., et al. (2010). Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress. *Plant Physiol.* 153, 1526–1538. doi: 10.1104/pp.110.157370

Ikegawa, T., Mayama, S., Nakayashiki, H., and Kato, H. (1996). Accumulation of diferulic acid during the hypersensitive response of oat leaves to *Puccinia coronate* f. sp. *avenae* and its role in the resistance of oat tissues to cell wall degrading enzymes. *Physiol. Mol. Plant Pathol.* 48, 245–256. doi: 10.1006/pmpp.1996.0021

Jiang, K., Frick-Cheng, A., Trusov, Y., Delgado-Cerezo, M., Rosenthal, D. M., Lorek, J., et al. (2012). Dissecting *Arabidopsis* G-β signal transduction on the protein surface. *Plant Physiol.* 159, 975–983. doi: 10.1104/pp.112.196337

Kim, H., Ralph, J., Yahiaoui, N., Pean, M., and Boudet, A.-M. (2000). Cross-coupling of hydroxycinnamyl aldehydes into lignins. *Org. Lett.* 2, 2197–2200. doi: 10.1021/ol005906o

Kliebenstein, D. J., Lim, J. E., Landry, L. G., and Last, R. L. (2002). *Arabidopsis* UVR8 regulates ultraviolet-B signal transduction and tolerance and contains sequence similarity to human regulator of chromatin condensation 1. *Plant Physiol.* 130, 234–243. doi: 10.1104/pp.005041

Klopffleisch, K., Phan, N., Augustin, K., Bayne, R. S., Booker, K. S., Botella, J. R. et al. (2011). *Arabidopsis* G-protein interactome reveals connections to cell wall carbohydrates and morphogenesis. *Mol. Syst. Biol.* 7, 532. doi: 10.1038/msb.2011.66

Kofalvi, S. A., and Nassuth, A. (1995). Influence of wheat streak mosaic virus infection on phenylpropanoid metabolism and the accumulation of phenolics and lignin in wheat. *Physiol. Mol. Plant Pathol.* 47, 365–377. doi: 10.1006/pmpp.1995.1065

König, S., Feussner, K., Kaever, A., Landesfeind, M., Thurow, C., Karlovsky, P., et al. (2014). Soluble phenylpropanoids are involved in the defense response of *Arabidopsis* against *Verticillium longisporum*. *New Phytol.* 202, 823–837. doi: 10.1111/nph.12709

Lauvergeat, V., Lacomme, C., Lacombe, E., Lasserre, E., Roby, D., and Grima-Pettenati, J. (2001). Two cinnamoyl-CoA reductase (CCR) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. *Phytochemistry* 57, 1187–1195. doi: 10.1016/S0031-9422(01)00053-X

Lee, C., Teng, Q., Zhong, R., and Ye, Z. H. (2011). The four *Arabidopsis* REDUCED WALL ACETYLATION genes are expressed in secondary wall-containing cells and required for the acetylation of xylan. *Plant Cell Physiol.* 52, 1289–1301. doi: 10.1093/pcp/pcr075

Lee, K., Dudley, M. W., Hess, K. M., Lynn, D. G., Joerger, R. D., and Binns, A. N. (1992). Mechanism of activation of *Agrobacterium* virulence genes: identification of phenol-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* 89, 8666–8670. doi: 10.1073/pnas.89.18.8666

Lee, Y. W., Jin, S., Sims, W. S., and Nester, E. W. (1995). Genetic evidence for direct sensing of phenolic compounds by the VirA protein of *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. U.S.A.* 92, 12245–12249. doi: 10.1073/pnas.92.26.12245

Lionetti, V., Cervone, F., and Bellincampi, D. (2012). Methyl esterification of pectin plays a role during plant–pathogen interactions and affects plant resistance to diseases. *J. Plant Physiol.* 169, 1623–1630. doi: 10.1016/j.jplph.2012.05.006

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., et al. (2007). Overexpression of pectin methylesterase inhibitors in *Arabidopsis* restricts fungal infection by *Botrytis cinerea*. *Plant Physiol.* 143, 1871–1880. doi: 10.1104/pp.106.090803

Liu, J., Ding, P., Sun, T., Nitta, Y., Dong, O., Huang, X., et al. (2013). Heterotrimeric G proteins serve as a converging point in plant defense signaling

activated by multiple receptor-like kinases. *Plant Physiol.* 161, 2146–2158. doi: 10.1104/pp.112.212431

Llorente, F., Alonso-Blanco, C., Sanchez-Rodriguez, C., Jorda, L., and Molina, A. (2005). ERECTA receptor-like kinase and heterotrimeric G protein from *Arabidopsis* are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. *Plant J.* 43, 165–180. doi: 10.1111/j.1365-313X.2005.02440.x

Lo, S. C. C., and Nicholson, R. L. (1998). Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls: implications for a compensatory role in the defense response. *Plant Physiol.* 116, 979–989. doi: 10.1104/pp.116.3.979

Lorek, J., Griebel, T., Jones, A. M., Kuhn, H., and Panstruga, R. (2013). The role of *Arabidopsis* heterotrimeric G-protein subunits in MLO2 function and MAMP-triggered immunity. *Mol. Plant Microbe. Interact.* 26, 991–1003. doi: 10.1094/MPMI-03-13-0077-R

Lozovaya, V. V., Lygin, A. V., Zernova, O. V., Ulanov, A. V., Li, S. X., Hartman, G. L., et al. (2007). Modification of phenolic metabolism in soybean hairy roots through down regulation of chalcone synthase or isoflavone synthase. *Planta* 225, 665–679. doi: 10.1007/s00425-006-0368-z

Lu, F., and Ralph, J. (2008). Novel tetrahydrofuran structures derived from β-β-coupling reactions involving sinapyl acetate in kenaf lignins. *Org. Biomol. Chem.* 6, 3681–3694. doi: 10.1039/b809464k

Macho, A. P., and Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Mol. Cell.* 54, 263–272. doi: 10.1016/j.molcel.2014.03.028

Maher, E. A., Bate, N. J., Ni, W., Elkind, Y., Dixon, R. A., and Lamb, C. J. (1994). Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. *Proc. Natl. Acad. Sci. U.S.A.* 91, 7802–7806. doi: 10.1073/pnas.91.16.7802

Malinovsky, F. G., Fangel, J. U., and Willats, W. G. T. (2014). The role of the cell wall in plant immunity. *Front. Plant Sci.* 5:178. doi: 10.3389/fpls.2014.00178

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., et al. (2011). Loss-of-function mutation of REDUCED WALL ACETYLATION in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. *Plant Physiol.* 155, 1068–1078. doi: 10.1104/pp.110.168989

Mandal, S., Das, R. K., and Mishra, S. (2011). Differential occurrence of oxidative burst and antioxidative mechanism in compatible and incompatible interactions of tomato and *Ralstonia solanacearum*. *Plant Physiol. Biochem.* 49, 117–123. doi: 10.1016/j.plaphy.2010.10.006

Mandal, S., Kar, I., Mukherjee, A. K., and Acharya, P. (2013). Elicitor-induced defense responses in *Solanum lycopersicum* against *Ralstonia solanacearum*. *Sci. World J.* 25:561056. doi: 10.1155/2013/561056

Martin, J. A., Solla, A., Woodward, S., and Gil, L. (2007). Detection of differential changes in lignin composition of elm xylem tissues inoculated with *Ophiostoma novoulmi* using Fourier transform-infrared spectroscopy. *For. Pathol.* 37, 187–191. doi: 10.1111/j.1439-0329.2007.00495.x

Maury, S., Delaunay, A., Mesnard, F., Cronier, D., Chabbert, B., Geoffroy, P., et al. (2010). O-methyltransferase(s)-suppressed plants produce lower amounts of phenolic vir inducers and are less susceptible to *Agrobacterium tumefaciens* infection. *Planta* 232, 975–986. doi: 10.1007/s00425-010-1230-x

Menden, B., Kohlhoff, M., and Moerschbacher, B. M. (2007). Wheat cells accumulate a syringyl-rich lignin during the hypersensitive resistance response. *Phytochemistry* 68, 513–520. doi: 10.1016/j.phytochem.2006.11.011

Meyer, K., Shirley, A. M., Cusumano, J. C., Bell-Lelong, D. A., and Chapple, C. (1998). Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6619–6623. doi: 10.1073/pnas.95.12.6619

Mohr, P. G., and Cahill, D. M. (2007). Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in *Arabidopsis* infected with *Pseudomonas syringae* pv. tomato. *Funct. Integr. Genomics* 7, 181–191. doi: 10.1007/s10142-006-0041-4

Morreel, K., Ralph, J., Kim, H., Lu, F., Goeminne, G., Ralph, S., et al. (2004). Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem. *Plant Physiol.* 136, 3537–3549. doi: 10.1104/pp.104.049304

Naoumkina, M. A., Zhao, Q., Gallego-Giraldo, L., Dai, X., Zhao, P. X., and Dixon, R. A. (2010). Genome-wide analysis of phenylpropanoid defense pathways. *Mol. Plant Pathol.* 11, 829–846. doi: 10.1111/j.1364-3703.2010.00648.x

Oliver, A. L., Pedersen, J. F., Grant, R. J., Klopfenstein, T. J., and Jose, H. (2005). Comparative effects of the sorghum bmr-6 and bmr-12 genes: II. Grain yield, stover yield, and stover quality in grain sorghum. *Crop Sci.* 45, 2240–2245. doi: 10.2135/cropsci2004.0660

Ong, R. G., Chundawat, S. P. S., Hodge, D. B., Keskar, S., and Dale, B. E. (2014). "Linking plant biology and pretreatment: understanding the structure and organization of the plant cell wall and interactions with cellulosic biofuel production," in *Plants and BioEnergy, Advances in Plant Biology*, eds M. C. McCann, M. S. Buckeridge, and N. C. Carpita (New York, NY: Springer), 231–253.

Pan, Q., Zhan, J., Liu, H., Zhang, J., Chen, J., Wen, P., et al. (2006). Salicylic acid synthesized by benzoic acid 2-hydroxylase participates in the development of thermotolerance in pea plants. *Plant Sci.* 171, 226–233. doi: 10.1016/j.plantsci.2006.03.012

Panstruga, R., Parker, J. E., and Schulze-Lefert, P. (2009). SnapShot: plant immune response pathways. *Cell* 136, 978–U976. doi: 10.1016/j.cell.2009.02.020

Parrott, D. L., Anderson, A. J., and Carman, J. G. (2002). *Agrobacterium* induces plant cell death in wheat (*Triticum aestivum* L.). *Physiol. Mol. Plant Pathol.* 60, 59–69. doi: 10.1006/pmpp.2002.0378

Pesquet, E., Ranocha, P., Legay, S., Digonnet, C., Barbier, O., Pichon, M., et al. (2005). Novel markers of xylogenesis in zinnia are differentially regulated by auxin and cytokinin. *Plant Physiol.* 139, 1821–1839. doi: 10.1104/pp.105.064337

Petersen, P. D., Lau, J., Ebert, B., Yang, F., Verhertbruggen, Y., Kim, J. S., et al. (2012). Engineering of plants with improved properties as biofuels feedstocks by vessel-specific complementation of xylan biosynthesis mutants. *Biotechnol. Biofuels* 26, 84. doi: 10.1186/1754-6834-5-84

Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leplé, J.-C., et al. (2002). Field and pulping performances of transgenic trees with altered lignification. *Nat. Biotechnol.* 20, 607–612. doi: 10.1038/nbt0602-607

Pogorelko, G., Lionetti, V., Fursova, O., Sundaram, R. M., Qi, M., and Whitham, S. A., et al. (2013). *Arabidopsis* and *Brachypodium distachyon* transgenic plants expressing *Aspergillus nidulans* acetylesterases have decreased degree of polysaccharide acetylation and increased resistance to pathogens. *Plant Physiol.* 162, 9–23. doi: 10.1104/pp.113.214460

Quentin, M., Allasia, V., Pegard, A., Allais, F., Ducrot, P. H., Favery, B., et al. (2009). Imbalanced lignin biosynthesis promotes the sexual reproduction of homothallic oomycete pathogens. *PLoS Pathog.* 5:e1000264. doi: 10.1371/journal.ppat.1000264

Raiola, A., Lionetti, V., Elmaghraby, I., Immerzeel, P., Mellerowicz, E. J., Salvi, G., et al. (2011). Pectin methylesterase is induced in *Arabidopsis* upon infection and is necessary for a successful colonization by necrotrophic pathogens. *Mol. Plant Microbe Interact.* 24, 432–440. doi: 10.1094/MPMI-07-10-0157

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P. F., et al. (2004). Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochem. Rev.* 3, 29–60. doi: 10.1023/B:PHYT.0000047809.65444.a4

Ramírez, V., Agorio, A., Coego, A., García-Andrade, J., Hernández, M. J., Balaguer, B., et al. (2011). MYB46 modulates disease susceptibility to *Botrytis cinerea* in *Arabidopsis*. *Plant Physiol.* 155, 1920–1935. doi: 10.1104/pp.110.171843

Ranocha, P., Denancé, N., Vanholme, R., Freydier, A., Martinez, Y., Hoffmann, L., et al. (2010). Walls are thin 1 (WAT1), an *Arabidopsis* homolog of *Medicago truncatula* NODULIN21, is a tonoplast-localized protein required for secondary wall formation in fibers. *Plant J.* 63, 469–483. doi: 10.1111/j.1365-313X.2010.04256.x

Ranocha, P., Dima, O., Nagy, R., Felten, J., Corratgé-Faillie, C., Novak, O., et al. (2013). *Arabidopsis* WAT1 is a vacuolar auxin transport facilitator required for auxin homeostasis. *Nat. Commun.* 4, 2625. doi: 10.1038/ncomms3625

Ringli, C. (2010). Monitoring the outside: cell wall-sensing mechanisms. *Plant Physiol.* 153, 1445–1452. doi: 10.1104/pp.110.154518

Rogers, L. A., Dubos, C., Surman, C., Willment, J., Cullis, I. F., Mansfield, S. D., et al. (2005). Comparison of lignin deposition in three ectopic lignification mutants. *New Phytol.* 168, 123–140. doi: 10.1111/j.1469-8137.2005.01496.x

Romero, D., Rivera, M. E., Cazorla, F. M., Codina, J. C., Fernández-Ortuño, D., Torés, J. A., et al. (2008). Comparative histochemical analyses of oxidative burst and cell wall reinforcement in compatible and incompatible melon-powdery mildew (*Podosphaera fusca*) interactions. *J. Plant Physiol.* 165, 1895–1905. doi: 10.1016/j.jplph.2008.04.020

Ruuhola, T., and Julkunen-Tiitto, R. (2003). Trade-off between synthesis of salicylates and growth of micropropagated *Salix pentandra*. *J. Chem. Ecol.* 29, 1565–1588. doi: 10.1023/A:1024266612585

Sampedro, J., Pardo, B., Gianzo, C., Guitián, E., Revilla, G., and Zarra, I. (2010). Lack of α-xylosidase activity in *Arabidopsis* alters xyloglucan composition and results in growth defects. *Plant Physiol.* 154, 1105–1115. doi: 10.1104/pp.110.163212

Sánchez-Rodríguez, C., Estevez, J. M., Llorente, F., Hernandez-Blanco, C., Jorda, L., Pagan, I., et al. (2009). The ERECTA Receptor-Like Kinase regulates cell wall mediated resistance to pathogens in *Arabidopsis thaliana*. *Mol. Plant Microbe. Interact.* 22, 953–963. doi: 10.1094/MPMI-22-8-0953

Sánchez-Vallet, A., Ramos, B., Bednarek, P., López, G., Piślewska-Bednarek, M., Schulze-Lefert, P., et al. (2010). Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. *Plant J.* 63, 115–127. doi: 10.1111/j.1365-313X.2010.04224.x

Sarkar, P., Bosneaga, E., and Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *J. Exp. Bot.* 60, 3615–3635. doi: 10.1093/jxb/erp245

Sattler, S. E., and Funnell-Harris, D. L. (2013). Modifying lignin to improve bioenergy feedstocks: strengthening the barrier against pathogens? *Front Plant Sci.* 4:70. doi: 10.3389/fpls.2013.00070

Sattler, S. E., Saathoff, A. J., Haas, E. J., Palmer, N. A., Funnell-Harris, D. L., Sarath, G., et al. (2009). A nonsense mutation in a cinnamyl alcohol dehydrogenase gene is responsible for the sorghum brown midrib 6 phenotype. *Plant Physiol.* 150, 584–595. doi: 10.1104/pp.109.136408

Schulze-Lefert, P. (2004). Knocking on the heaven's wall: pathogenesis of and resistance to biotrophic fungi at the cell wall. *Curr. Opin. Plant Biol.* 7, 377–383. doi: 10.1016/j.pbi.2004.05.004

Shadle, G. L., Wesley, S. W., Korth, K. L., Chen, F., Lamb, C., and Dixon, R. A. (2003). Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of l-phenylalanine ammonia-lyase. *Phytochemistry* 64, 153–161. doi: 10.1016/S0031-9422(03)00151-1

Shi, H., Liu, Z., Zhu, L., Zhang, C., Chen, Y., Zhou, Y., et al. (2012). Overexpression of cotton (*Gossypium hirsutum*) dirigent1 gene enhances lignification that blocks the spread of *Verticillium dahliae*. *Acta Biochim. Biophys. Sin.* 44, 555–564. doi: 10.1093/abbs/gms035

Simmons, B. A., Loqué, D., and Ralph, J. (2010). Advances in modifying lignin for enhanced biofuel production. *Curr. Opin. Plant Biol.* 13, 313–320. doi: 10.1016/j.pbi.2010.03.001

Smith, A. H., Gill, W. M., Pinkard, E. A., and Mohammed, C. L. (2007). Anatomical and histochemical defence responses induced in juvenile leaves of *Eucalyptus globulus* and *Eucalyptus nitens* by *Mycosphaerella* infection. *For. Pathol.* 37, 361–373. doi: 10.1111/j.1439-0329.2007.00502.x

Torres, M. A., Morales, J., Sánchez-Rodríguez, C., Molina, A., and Dangl, J. L. (2013). Functional interplay between *Arabidopsis* NADPH oxidases and heterotrimeric G protein. *Mol. Plant Microbe Interact.* 26, 686–694. doi: 10.1094/MPMI-10-12-0236-R

Tronchet, M., Balagué, C., Kroj, T., Jouanin, L., and Roby, D. (2010). Cinnamyl alcohol dehydrogenases C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in *Arabidopsis*. *Mol. Plant Pathol.* 11, 83–92. doi: 10.1111/j.1364-3703.2009.00578.x

Trusov, Y., Jordá, L., Molina, A., and Botella, J. R. (2010). "G proteins and plant innate immunity," in *Integrated G Proteins Signaling in Plants*, eds S. Yalovsky, F. Baluška, and A. Jones (Berlin, Heidelberg: Springer), 221–250.

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Front. Plant Sci.* 3:85. doi: 10.3389/fpls.2012.00085

Van Acker, R., Leplé, J.-C., Aerts, D., Storme, V., Goeminne, G., Ivens, B., et al. (2014). Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase. *Proc. Natl. Acad. Sci. U.S.A.* 111, 845–850. doi: 10.1073/pnas.1321673111

Van Acker, R., Vanholme, R., Storme, V., Mortimer, J. C., Dupree, P., and Boerjan, W. (2013). Lignin biosynthesis perturbations affect secondary cell wall composition and saccharification yield in *Arabidopsis thaliana*. *Biotechnol. Biofuels* 6:46. doi: 10.1186/1754-6834-6-46

Vanholme, R., Cesarino, I., Rataj, K., Xiao, Y., Sundin, L., Goeminne, G., et al. (2013). Caffeoyl shikimate esterase (CSE) is an enzyme in the lignin biosynthetic pathway in *Arabidopsis*. *Science* 341, 1103–1106. doi: 10.1126/science.1241602

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., and Boerjan, W. (2010). Lignin biosynthesis and structure. *Plant Physiol.* 153, 895–905. doi: 10.1104/pp.110.155119

Vanholme, R., Morreel, K., Darrah, C., Oyarce, P., Grabber, J. H., Ralph, J., et al. (2012a). Metabolic engineering of novel lignin in biomass crops. *New Phytol.* 196, 978–1000. doi: 10.1111/j.1469-8137.2012.04337.x

Vanholme, R., Storme, V., Vanholme, B., Sundin, L., Christensen, J. H., Goeminne, G., et al. (2012b). A systems biology view of responses to lignin biosynthesis perturbations in *Arabidopsis*. *Plant Cell* 24, 3506–3529. doi: 10.1105/tpc.112.102574

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. C. (2002). PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis*. *Plant Cell* 14, 2095–2106. doi: 10.1105/tpc.003509

Vogel, J. P., Raab, T. K., Somerville, C. R., and Somerville, S. C. (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. *Plant J.* 40, 968–978. doi: 10.1111/j.1365-313X.2004.02264.x

Volpi, C., Janni, M., Lionetti, V., Bellincampi, D., Favaron, F., and D'Ovidio, R. (2011). The ectopic expression of a pectin methyl esterase inhibitor increases pectin methyl esterification and limits fungal diseases in wheat. *Mol. Plant Microbe. Interact.* 24, 1012–1019. doi: 10.1094/MPMI-01-11-0021

Vorwerk, S., Somerville, S., and Somerville, C. (2004). The role of plant cell wall polysaccharide composition in plant disease resistance. *Trends Plant Sci.* 9, 203–209. doi: 10.1016/j.tplants.2004.02.005

Way, H. M., Birch, R. G., and Manners, J. M. (2011). A comparison of individual and combined l-phenylalanine ammonia lyase and cationic peroxidase transgenes for engineering resistance in tobacco to necrotrophic pathogens. *Plant Biotechnol. Rep.* 5, 301–308. doi: 10.1007/s11816-011-0183-2

Way, H. M., Kazan, K., Mitter, N., Goulter, K. C., Birch, R. G., and Manners, J. M. (2002). Constitutive expression of a phenylalanine ammonia-lyase gene from *Stylosanthes humilis* in transgenic tobacco leads to enhanced disease resistance but impaired plant growth. *Physiol. Mol. Plant Pathol.* 60, 275–282. doi: 10.1016/S0885-5765(02)90407-8

Weiergang, I., Hipskind, J. D., and Nicholson, R. L. (1996). Synthesis of 3-deoxyanthocyanidin phytoalexins in sorghum occurs independent of light. *Physiol. Mol. Plant Pathol.* 49, 377–388. doi: 10.1006/pmpp.1996.0060

Wolf, S., Hématy, K., and Höfte, H. (2012). Growth control and cell wall signaling in plants. *Annu. Rev. Plant Biol.* 63, 381–407. doi: 10.1146/annurev-arplant-042811-105449

Wróbel-Kwiatkowska, M., Starzycki, M., Zebrowski, J., Oszmiański, J., and Szopa, J. (2007). Lignin deficiency in transgenic flax resulted in plants with improved mechanical properties. *J. Biotechnol.* 128, 919–934. doi: 10.1016/j.jbiotec.2006.12.030

Xu, L., Zhu, L., Tu, L., Liu, L., Yuan, D., Jin, L., et al. (2011). Lignin metabolism has a central role in the resistance of cotton to the wilt fungus *Verticillium dahliae* as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry. *J. Exp. Bot.* 62, 5607–5621. doi: 10.1093/jxb/err245

Yang, F., Mitra, P., Zhang, L., Prak, L., Verhertbruggen, Y., Kim, J. S., et al. (2013). Engineering secondary cell wall deposition in plants. *Plant Biotechnol. J.* 11, 325–335. doi: 10.1111/pbi.12016

Yu, C. K., Springob, K., Schmidt, J., Nicholson, R. L., Chu, I. K., Yip, W. K., et al. (2005). A stilbene synthase gene (SbSTS1) is involved in host and nonhost defense responses in sorghum. *Plant Physiol.* 138, 393–401. doi: 10.1104/pp.105.059337

Yu, O., Jung, W., Shi, J., Croes, R. A., Fader, G. M., McGonigle, B., et al. (2000). Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. *Plant Physiol.* 124, 781–794. doi: 10.1104/pp.124.2.781

Zhang, S. H., Yang, Q., and Ma, R. C (2007). *Erwinia carotovora* ssp. carotovora infection induced "defense lignin" accumulation and lignin biosynthetic gene expression in Chinese cabbage (*Brassica rapa* L. ssp. pekinensis). *J. Integr. Plant Biol.* 49, 993–1002. doi: 10.1111/j.1672-9072.2007.00478.x

Zhao, J., Buchwaldt, L., Rimmer, S. R., Sharpe, A., Mcgregor, L., Bekkoui, D., et al. (2009). Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotiorum*. *Mol. Plant Pathol.* 10, 635–649. doi: 10.1111/j.1364-3703.2009.00558.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 May 2014; paper pending published: 13 June 2014; accepted: 04 July 2014; published online: 05 August 2014.

*Citation: Miedes E, Vanholme R, Boerjan W and Molina A (2014) The role of the secondary cell wall in plant resistance to pathogens. Front. Plant Sci. **5**:358. doi: 10.3389/fpls.2014.00358*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 19 March 2014
doi: 10.3389/fpls.2014.00089

The plant cell wall in the feeding sites of cyst nematodes

Holger Bohlmann[1]* and Miroslaw Sobczak[2]

[1] *Division of Plant Protection, Department of Crop Sciences, University of Natural Resources and Life Sciences, Vienna, Austria*
[2] *Department of Botany, Warsaw University of Life Sciences, Warsaw, Poland*

Edited by:
Vincenzo Lionetti, Sapienza – Università di Roma, Italy

Reviewed by:
Daguang Cai, Christian Albrechts University of Kiel, Germany
Carolina Escobar, Universidad de Castilla-La Mancha, Spain

****Correspondence:***
Holger Bohlmann, Division of Plant Protection, Department of Crop Sciences, University of Natural Resources and Life Sciences, UFT Tulln, Konrad Lorenz Str. 24, Vienna, 3430 Tulln, Austria
e-mail: holger.bohlmann@boku.ac.at

Plant parasitic cyst nematodes (genera *Heterodera* and *Globodera*) are serious pests for many crops. They enter the host roots as migratory second stage juveniles (J2) and migrate intracellularly toward the vascular cylinder using their stylet and a set of cell wall degrading enzymes produced in the pharyngeal glands. They select an initial syncytial cell (ISC) within the vascular cylinder or inner cortex layers to induce the formation of a multicellular feeding site called a syncytium, which is the only source of nutrients for the parasite during its entire life. A syncytium can consist of more than hundred cells whose protoplasts are fused together through local cell wall dissolutions. While the nematode produces a cocktail of cell wall degrading and modifying enzymes during migration through the root, the cell wall degradations occurring during syncytium development are due to the plants own cell wall modifying and degrading proteins. The outer syncytial cell wall thickens to withstand the increasing osmotic pressure inside the syncytium. Furthermore, pronounced cell wall ingrowths can be formed on the outer syncytial wall at the interface with xylem vessels. They increase the surface of the symplast-apoplast interface, thus enhancing nutrient uptake into the syncytium. Processes of cell wall degradation, synthesis and modification in the syncytium are facilitated by a variety of plant proteins and enzymes including expansins, glucanases, pectate lyases and cellulose synthases, which are produced inside the syncytium or in cells surrounding the syncytium.

Keywords: cell wall ingrowths, cell wall openings, cyst nematodes, feeding site, syncytium

INTRODUCTION

Various species of plant parasitic nematodes attack the roots of crop plants, leading to serious agricultural losses, which have been estimated to be between US $ 125 and 157 billion per year (Chitwood, 2003; Abad et al., 2008). The sedentary cyst nematodes can be especially difficult to eradicate because they can survive in the soil for many years as dormant second stage juveniles (J2) in eggs protected by dead female bodies turned into cysts. Agriculturally the most important cyst nematode species are the potato cyst nematodes (*Globodera rostochiensis* and *G. pallida* with yellow/golden and white cysts, respectively) and the soybean cyst nematode (*Heterodera glycines*), which causes serious losses in soybean. Several other *Heterodera* species are found on cereals: *H. avenae* and *H. filipjevi* on oat, wheat and barley, and *H. zeae* on maize. The beet cyst nematode (*H. schachtii*) poses a serious problem for sugar beet production, but interestingly it is able to infect also species of the family *Brassicaceae* including *Arabidopsis thaliana* and this interaction is widely accepted as a model system in plant-nematode research (Sijmons et al., 1991). Upon stimulation by mostly unknown triggers, the migratory J2s hatch from the eggs and migrate toward roots. They enter an epidermal cell of host plant roots making numerous perforations in the cell wall with the aid of their stylet. Afterwards, they migrate intracellularly through the root cortical cells toward the vascular cylinder. J2s of *Globodera* sp. usually select an ISC among the inner cortex or the endodermal cells (Sobczak et al., 2005; Lozano-Torres et al., 2012) while *H. schachtii* selects an ISC among cambial or procambial cells inside the vascular cylinder (Golinowski et al., 1996; Sobczak et al., 1999). The cell wall of the ISC is pierced by the J2 with its stylet and secretions are injected directly into the plant cell cytoplasm through the hollow stylet (Wyss and Zunke, 1986; Wyss, 1992; Sobczak et al., 1999). These secretions are produced in the esophageal glands and although their nature and composition is still largely unknown, there is a common agreement that they contain effector proteins, which modify plant morphogenetic pathways thus facilitating the development of the syncytia (Hewezi and Baum, 2013). Neighboring cells fuse successively with the ISC through local cell wall dissolutions (Grundler et al., 1998) and thus a multinuclear syncytium composed of a large number of syncytial elements (=former root cells) is formed (Wyss and Grundler, 1992). The syncytium keeps expanding centripetally into the vascular cylinder by incorporation of pro-/cambial cells located between xylem and phloem bundles (**Figures 1A,B**; Golinowski et al., 1996; Sobczak et al., 1997) and acro- and basipetally along the root. The nematodes remain sedentary during their J2 and J3 developmental stages (Wyss, 1992). At the short-lasting J4 stage male nematodes cease food withdrawal and after the next molt they leave the roots as adult nematodes to search females for mating. The females continue to feed on syncytia during J4 and adult stages, and they never leave their feeding site. After insemination, they deposit hundreds of eggs mainly inside their body, which hardens to form the protective cyst (Wyss and Grundler, 1992).

The syncytium is the only source of nutrients for the cyst nematodes during their entire life cycle and thus it constitutes a severe sink in the plant because it has to be continuously "refilled"

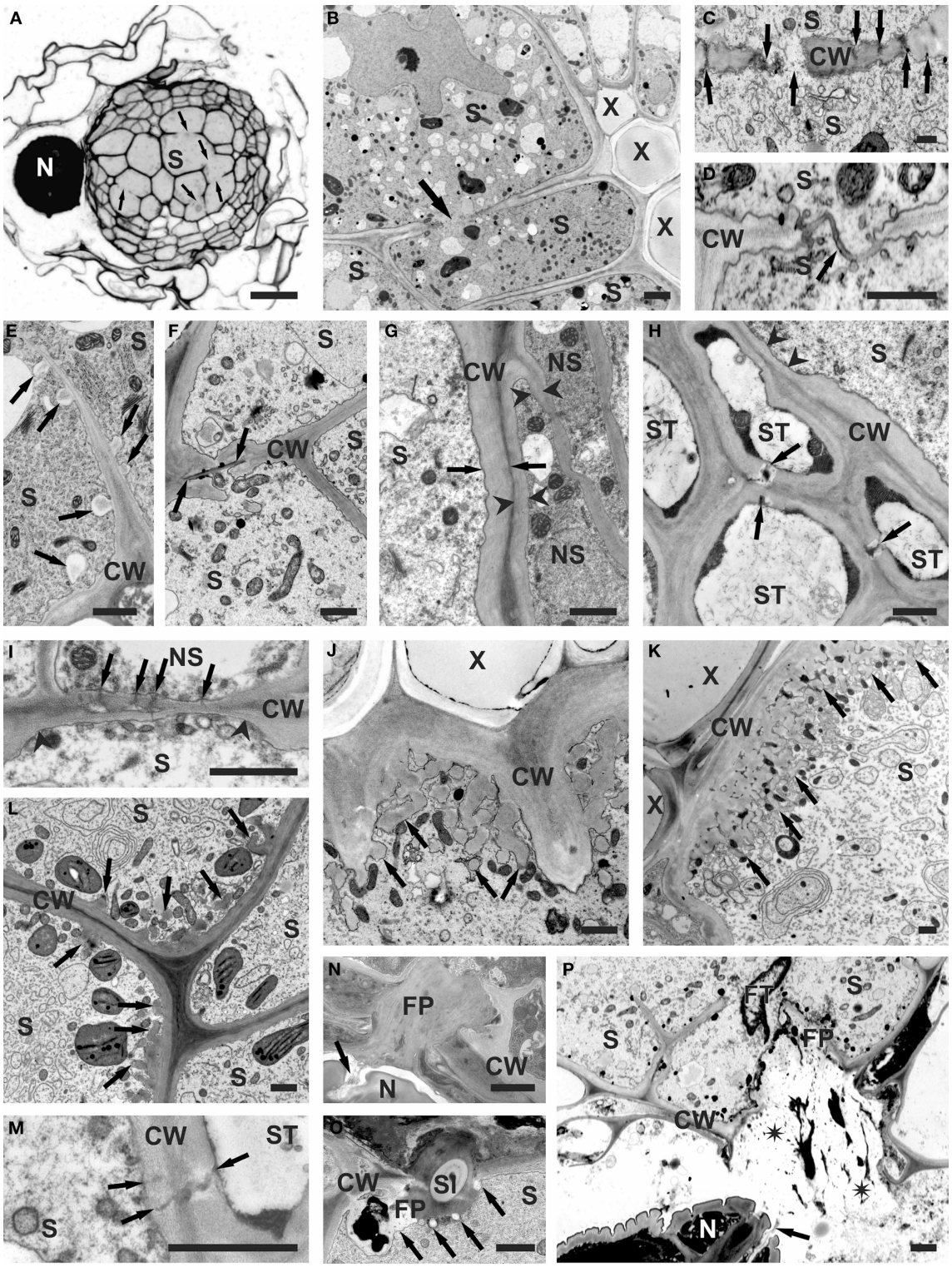


FIGURE 1 | Structural features of cell walls of syncytia induced by *H. schachtii* in *A. thaliana* roots. (A) Anatomy of root containing syncytium. Arrows indicate cell wall openings. **(B)** Ultrastructure of root containing syncytium. Arrow indicates cell wall opening. **(C)** Cell wall openings formed by widening of plasmodesmata (arrows). **(D)** Cell wall openings formed by local dissolution of cell wall without involvement of plasmodesmata. Arrow indicates middle lamella covered with plasmalemma. **(E)** Paramural bodies (arrows) formed at extensively digested part of internal cell wall. **(F)** Casparian stripe (between arrows) covered with newly deposited cell wall in untypical syncytium induced in the endodermis. **(G)** Comparison of thickness of outer syncytial cell wall (between arrows) and cell wall of non-syncytial cells (between arrowheads). **(H)** Thin part of outer syncytial cell wall (arrowheads) facing sieve tube. Arrows indicate plasmodesmata between sieve tubes.

(Continued)

FIGURE 1 | Continued
(I) A group of plasmodesmata (arrows) at thin part of outer syncytial cell wall (between arrowheads) facing non-syncytial parenchymatous cell. **(J)** Single cell wall ingrowths (arrows) formed at syncytial wall facing vessels. **(K)** Well-developed system of cell wall ingrowths (arrows) formed at syncytial wall facing vessels. **(L)** Unusual localization of poorly developed cell wall ingrowths (arrows) on wall between syncytial elements. **(M)** Plasmodesmata (arrows) between syncytial element and sieve tube. **(N)** Feeding plug in syncytial cell wall. Secretions emanating from nematode amphids are marked with arrow. **(O)** Feeding plug with inserted cross-sectioned nematode stylet. Callose depositions are indicated with arrows. **(P)** Broken feeding plug in syncytial wall. Spilled syncytial cytoplasm is marked with asterisks. Arrow points to amphidal secretions. Light microscopy **(A)** and transmission electron microscopy micrograms **(B–P)** of syncytia at 2 **(E,O)**, 5 **(A,B,D,F,N,P)**, 10 **(G,H,I,J,M)**, and 13 **(C,K,L)** days post inoculation. CW, cell wall; FP, feeding plug; FT, feeding tube; N, nematode; NS, non-syncytial cell; S, syncytium; SI, stylet; ST, sieve tube; X, xylem vessel. Bars = 20 μm **(A)** and 1 μm **(B–P)**.

with nutrients necessary for the developing nematode. The nuclei of the syncytial elements enlarge and undergo endoreduplication (De Almeida Engler and Gheysen, 2013). Furthermore, also other ultrastructural features of the syncytial elements differ drastically from the ultrastructure of typical pro-/cambial cells. The large central vacuole is replaced by several small vacuoles and proliferating syncytial cytoplasm contains numerous plastids, mitochondria, ribosomes and structures of endoplasmic reticulum (Jones and Northcote, 1972; Bleve-Zacheo and Zacheo, 1987; Golinowski et al., 1996). In the course of these processes, the osmotic pressure in syncytia increases and becomes higher than in adjacent cells (Böckenhoff and Grundler, 1994). The ultrastructural features of syncytia implicate their high metabolic activity, which is confirmed by transcriptome analyses indicating up-regulation of genes related to high metabolic activity (Szakasits et al., 2009).

THE MIGRATORY PHASE

The invasion of the root and the development of the syncytium require both, destructive and constructive modifications of plant cell walls. It was originally believed that during invasion of the root, the cyst nematodes use only their stylet to make a series of punctures in the cell wall to break it solely with mechanical force of its stylet and lips (Wyss and Zunke, 1986; Wyss, 1992). However, several years ago it was evidenced that in addition to mechanical force also cell wall degrading enzymes are produced and secreted by the migratory J2s of cyst nematodes during their intracellular migration (Smant et al., 1998). The secreted cell wall degrading enzymes are produced in the two subventral gland cells, which are highly active in infective J2s, but atrophy when the nematodes become sedentary after induction of the ISC (Tytgat et al., 2002). In sedentary juveniles the dorsal gland cell becomes enlarged and more active thus it seems to be involved in further syncytium development and maintenance.

The plant cell wall is primarily composed of a variety of polysaccharides. Its exact composition is highly variable and depends on many factors such as the plant species, the cell type, the cell developmental stage as well as on external biotic and abiotic factors (Keegstra, 2010). Its main component is cellulose, a β-1,4-linked glucan, which forms crystalline microfibrils cross-linked by hemicelluloses: xyloglucan, glucomannan, xylan, and mixed-linkage glucans (Scheller and Ulvskov, 2010). These are embedded in a complex polysaccharide matrix of pectin: homogalacturonan and rhamnogalacturonan I and II (Harholt et al., 2010). In addition, cell walls can be lignified (Li and Chapple, 2010) and they can also contain a variety of different proteins and glycoproteins (Rose and Lee, 2010).

The plant cell wall is a barrier for many pathogens and various cell wall polysaccharide degrading enzymes have been described for many different pathogens (for review see Hematy et al., 2009). Cyst nematodes also produce a variety of cell wall polysaccharide degrading enzymes during migration through the root. However, in contrast to fungal pathogens, which usually secrete first pectin degrading enzymes, there is no indication that nematode secreted cell wall degrading enzymes are released in chronological or sequential order. Rather, it seems that a cocktail of various polysaccharide degrading enzymes is produced in the nematode esophageal glands and secreted through the stylet orifice (for review see Davis et al., 2011).

Two groups of pectin degrading enzymes are produced by plant parasitic nematodes, which are specifically active on unmethylated polysaccharides: polygalacturonases and pectate lyases (see **Table 1** for the genes/proteins from nematodes mentioned in this review). The former cleave bonds between galacturonic acids in (unmethylated) homogalaturonan and they were found in secretions of root knot nematodes (Jaubert et al., 2002), but not in cyst nematodes. The latter have been identified in several cyst nematode species, including *H. glycines* (De Boer et al., 2002), *H. schachtii* (Vanholme et al., 2007), and *G. rostochiensis* (Popeijus et al., 2000; Kudla et al., 2007). Down-regulation of one of the two pectate lyase genes present in the *H. schachtii* genome by RNAi resulted in a lower number of infecting juveniles, thus clearly demonstrating the importance of pectate lyases for root invasion and successive migration of the nematode inside the host root (Vanholme et al., 2007).

Cellulases (β-1,4-endoglucanases) found in *G. rostochiensis* and *H. glycines* were the first cell wall degrading enzymes encoded by animal genomes that were discovered, which was surprising at that time because it was a general thought that animals are unable to produce these enzymes themselves but depended on symbiotic microorganisms for that purpose (Smant et al., 1998). These proteins are produced in the subventral gland cells and they are secreted during nematode migration as indicated by their immunolocalization in plant tissues (Wang et al., 1999). Secreted cellulases can be involved in the softening and weakening of the cellulose network in plant cell walls during the root invasion and intracellular migration phases making mechanical cell wall rupture easier. Their importance for cyst nematode parasitism was demonstrated by RNAi silencing of their expression in *G. rostochiensis*. Incubation of invasive J2s with dsRNA targeting the mRNA of secreted cellulases resulted in fewer nematodes that could invade and induce syncytia in the host roots (Chen et al., 2005).

Table 1 | Nematode proteins acting on plant cell walls.

Gene/Protein	Nematode	Class	Function	References
Hg-pel-1	*H. glycines*	Pectate lyase		De Boer et al., 2002
Hspel1	*H. schachtii*	Pectate lyase		Vanholme et al., 2007
Hspel2			Silencing results in low infection efficiency	
Gr-PEL1	*G. rostochiensis*	Pectate lyase		Popeijus et al., 2000
Gr-PEL2			Transient expression in *Nicotiana benthamiana* resulted in severe malformations of infiltrated tissues	Kudla et al., 2007
GR-ENG-1	*G. rostochiensis*	Cellulase	Hydrolysis of carboxymethylcellulose	Smant et al., 1998
GR-ENG-2	*G. rostochiensis*			
HG-ENG-1	*H. glycines*			
HG-ENG-2	*H. glycines*			
GR-ENG-1/2/3/4	*G. rostochiensis*	Cellulase	Targeting the mRNAs of secreted cellulases with dsRNA resulted in lower nematode invasion	Chen et al., 2005
Gr-EXPB1	*G. rostochiensis*	Expansin	Cell-wall-extension activity	Qin et al., 2004; Kudla et al., 2005
Hs CBP	*H. schachtii*	Cellulose binding protein	Interacts with a plant pectin methylesterase (PME3) to aid cyst nematode parasitism	Hewezi et al., 2008

Genes are shown in italic for those cases where the proteins have not been reported.

Genomes of sedentary plant parasitic nematodes encode also expansins (Qin et al., 2004; Kudla et al., 2005). Expansins are widespread in plants, but had not been found outside the plant kingdom before. They have no enzymatic activity but function by loosening or weakening of the non-covalent bonds between cellulose microfibrills and associated hemicelluloses, thus making the cell wall more accessible for cell wall degrading enzymes, and more responsive to turgor-driven plant cell growth (McQueen-Mason and Cosgrove, 1994). Loosening of the plant cell walls by expansins produced by migratory juveniles would result in better accessibility for nematode secreted cell wall degrading enzymes and could also facilitate mechanical disruption with the aid of the nematode stylet and lips (Qin et al., 2004).

We can conclude that there is a bulk of evidence indicating that during root invasion and migration through the root tissues the cyst nematodes use mechanical force in combination with a sophisticated set of enzymes that soften the rigidity of plant cell walls through polysaccharide degrading enzymes and cell wall modifying proteins. Once the J2 has selected an ISC, it becomes sedentary and changes its strategy. Now, the dorsal gland cell becomes more active, indicating that the nematode is producing a different set of parasitism-related proteins (effectors). The ISC develops into a syncytium via hypertrophy of affected plant cells and formation of partial cell wall dissolutions between them leading to the formation of a confluent protoplast inside the entire feeding site. This again requires activities of cell wall degrading enzymes and modifying proteins such as expansins, but now they are apparently produced by the plant, not by the nematode. There have been no reports that cell wall degrading enzymes produced by the nematode are secreted into the syncytium, although, this cannot be excluded. Instead, the sedentary plant parasitic nematodes have developed a sophisticated system of effectors released into the feeding site, which allow them now to avoid plant defense responses and to "persuade" the plant to work for parasite benefits (Quentin et al., 2013).

THE SEDENTARY PHASE

The incorporation of root cells into syncytia requires a local dissolution of cell walls (**Figures 1A,B**; Grundler et al., 1998) and it has been shown that several plant proteins are involved in this process, including expansins, cellulases, and pectinases. The expression of these proteins has to be tightly regulated and some of the genes encoding expansins and cell wall degrading enzymes are specifically temporally and spatially regulated in syncytia or in the surrounding cells (Wieczorek et al., 2006, 2008; Szakasits et al., 2009). Most of the knowledge concerning cell wall modifications has been gained from the research on the interaction between *H. schachtii* and *A. thaliana* roots; therefore we will concentrate on this interaction (see **Table 2** for the genes mentioned in this review). Further information on other host plants and other cyst (and root knot nematodes) can be found in a review by Sobczak et al. (2011).

The formation of syncytia requires the fusion of root cell protoplasts by partial cell wall dissolution (**Figures 1A,B**; Grundler et al., 1998). During early stages of syncytium development it is achieved by widening of plasmodesmata between the ISC and neighboring cells (**Figure 1C**; see also Figures 3, 4 in Grundler et al., 1998), while at later stages cell wall openings are mostly formed by dissolution of cell wall fragments without the involvement of plasmodesmata (**Figure 1D**; see also Figures 8, 9 in Grundler et al., 1998). However, when a group of cells is incorporated into a syncytium, cell wall dissolutions can be formed among distal cells by widening of plasmodesmata. Expansins are apparently helper-proteins in this process. As it has been mentioned, expansins do not have enzymatic activity, but they are involved in all processes that require the expansion of plant cells or the degradation of cell walls. They act by weakening the hydrogen bonds between cell wall polysaccharides (McQueen-Mason and Cosgrove, 1994). The *A. thaliana* genome contains 26 genes coding for α-expansins and 5 genes coding for β-expansins, which are differentially regulated in different plant organs and in feeding

Table 2 | Plant genes involved in syncytium formation.

Gene	Name	Class	Function/Expression	References
At2g37640 *At3g55500*	AtEXPA3 AtEXPA16	Expansin	Up-regulated in syncytia, loosening of cell walls	Wieczorek et al., 2006
At1g70710	AtCel1	Cellulase	Expressed in young syncytia. Implicated in cell wall softening during early stages of syncytium development	Mitchum et al., 2004 Wieczorek et al., 2008
At1g02800 *At4g24260*	AtCel2 AtKor3	Cellulase	Degradation of cell walls. Decreased numbers of females develops on roots of T-DNA mutants	Wieczorek et al., 2008
At3g14310	Pme3	Pectin methylesterase	Interacts with a cellulose binding protein secreted by *H. schachtii* during early phase of syncytium development	Hewezi et al., 2008
At3g05910	PAE (DiDi 9C-12)	Pectin acetylesterase	Up-regulated in and around young expanding syncytia induced by *H. schachtii*	Vercauteren et al., 2002
At3g27400 *At4g24780*	PLL18 PLL19	Pectate lyase-like	Up-regulated in syncytia. Important for proper development of the cyst nematode *H. schachtii*	Wieczorek et al., unpublished
At3g54920	PMR6	Pectate lyase-like	Up-regulated in syncytia. Known also as powdery mildew susceptibility gene	Vogel et al., 2002 Szakasits et al., 2009
At1g14520 *At2g19800* *At4g26260* *At5g56640*	MIOX1 MIOX2 MIOX4 MIOX5	Myo-inositol oxygenase	Strong expression in syncytia. Important for syncytium and nematode development, probably due to removal of excess myo-inositol to decrease the level of galactinol	Siddique et al., 2009, 2014
At5g39320 *At3g29360* *At5g15490* *At1g26570*	UGD1 UGD2 UGD3 UGD4	UDP-glucose dehydrogenase	Expressed in syncytia Expressed in syncytia. In mutant plants cell wall ingrowths are not formed in syncytia Expressed in syncytia	Siddique et al., 2012

sites of the cyst nematode *H. schachtii* (Wieczorek et al., 2006). Some expansin genes are expressed in roots and syncytia while some others are expressed in roots but down-regulated or silenced in syncytia. Genes, such as *AtEXPA3* (*At2g37640*) and *AtEXPA16* (*At3g55500*), which are expressed in shoots but not in roots in uninfected plants, are specifically up-regulated in syncytia, indicating that they are important for syncytium development.

When the texture of plant cell walls is loosened by expansins, it becomes more accessible for a variety of plant derived enzymes, which are involved in cell wall degradation. These enzymes include cellulases (endo-1,4-β-glucanases) and different pectin degrading enzymes. Goellner et al. (2001) were the first who showed that plant cellulase genes are expressed in tobacco roots infected with *G. tabacum*. A detailed analysis of the cellulase gene expression pattern in *Arabidopsis* roots infected with *H. schachtii* showed a complex picture resembling the expression pattern of expansin genes (Wieczorek et al., 2008). The *A. thaliana* genome contains 25 genes annotated as cellulases. Seven of them were up-regulated and seven were down-regulated in syncytia. GeneChip analysis indicated the *AtCel1* gene (*At1g70710*) as being up-regulated in syncytia at 5 days post inoculation (dpi), while GUS analysis did not detect expression of the *AtCel1* promoter in 10 dpi syncytia (Mitchum et al., 2004). The reason for these divergent results might be that this gene is apparently expressed only in young syncytia. Other up-regulated genes are *AtCel2* (*At1g02800*) and *AtKor3* (*At4g24260*). Their importance for feeding site and nematode development was demonstrated with mutant studies. Only about half of the number of females developed on roots of *AtCel2* or *AtKor3* mutants in comparison to wild type plants (Wieczorek et al., 2008).

Another important group of enzymes involved in degradation of cell walls during the development of feeding sites induced by the cyst nematodes are enzymes taking part in degradation and modifications of pectins. These enzymes are encoded by large gene families in the *A. thaliana* genome (see supplementary tables). There are 66 genes coding for polygalacturonases (Kim et al., 2006), 66 genes encoding pectin methylesterases (Louvet et al., 2006), 12 genes encoding pectin acetylesterases (Gou et al., 2012), 26 genes annotated as pectate lyases or pectate lyase-like genes (Palusa et al., 2007; Sun and van Nocker, 2010) and 67 genes coding for pectin lyases (Cao, 2012). However, it has to be kept in mind that this classification is based at the moment on *in silico* analyses and that the enzymatic activity of most proteins has not been tested *in vivo* or *in vitro*. During syncytium development the action of these enzymes seems to be of special importance to allow intrusive growth of enlarging syncytial elements among surrounding parenchymatous cells (**Figures 1A,B**). Their action seems also necessary for proper formation of cell

wall openings when neighboring protoplasts become separated by middle lamella only (**Figure 1D**; see also Grundler et al., 1998). It has to be digested to allow formation of confluent syncytial cytoplasm. However, during enlargement of syncytial elements the exposed middle lamella might also be torn apart mechanically. When the cell wall openings expand numerous paramural bodies of different sizes are formed (**Figure 1E**; see also Figure 11 in Golinowski et al., 1996).

Very little is currently known about the expression of genes coding for pectin-degrading enzymes in feeding sites of *H. schachtii*. The transcriptome analysis conducted by Szakasits et al. (2009) showed that among 67 genes coding for pectin lyases four were up-regulated and four were down-regulated in syncytia as compared to control root segments (a false discovery rate at <5% was used). None of these genes was expressed at a high level, indicating that pectin lyases might be less important for the degradation of cell walls leading to incorporation of new root cells into the growing syncytium than pectate lyases (discussed below).

While pectin lyases are able to hydrolyze methylated pectin, polygalacturonases and pectate lyases are unable to degrade methylated pectin. These enzymes can only work once the pectin is demethylated or deacetylated by pectin methylesterases or acetylesterases. Out of the 66 genes encoding pectin methylesterases three were up-regulated and 10 were down-regulated in syncytia induced by *H. schachtii* according to data reported by Szakasits et al. (2009). The protein encoded by *Pme3* (*At3g14310*) interacts with a cellulose binding protein secreted by *H. schachtii* during early phases of syncytium development. Transgenic plants overexpressing this pectin methylesterase were more susceptible to infection with *H. schachtii* while a knockout mutant showed the opposite effect, indicating the importance of Pme3 for nematode parasitism (Hewezi et al., 2008).

Among 12 putative pectin acetylesterase genes, two were down-regulated and three were up-regulated in syncytia according to data reported by Szakasits et al. (2009). One of the latter genes (*At3g05910*) was up-regulated both in young giant cells induced by the root-knot nematode *M. incognita* as well as in syncytia induced by *H. schachtii* (Vercauteren et al., 2002). Deacetylation and demethylation makes the pectin accessible for degradation by polygalacturonases and pectate lyases. Four out of the 66 genes encoding putative polygalacturonases in the *A. thaliana* genome were slightly up-regulated while five were down-regulated in syncytia induced by *H. schachtii*. Additionally, there are also 26 genes in the *A. thaliana* genome coding for pectate lyase-like (PLL) enzymes (Palusa et al., 2007). Six of them were up-regulated and four were down-regulated in syncytia when compared to uninfected *Arabidopsis* roots according to data reported by Szakasits et al. (2009). These results, with all due precautions, indicate that pectate lyases might be more important for the degradation of cell walls in syncytia than polygalacturonases and recent work by Wieczorek et al. (pers. comm.) supports this assumption. They studied two PLL genes (*PLL18*; *At3g27400* and *PLL19*; *At4g24780*), which are up-regulated in syncytia. Analyses of T-DNA mutants showed that both genes are important for proper development of the cyst nematode *H. schachtii*, but not for the root knot nematode *M. incognita*. It should also be stressed that one pectate lyase-like gene (*PMR6*; *At3g54920*), which is also up-regulated in syncytia was identified as a powdery mildew susceptibility gene in *A. thaliana* (Vogel et al., 2002). With a few exceptions, our information about the role of the different pectin modifying/degrading enzymes for syncytium development is still very limited and fragmentary.

It seems that plant parasitic nematodes do not have enzymes degrading suberin as well as they are not able to modify expression of plant genes coding for enzymes degrading it. The juveniles of the root knot nematode *M. incognita* invade plant roots close to the root tip. They enter the root and migrate toward the vascular cylinder intercellularly dissolving the middle lamellae. When they reach the endodermis with developed Casparian stripes (narrow bands of suberinized cell wall) they turn first toward the root tip and then turn again toward the center of the vascular cylinder just above the root tip meristem where the Casparian stripes are not yet formed (Wyss et al., 1992). In the case of syncytia induced by *Globodera* sp., which are induced in the root cortex, the cell wall openings were never formed at parts of endodermal cell walls with Casparian stripes. In general, suberinized Casparian stripes are not a problem for *Heterodera* sp., which usually induce syncytia among cells of the vascular cylinder and migrate through the endodermis intracellularly. However, in a very few cases, syncytia induced in young parts of *A. thaliana* roots incorporated also endodermal cells with developed Casparian stripes (**Figure 1F**). In such cases the suberinized part of the plant cell wall remained undigested and was covered by thick layers of newly deposited cell wall material. It indicates that the cyst nematodes are unable to regulate the expression of plant suberin-digesting enzymes and do not produce these enzymes themselves.

Concomitantly with processes of cell wall degradation, the development of syncytia also requires the synthesis of new cell wall materials. The outer syncytial cell wall is thickened (**Figures 1B,G**; see also: Figures 25, 26 in Sobczak et al., 1997; Figure 10 in Golinowski et al., 1996) to withstand and counteract the high osmotic pressure inside the syncytia, which can reach 10,000 hPa in syncytia induced by *H. schachtii* in *A. thaliana* roots (Böckenhoff and Grundler, 1994). While the outer cell wall is generally thickened (**Figure 1G**; see also: Figures 25, 26 in Sobczak et al., 1997; Figure 10 in Golinowski et al., 1996), those parts of the syncytial wall that face sieve tubes (**Figure 1H**; see also: Figure 5 in Grundler et al., 1998; Figure 19.1A in Sobczak et al., 2011) or cells being incorporated into syncytia (**Figure 1I**; see also Figure 19.1B in Sobczak et al., 2011) often remain thin while the sieve tube wall becomes thickened. This might favor the uptake of solutes into the syncytium. In syncytia associated with female nematodes cell wall ingrowths are developed at the interface with xylem vessels (Jones and Northcote, 1972; Golinowski et al., 1996). The first cell wall ingrowths are formed as dispersed finger-like protrusions (**Figure 1J**; see also Figure 5E in Siddique et al., 2012) in 5–7 dpi syncytia. Later they ramify and expand forming pronounced cell wall labyrinths imprisoning mitochondria and vesicles (**Figure 1K**; see also: Figures 5–7, 27 in Jones and Northcote, 1972; Figure 20 in Golinowski et al., 1996). In syncytia associated with male juveniles, which have only two developmental stages of active food uptake and thus lower nutritional demands than female juveniles (Müller et al., 1981), and in syncytia induced in plants grown under adverse conditions the

disperse and single cell wall ingrowths are formed at different locations, for instance between syncytial elements or on the outer syncytial wall (**Figure 1L**; see also Figures 28, 29 in Sobczak et al., 1997). Cell wall ingrowths are a characteristic feature of transfer cells differentiating at various locations in plant organs. They are ubiquitous in plants and are also found in fungi and algae (Talbot et al., 2002; Offler et al., 2003). It has been proposed that the role of cell wall ingrowths is to increase the surface of the interface between symplast and apoplast thus allowing a higher exchange of solutes. As the cell wall ingrowths in syncytia are formed at the xylem interface it might be assumed that they enhance the uptake of water from the vessels because it was calculated that female juveniles withdraw four times the syncytium volume daily (Müller et al., 1981).

Thickening of the outer syncytial wall begins soon after syncytium induction (Sobczak et al., 1999). It usually leads to occlusion of plasmodesmata, if they have not been widened and turned into cell wall openings (**Figure 1C**; see also Figures 3, 4 in Grundler et al., 1998). Thus, for a long time it was generally accepted that syncytia are symplasmically isolated. However, recently several papers have been published providing indirect evidence that plasmodesmata are formed *de novo* in outer syncytial cell walls and that the syncytium is uploaded simplasmically (Hoth et al., 2005; Hofmann and Grundler, 2006; Hofmann et al., 2007). However, there is no ultrastructural support for functional plasmodesmata in the outer cell wall of syncytia. Plasmodesmata in the outer syncytial wall can be found only occasionally in cell walls facing sieve tubes (**Figure 1M**; see also: Figure 5 in Grundler et al., 1998; Figures 19.2C,D in Sobczak et al., 2011) or parenchymatous cells being incorporated into syncytia (**Figure 1I**; see also Figures 19.2E,F in Sobczak et al., 2011). If present, they usually seem to be occluded by deposited cell wall material from the syncytial side. However, plasmodesmata are present abundantly between parenchymatous cells surrounding syncytia (see also Figure 19.3A in Sobczak et al., 2011).

The feeding plug is another structure characteristic for syncytial cell walls, which is not found in giant cells induced by root knot nematodes. It is embedded into the outer syncytial cell wall at the place where the nematode stylet is inserted into the syncytium (**Figures 1N,O**; see also Figures 5, 6, 8 in Sobczak et al., 1999). Presumably, this structure is important as a seal preventing leakage of the syncytial cytoplasm along the inserted stylet (**Figure 1O**; see also Figures 6, 8 in Sobczak et al., 1999). It must be soft enough to allow stylet insertion, but also strong enough to prevent cell wall rupture when the cell wall is punctured with the nematode stylet. If the stylet is inserted outside the feeding plug it can lead to cell wall rupture and syncytium collapse, although a callose-based repair mechanism is activated (**Figure 1P**). The origin and chemical composition of the feeding plug remain obscure. A small feeding plug is observed in syncytia already several hours after ISC selection. It enlarges and often becomes multipartite during syncytium development. The feeding plug is probably mostly composed of callose that is abundantly deposited around the tip (except the canal orifice) of the inserted stylet (**Figure 1O**; see also Figure 8 in Sobczak et al., 1999). The stylet is withdrawn in every feeding cycle, approximately every 3–4 h (Wyss, 1992), which could lead to leakage from the syncytium. This is prevented by the callose, which is pulled into the opening in the cell wall. However, active formation of the feeding plug by the secretions originating from nematode amphids cannot be ruled out (**Figures 1N,P**; see also: Figure 3 in Endo, 1978; Figure 7 in Sobczak et al., 1999).

The major precursor for synthesis of cell wall polysaccharides in *Arabidopsis* is UDP-glucuronic acid, which can be produced through two different pathways. Under normal growth conditions UDP-glucose dehydrogenase (UGD) supplies the majority of UDP-glucuronic acid from UDP-glucose. A second pathway involves the enzyme myo-inositol oxygenase (MIOX) converting myo-inositol to D-glucuronic acid, which is thereafter converted to D-glucuronic acid-1 phosphate by glucuronokinase, and finally to UDP-glucuronic acid by UDP-sugar pyrophosphorylase (Kanter et al., 2005; Klinghammer and Tenhaken, 2007). Myo-inositol is produced from glucose 6-phosphate through the rate-limiting conversion to myo-inositol-3-phosphate, which is catalyzed by the enzyme myo-inositol-phosphate synthase (MIPS). Myo-inositol-3-phosphate is then dephosphorylated to myo-inositol by myo-inositol monophosphatases (Loewus and Murthy, 2000).

MIOX is encoded by 4 genes in the *A. thaliana* genome. *MIOX1* and *MIOX2* are expressed preferentially in seedlings while *MIOX4* and *MIOX5* are highly expressed in pollen grains (Kanter et al., 2005). Expression of all 4 *MIOX* genes is strongly elevated in syncytia induced by *H. schachtii* (Siddique et al., 2009). Double mutants of the four *MIOX* genes supported development of a significantly reduced number of *H. schachtii* females indicating the importance of this pathway for the proper development of syncytia and associated nematodes (Siddique et al., 2009). However, no differences could be detected between *miox* double mutants, a quadruple mutant and wild type plants in biochemical composition and ultrastructure of syncytial cell walls (Siddique et al., 2009, 2014). The importance of the MIOX pathway for syncytium development is probably not in the production of cell wall precursors, but rather in the removal of excess myo-inositol from syncytia. A high level of myo-inositol would lead to a higher level of galactinol and there is evidence that the high galactinol level leads to decreased susceptibility of *miox* mutants against *H. schachtii* (Siddique et al., 2014).

There is also a small gene family in the *A. thaliana* genome consisting of four genes that encode UGD (*UGD1*, *UGD2*, *UGD3*, and *UGD4*) (Klinghammer and Tenhaken, 2007). *UGD1* is only weakly expressed in roots while *UGD2*, *UGD3*, and *UGD4* are expressed at high levels in roots. We have studied the expression pattern of these genes in syncytia using promoter::GUS lines (Siddique et al., 2012). All four genes are expressed in syncytia; *UGD2* and *UGD3* as early as 1 dpi while expression of *UGD1* and *UGD4* was detected starting at 2 dpi. A mutant analysis revealed that single *UGD* mutants (Δ*ugd2* and Δ*ugd3*) support development of fewer and smaller females, which feed from smaller syncytia as compared to wild type plants. The double mutant Δ*Δugd23* had an even stronger effect than the single mutants. Ultrastructural examination of syncytia induced in the Δ*Δugd23* double mutant revealed that syncytia usually contained an electron translucent cytoplasm with degenerating organelles and cell wall ingrowths were absent. These results showed that *UGD2* and

UGD3 are needed for the formation of cell wall ingrowths in syncytia (Siddique et al., 2012).

Knowledge concerning the chemical composition of syncytial cell walls is very limited. The basic reason for this situation is the general difficulty to obtain a sufficient amount of purified material for biochemical analyses. Therefore, immunological methods are the only reasonable method to study the chemical composition of syncytial cell walls. Using monoclonal antibodies, which target specific components of plant cell wall polysaccharides, cell wall composition was examined in syncytia induced by *H. schachtii* in *A. thaliana* roots (Davies et al., 2012). These analyses demonstrated the presence of cellulose and hemicelluloses such as xyloglucan and heteromannan. Xylan was not detected, indicating that syncytia are devoid of secondary cell walls although outer cell walls of syncytia are strongly thickened. The pectin in syncytial walls appeared to be heavily methyl-esterified, which could explain the rather low expression of pectin lyases in syncytia as these enzymes are able to degrade only methylated pectin, but not de-esterified one. There is at the moment no explanation why the higher expression of *AtPme3* was positively correlated with susceptibility because a higher expression of pectin methylesterases might not only result in an increased degradation of cell walls between syncytial elements, which might favor syncytium development, but also should lead to an enhanced degradation of the outer syncytial cell wall and thus have detrimental effects on the syncytium. One might therefore speculate that there are certain chemical differences between inner and outer cell walls of syncytia, however, the inner cell walls (those between syncytial elements) are also not completely degraded but only locally dissolved. An explanation for this phenomenon could be that cell wall degrading enzymes produced in syncytia are very specifically targeted to only those parts of the cell walls between syncytial elements that are scheduled to be degraded or that the outer cell walls of syncytia are protected through the deposition of enzyme inhibitors.

Examination of the chemical composition of the outer syncytial cell wall (Davies et al., 2012) indicates that it is on the one hand strong enough to withstand the high internal turgor pressure of the syncytium and on the other hand it remains flexible (Davies et al., 2012). This flexibility of outer syncytial walls seems to be especially important when we consider that feeding of the cyst nematodes occurs in cycles (Wyss and Zunke, 1986; Wyss, 1992). During each feeding cycle a substantial part of the syncytium protoplast is withdrawn by the nematode. It has been calculated that an adult female of *H. schachtii* can withdraw four times the volume of the syncytium through its stylet per day (Müller et al., 1981). The flexibility of the outer syncytial cell wall allows the syncytium to contract during the active food withdrawal stage when the nematode feeds and takes up a large part of the syncytial volume with solutes and to expand when no food is withdrawn and the syncytium is "refilled" from conductive elements.

ACKNOWLEDGMENTS

We gratefully acknowledge Christiane Matera and Shahid Siddique for providing probes, which were used to produce figures, and Elzbieta Rozanska and Ewa Znojek for excellent utramicrotomy work. Research of Holger Bohlmann was supported by the Austrian Science Foundation (FWF, Grant P20471-B11). Research of Miroslaw Sobczak was supported by the Polish Ministry of Science and Higher Education (grant no 116/N-COST/2008/0).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00089/abstract

REFERENCES

Abad, P., Gouzy, J., Aury, J. M., Castagnone-Sereno, P., Danchin, E. G., Deleury, E., et al. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nat. Biotechnol.* 26, 909–915. doi: 10.1038/nbt.1482

Bleve-Zacheo, T., and Zacheo, G. (1987). Cytological studies of the susceptible reaction of sugarbeet roots to *Heterodera schachtii*. *Physiol. Mol. Plant Pathol.* 30, 13–25. doi: 10.1016/0885-5765(87)90079-8

Böckenhoff, A., and Grundler, F. M. W. (1994). Studies on the nutrient uptake by the beet cyst nematode *Heterodera schachtii* by *in situ* microinjection of fluorescent probes into the feeding structures in *Arabidopsis thaliana*. *Parasitology* 109, 249–254. doi: 10.1017/S003118200007637X

Cao, J. (2012). The pectin lyases in *Arabidopsis thaliana*: evolution, selection and expression profiles. *PLoS ONE* 7:e46944. doi: 10.1371/journal.pone.0046944

Chen, Q., Rehman, S., Smant, G., and Jones, J. T. (2005). Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi. *Mol. Plant Microbe. Interact.* 18, 621–625. doi: 10.1094/MPMI-18-0621

Chitwood, D. J. (2003). Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture-Agricultural Research Service. *Pest Manag. Sci.* 59, 748–753. doi: 10.1002/ps.684

Davies, L. J., Lilley, C. J., Knox, P. J., and Urwin, P. E. (2012). Syncytia formed by adult female *Heterodera schachtii* in *Arabidopsis thaliana* roots have a distinct cell wall molecular architecture. *New Phytol.* 196, 238–246. doi: 10.1111/j.1469-8137.2012.04238.x

Davis, E. L., Haegeman, A., and Kikuchi, T. (2011). "Degradation of the plant cell wall by nematodes," in *Genomics and Molecular Genetics of Plant-Nematode Interactions*, eds J. Jones, G. Gheysen, and C. Fenoll (Dordrecht: Springer), 255–272. doi: 10.1007/978-94-007-0434-3_12

De Almeida Engler, J., and Gheysen, G. (2013). Nematode-induced endoreduplication in plant host cells: why and how? *Mol. Plant Microbe. Interact.* 26, 17–24. doi: 10.1094/MPMI-05-12-0128-CR

De Boer, J. M., Davis, E. L., Hussey, R. S., Popeijus, H., Smant, G., and Baum, T. J. (2002). Cloning of a putative pectate lyase gene expressed in the subventral esophageal glands of *Heterodera glycines*. *J. Nematol.* 34, 9–11.

Endo, B. Y. (1978). Feeding plug formation in soybean roots infected with the soybean cyst nematode. *Phytopathology* 68, 1022–1031. doi: 10.1094/Phyto-68-1022

Goellner, M., Wang, X., and Davis, E. L. (2001). Endo-ß-1,4-glucanase expression in compatible plant-nematode interactions. *Plant Cell* 13, 2241–2255. doi: 10.1105/tpc.010219

Golinowski, W., Grundler, F. M. W., and Sobczak, M. (1996). Changes in the structure of *Arabidopsis thaliana* during female development of the plant-parasitic nematode *Heterodera schachtii*. *Protoplasma* 194, 103–116. doi: 10.1007/BF01273172

Gou, J. Y., Miller, L. M., Hou, G., Yu, X. H., Chen, X. Y., and Liu, C. J. (2012). Acetylesterase-mediated deacetylation of pectin impairs cell elongation, pollen germination, and plant reproduction. *Plant Cell* 24, 50–65. doi: 10.1105/tpc.111.092411

Grundler, F. M. W., Sobczak, M., and Golinowski, W. (1998). Formation of wall openings in root cells of *Arabidopsis thaliana* following infection by the plant-parasitic nematode *Heterodera schachtii*. *Eur. J. Plant Pathol.* 104, 545–551. doi: 10.1023/A:1008692022279

Harholt, J., Suttangkakul, A., and Vibe Scheller, H. (2010). Biosynthesis of pectin. *Plant Physiol.* 153, 384–395. doi: 10.1104/pp.110.156588

Hematy, K., Cherk, C., and Somerville, S. (2009). Host-pathogen warfare at the plant cell wall. *Curr. Opin. Plant Biol.* 12, 406–413. doi: 10.1016/j.pbi.2009.06.007

Hewezi, T., and Baum, T. J. (2013). Manipulation of plant cells by cyst and root-knot nematode effectors. *Mol. Plant Microbe. Interact.* 26, 9–16. doi: 10.1094/MPMI-05-12-0106-FI

Hewezi, T., Howe, P., Maier, T. R., Hussey, R. S., Mitchum, M. G., Davis, E. L., et al. (2008). Cellulose binding protein from the parasitic nematode *Heterodera schachtii* interacts with Arabidopsis pectin methylesterase: cooperative cell wall modification during parasitism. *Plant Cell* 20, 3080–3093. doi: 10.1105/tpc.108.063065

Hofmann, J., and Grundler, F. M. W. (2006). Females and males of root-parasitic cyst nematodes induce different symplasmic connections between their syncytial feeding cells and the phloem in *Arabidopsis thaliana*. *Plant Physiol. Biochem.* 44, 430–433. doi: 10.1016/j.plaphy.2006.06.006

Hofmann, J., Wieczorek, K., Blöchl, A., and Grundler, F. M. W. (2007). Sucrose supply to nematode-induced syncytia depends on the apoplasmic and symplasmic pathways. *J. Exp. Bot.* 58, 1591–1601. doi: 10.1093/jxb/erl285

Hoth, S., Schneidereit, A., Lauterbach, C., Scholz-Starke, J., and Sauer, N. (2005). Nematode infection triggers the de novo formation of unloading phloem that allows macromolecular trafficking of green fluorescent protein into syncytia. *Plant Physiol.* 138, 383–392. doi: 10.1104/pp.104.058800

Jaubert, S., Laffaire, J. B., Abad, P., and Rosso, M. N. (2002). A polygalacturonase of animal origin isolated from the root-knot nematode *Meloidogyne incognita*. *FEBS Lett.* 522, 109–112. doi: 10.1016/S0014-5793(02)02906-X

Jones, M. G., and Northcote, D. H. (1972). Nematode-induced syncytium-a multinucleate transfer cell. *J. Cell Sci.* 10, 789–809.

Kanter, U., Usadel, B., Guerineau, F., Li, Y., Pauly, M., and Tenhaken, R. (2005). The inositol oxygenase gene family of Arabidopsis is involved in the biosynthesis of nucleotide sugar precursors for cell-wall matrix polysaccharides. *Planta* 221, 243–254. doi: 10.1007/s00425-004-1441-0

Keegstra, K. (2010). Plant cell walls. *Plant Physiol.* 154, 483–486. doi: 10.1104/pp.110.161240

Kim, J., Shiu, S. H., Thoma, S., Li, W. H., and Patterson, S. E. (2006). Patterns of expansion and expression divergence in the plant polygalacturonase gene family. *Genome Biol.* 7, R87. doi: 10.1186/gb-2006-7-9-r87

Klinghammer, M., and Tenhaken, R. (2007). Genome-wide analysis of the UDP-glucose dehydrogenase gene family in Arabidopsis, a key enzyme for matrix polysaccharides in cell walls. *J. Exp. Bot.* 58, 3609–3621. doi: 10.1093/jxb/erm209

Kudla, U., Milac, A. L., Qin, L., Overmars, H., Roze, E., Holterman, M., et al. (2007). Structural and functional characterization of a novel, host penetration-related pectate lyase from the potato cyst nematode *Globodera rostochiensis*. *Mol. Plant Pathol.* 8, 293–305. doi: 10.1111/j.1364-3703.2007.00394.x

Kudla, U., Qin, L., Milac, A., Kielak, A., Maissen, C., Overmars, H., et al. (2005). Origin, distribution and 3D-modeling of Gr-EXPB1, an expansin from the potato cyst nematode *Globodera rostochiensis*. *FEBS Lett.* 579, 2451–2457. doi: 10.1016/j.febslet.2005.03.047

Li, X., and Chapple, C. (2010). Understanding lignification: challenges beyond monolignol biosynthesis. *Plant Physiol.* 154, 449–452. doi: 10.1104/pp.110.162842

Loewus, F. A., and Murthy, P. P. N. (2000). Myo-inositol metabolism in plants. *Plant Sci.* 150, 1–19. doi: 10.1016/S0168-9452(99)00150-8

Louvet, R., Cavel, E., Gutierrez, L., Guénin, S., Roger, D., Gillet, F., et al. (2006). Comprehensive expression profiling of the pectin methylesterase gene family during silique development in *Arabidopsis thaliana*. *Planta* 224, 782–791. doi: 10.1007/s00425-006-0261-9

Lozano-Torres, J. L., Wilbers, R. H. P., Gawronski, P., Boshoven, J. C., Finkers-Tomczak, A., Cordewener, J. H. G., et al. (2012). Dual disease resistance mediated by the immune receptor Cf-2 in tomato requires a common virulence target of a fungus and a nematode. *Proc. Natl. Acad. Sci. U.S.A.* 109, 10119–10124. doi: 10.1073/pnas.1202867109

McQueen-Mason, S., and Cosgrove, D. J. (1994). Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Proc. Natl. Acad. Sci. U.S.A.* 91, 6574–6578. doi: 10.1073/pnas.91.14.6574

Mitchum, M. G., Sukno, S., Wang, X., Shani, Z., Tsabary, G., Shoseyov, O., et al. (2004). The promoter of the *Arabidopsis thaliana Cel1* endo-1,4-ßglucanase gene is differentially expressed in plant feeding cells induced by root-knot and cyst nematodes. *Mol. Plant Pathol.* 5, 175–181. doi: 10.1111/j.1364-3703.2004.00216.x

Müller, J., Rehbock, K., and Wyss, U. (1981). Growth of *Heterodera schachtii* with remarks on amounts of food consumed. *Rev. Nématol.* 4, 227–234.

Offler, C. E., McCurdy, D. W., Patrick, J. W., and Talbot, M. J. (2003). Transfer cells: cells specialized for special purpose. *Annu. Rev. Plant Biol.* 54, 431–454. doi: 10.1146/annurev.arplant.54.031902.134812

Palusa, S. P., Golovkin, M., Shin, S.-B., Richardson, D. N., and Reddy, A. S. N. (2007). Organ-specific, developmental, hormonal and stress regulation of expression of putative pectate lyase genes in Arabidopsis. *New Phytol.* 174, 537–550. doi: 10.1111/j.1469-8137.2007.02033.x

Popeijus, H., Overmars, H., Jones, J., Blok, V., Goverse, A., Helder, J., et al. (2000). Enzymology - Degradation of plant cell walls by a nematode. *Nature* 406, 36–37. doi: 10.1038/35017641

Qin, L., Kudla, U., Roze, E. H. A., Goverse, A., Popeijus, H., Nieuwland, J., et al. (2004). Plant degradation: a nematode expansin acting on plants. *Nature* 427, 30–30. doi: 10.1038/427030a

Quentin, M., Abad, P., and Favery, B. (2013). Plant parasitic nematode effectors target host defense and nuclear functions to establish feeding cells. *Front. Plant Sci.* 4:53. doi: 10.3389/fpls.2013.00053

Rose, J. K., and Lee, S. J. (2010). Straying off the highway: trafficking of secreted plant proteins and complexity in the plant cell wall proteome. *Plant Physiol.* 153, 433–436. doi: 10.1104/pp.110.154872

Scheller, H. V., and Ulvskov, P. (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289. doi: 10.1146/annurev-arplant-042809-112315

Siddique, S., Endres, S., Atkins, J. M., Szakasits, D., Wieczorek, K., Hofmann, J., et al. (2009). Myo-inositol oxygenase genes are involved in the development of syncytia induced by *Heterodera schachtii* in Arabidopsis roots. *New Phytol.* 184, 457–472. doi: 10.1111/j.1469-8137.2009.02981.x

Siddique, S., Endres, S., Sobczak, M., Radakovic, Z. S., Fragner, L., Grundler, F. M., et al. (2014). Myo-inositol oxygenase is important for the removal of excess myo-inositol from syncytia induced by *Heterodera schachtii* in Arabidopsis roots. *New Phytol.* 201, 476–485. doi: 10.1111/nph.12535

Siddique, S., Sobczak, M., Tenhaken, R., Grundler, F. M., and Bohlmann, H. (2012). Cell wall ingrowths in nematode induced syncytia require UGD2 and UGD3. *PLoS ONE* 7:e41515. doi: 10.1371/journal.pone.0041515

Sijmons, P. C., Grundler, F. M. W., von Mende, N., Burrows, P. R., and Wyss, U. (1991). *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *Plant J.* 1, 245–254. doi: 10.1111/j.1365-313X.1991.00245.x

Smant, G., Stokkermans, J. P., Yan, Y., De Boer, J. M., Baum, T. J., Wang, X., et al. (1998). Endogenous cellulases in animals: isolation of ß-1, 4-endoglucanase genes from two species of plant-parasitic cyst nematodes. *Proc. Natl. Acad. Sci. U.S.A.* 95, 4906–4911. doi: 10.1073/pnas.95.9.4906

Sobczak, M., Avrova, A., Jupowicz, J., Phillips, M. S., Ernst, K., and Kumar, A. (2005). Characterization of susceptibility and resistance responses to potato cyst nematode (*Globodera* spp.) infection of tomato lines in the absence and presence of the broad-spectrum nematode resistance *Hero* gene. *Mol. Plant Microbe. Interact.* 18, 158–168. doi: 10.1094/MPMI-18-0158

Sobczak, M., Fudali, S., and Wieczorek, K. (2011). "Cell wall modifications induced by nematodes," in *Genomics and Molecular Genetics of Plant-Nematode Interactions,* eds J. Jones, G. Gheysen, and C. Fenoll (Dordrecht: Springer), 395–422. doi: 10.1007/978-94-007-0434-3_19

Sobczak, M., Golinowski, W., and Grundler, F. M. W. (1997). Changes in the structure of *Arabidopsis thaliana* roots induced during development of males of the plant parasitic nematode *Heterodera schachtii*. *Eur. J. Plant Pathol.* 103, 113–124. doi: 10.1023/A:1008609409465

Sobczak, M., Golinowski, W. A., and Grundler, F. M. W. (1999). Ultrastructure of feeding plugs and feeding tubes formed by *Heterodera schachtii*. *Nematology* 1, 363–374. doi: 10.1163/156854199508351

Sun, L., and van Nocker, S. (2010). Analysis of promoter activity of members of the PECTATE LYASE-LIKE (PLL) gene family in cell separation in Arabidopsis. *BMC Plant Biol.* 10, 152. doi: 10.1186/1471-2229-10-152. Available online at: http://www.ncbi.nlm.nih.gov/pubmed/20649977

Szakasits, D., Heinen, P., Wieczorek, K., Hofmann, J., Wagner, F., Kreil, D. P., et al. (2009). The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in Arabidopsis roots. *Plant J.* 57, 771–784. doi: 10.1111/j.1365-313X.2008.03727.x

Talbot, M. J., Offler, C. E., and McCurdy, D. W. (2002). Transfer cell wall architecture: a contribution towards understanding localized wall deposition. *Protoplasma* 219, 197–209. doi: 10.1007/s007090200021

Tytgat, T., De Meutter, J., Vanholme, B., Claeys, M., Verreijdt, L., Gheysen, G., et al. (2002). Development and pharyngeal gland activities of *Heterodera schachtii* infecting *Arabidopsis thaliana* roots. *Nematology* 4, 899–908. doi: 10.1163/156854102321122511

Vanholme, B., Van Thuyne, W., Vanhouteghem, K., De Meutter, J., Cannoot, B., and Gheysen, G. (2007). Molecular characterization and functional importance of pectate lyase secreted by the cyst nematode *Heterodera schachtii*. *Mol. Plant Pathol.* 8, 267–278. doi: 10.1111/j.1364-3703.2007.00392.x

Vercauteren, I., De Almeida Engler, J., De Groodt, R., and Gheysen, G. (2002). An Arabidopsis thaliana pectin acetylesterase gene is upregulated in nematode feeding sites induced by root-knot and cyst nematodes. *Mol. Plant Microbe. Interact.* 15, 404–407. doi: 10.1094/MPMI.2002.15.4.404

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. C. (2002). *PMR6*, a pectate lyase-like gene required for powdery mildew susceptibility in Arabidopsis. *Plant Cell* 14, 2095–2106. doi: 10.1105/tpc.003509

Wang, X., Meyers, D., Yan, Y., Baum, T., Smant, G., Hussey, R., et al. (1999). In planta localization of a ß-1,4-endoglucanase secreted by *Heterodera glycines*. *Mol. Plant Microbe. Interact.* 12, 64–67. doi: 10.1094/MPMI.1999.12.1.64

Wieczorek, K., Golecki, B., Gerdes, L., Heinen, P., Szakasits, D., Durachko, D. M., et al. (2006). Expansins are involved in the formation of nematode-induced syncytia in roots of *Arabidopsis thaliana*. *Plant J.* 48, 98–112. doi: 10.1111/j.1365-313X.2006.02856.x

Wieczorek, K., Hofmann, J., Blochl, A., Szakasits, D., Bohlmann, H., and Grundler, F. M. (2008). Arabidopsis endo-1,4-ß-glucanases are involved in the formation of root syncytia induced by *Heterodera schachtii*. *Plant J.* 53, 336–351. doi: 10.1111/j.1365-313X.2007.03340.x

Wyss, U. (1992). Observations on the feeding behaviour of *Heterodera schachtii* throughout development, including events during moulting. *Fund. Appl. Nematol.* 15, 75–89.

Wyss, U., and Grundler, F. M. W. (1992). *Heterodera schachtii* and *Arabidopsis thaliana*, a model host-parasite interaction. *Nematologica* 38, 488–493. doi: 10.1163/187529292X00450

Wyss, U., Grundler, F., and Münch, A. (1992). The parasitic behaviour of second-stage juveniles of *Meloidogyne incognita* in roots of *Arabidopsis thaliana*. *Nematologica* 38, 98–111. doi: 10.1163/187529292X00081

Wyss, U., and Zunke, U. (1986). Observations on the behaviour of second stage juveniles of *Heterodera schachtii* inside host roots. *Rev. Nématol.* 9, 153–165.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 December 2013; paper pending published: 17 January 2014; accepted: 24 February 2014; published online: 19 March 2014.

*Citation: Bohlmann H and Sobczak M (2014) The plant cell wall in the feeding sites of cyst nematodes. Front. Plant Sci. **5**:89. doi: 10.3389/fpls.2014.00089*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 02 June 2014
doi: 10.3389/fpls.2014.00238

The role of the cell wall compartment in mutualistic symbioses of plants

Mélanie K. Rich, Martine Schorderet and Didier Reinhardt*

Department of Biology, University of Fribourg, Fribourg, Switzerland

Edited by:
Jean-Pierre Metraux, Université de Fribourg, Switzerland

Reviewed by:
Andrea Genre, University of Turin, Italy
Christian Staehelin, Sun Yat-sen University, China

***Correspondence:**
Didier Reinhardt, Department of Biology, University of Fribourg, Rte Albert Gockel 3, Fribourg CH-1700, Switzerland
e-mail: didier.reinhardt@unifr.ch

Plants engage in mutualistic interactions with microbes that improve their mineral nutrient supply. The most wide-spread symbiotic association is arbuscular mycorrhiza (AM), in which fungi of the order *Glomeromycota* invade roots and colonize the cellular lumen of cortical cells. The establishment of this interaction requires a dedicated molecular-genetic program and a cellular machinery of the plant host. This program is partially shared with the root nodule symbiosis (RNS), which involves prokaryotic partners collectively referred to as rhizobia. Both, AM and RNS are endosymbioses that involve intracellular accommodation of the microbial partner in the cells of the plant host. Since plant cells are surrounded by sturdy cell walls, root penetration and cell invasion requires mechanisms to overcome this barrier while maintaining the cytoplasm of the two partners separate during development of the symbiotic association. Here, we discuss the diverse functions of the cell wall compartment in establishment and functioning of plant symbioses with the emphasis on AM and RNS, and we describe the stages of the AM association between the model organisms *Petunia hybrida* and *Rhizophagus irregularis*.

Keywords: symbiosis, *Petunia*, *Rhizophagus*, *Rhizobium*, arbuscular mycorrhiza, nodulation, cell wall, symbiotic interface

INTRODUCTION

The plant cell wall is the outermost border of the plant body, and therefore the interface for interactions with the biotic and abiotic environment. Water and nutrients taken up from the soil have to cross the cell wall of the epidermis and the tissues between the epidermis and the vascular stele before they can enter the xylem for transport to the shoot with the transpiration stream (Kim et al., 2014). Together with the internal turgor pressure, the cell walls confer to the plant organs their particular shape (Zonia and Munnik, 2007), and in the case of dead tissues (xylem, wood), the walls represent the only remnant of the cells.

In interactions with pathogenic microbes, the cell wall is the first line of defense which provides a high level of general (non-host) resistance to plants. However, in mutualistic interactions such as root nodule symbiosis (RNS) and arbuscular mycorrhiza (AM), the cell walls of the host have to give way to the microbial partner in order to establish the intimate interfaces for developmental coordination and nutrient exchange. Intracellular accommodation of microbial symbionts thus involves dedicated pathways that evolved since the occurrence of the first symbiotic land plants over 400 Ma ago (Parniske, 2000; Brundrett, 2002). While the molecular-genetic program involved in mutual recognition and communication between the partners has recently been elucidated in considerable detail based on intense genetic and genomic analysis (Gutjahr and Parniske, 2013; Oldroyd, 2013), the later stages involving cellular coordination and establishment of the symbiotic interface are less well understood.

Here, we review recent developments in the domain of plant symbioses with the emphasis on the role of the cell wall compartment during penetration and establishment of the symbiotic interface. We discuss classical concepts and recent insight from genetics, genomics, and transcriptomics analysis concerning the mechanisms involved in symbiotic signaling, cell wall loosening, and penetration, and in the nutrition of the microbial endosymbiont. Finally, we provide a detailed description of the infection process in AM, the oldest and most widespread symbiotic association of plants, with the example of the interaction between *Petunia hybrida* and *Rhizophagus irregularis*.

ANATOMY OF PLANT ROOTS

The cell walls of roots have to comply with two very different tasks. On the one hand, the root is dedicated to the acquisition of water and mineral nutrients. For this, the epidermis (the epidermis of the root) has to be maximally permeable. On the other hand, the root surface has to be protected from harmful microbes (pathogens), toxic solutes, and, under conditions of drought, water loss. To comply with these two seemingly contradictory requirements, the root has specialized cell layers for nutrient absorption and protection.

With its thin cell walls, and the root hairs as surface extensions (**Figure 1A**), the epidermis is optimally suited for efficient nutrient uptake. In addition, the surface of the root system is increased by repeated lateral root formation and by the formation of tubular extensions of the epidermal cells, the root hairs. Further inside the root, the endodermis with its tight Casparian strip seals the vascular tissues in the stele from the cortex (Geldner, 2013b; **Figure 1A**). Water and solutes therefore have to pass through the endodermal cell layer exclusively symplastically, allowing this cell layer to control and filter the nutrient solution before it enters the xylem for the transfer to the shoot.

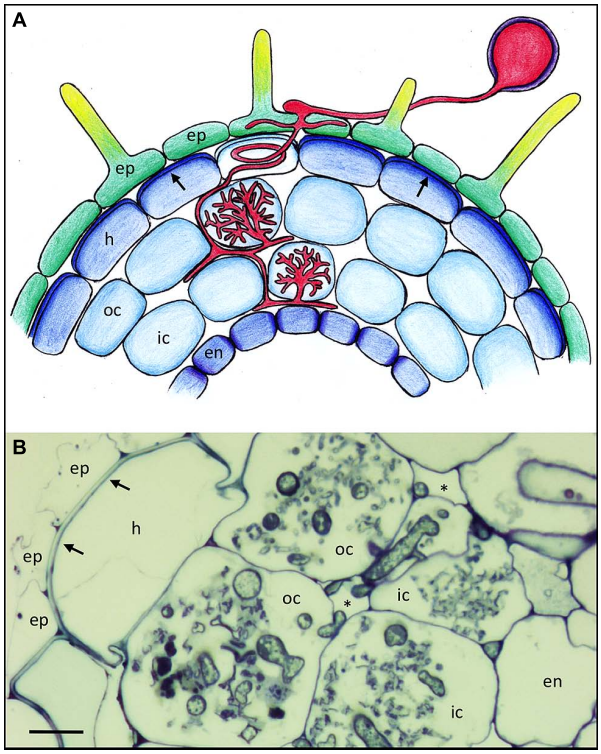


FIGURE 1 | Basic root anatomy and colonization pattern of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* in *Petunia hybrida*. (A) Schematic representation of a *Petunia* root with the different cell types and with an AM fungus (red). Arrows point to the reinforced outer cell wall of the hypodermis. Lighter coloration of the penetrated cell signifies a hypodermal passage cell. **(B)** Cross section of a mycorrhizal *Petunia* root. Arrows indicate the thick outer cell walls of the hypodermis. Asterisks indicate the intercellular air space between cortical cells. Fungal colonization is visible only in the cortex. ep, epidermis; h, hypodermis; oc, outer cortex; ic, inner cortex; en, endodermis. Size bar 10 μm.

Many plant species have a second protective cell layer below the epidermis, referred to as hypodermis, that is often reinforced with thickened cell walls and hydrophobic impregnations (**Figure 1A**; Meyer and Peterson, 2013), and which can have a Casparian strip like the endodermis (Enstone et al., 2002; Schreiber, 2010), in which case it is referred to as exodermis (Meyer and Peterson, 2013). Hence, like the endodermis, the hypodermis can contribute to the filtering of the soil solution, and to protection of the root from pathogens. Some plants have a so-called dimorphic hypodermis that consists of two cell types, the predominant impregnated cell type and individual interspersed cells that are characterized by higher permeability than their neighbors (Shishkoff, 1987). These are thought to function as dedicated passage cells for water and solutes, and interestingly, these cells are the preferred entry points for AM fungi. (Sharda and Koide, 2008). After they have passed beyond the hypodermis, AM fungi colonize the cortex by colonizing individual cells with their arbuscules (**Figures 1A,B**).

Besides the evolutionary differences in root structures between plant taxa (Shishkoff, 1987), a single species can have a heterogeneous root system. For example, rice first forms large crown roots (CR) from which large lateral roots (LLR) emanate, which in turn carry small lateral roots (SLR; Gutjahr and Paszkowski, 2013). AM colonization in rice is restricted to CR and LLR, presumably because SLRs lack a cortex and therefore cannot host AM fungi (see The Infection Process: Overcoming the Cell Wall).

STRUCTURE OF CELL WALLS IN ROOTS

In general, plant cell walls are assemblies of polysaccharides of different composition and properties. The main components are cellulose, hemicelluloses, and pectins. The cellulose component is chemically homogenous and consists of linear β-1,4-linked glucose moieties. Individual linear cellulose molecules are assembled to thicker cellulose microfibrils that represent the primary load-bearing component of the cell wall (McFarlane et al., 2014). The cellulose microfibrils are interlinked with hemicelluloses that consist of a linear polymer of glucose (like cellulose), but with heterogeneous side chains made of various sugars such as xylose, arabinose, and others (Scheller and Ulvskov, 2010). This complex three-dimensional network is embedded in a matrix of pectins, that represent highly acidic linear polymers of primarily galacturonic acid, which is interlinked by complexation with calcium (Caffall and Mohnen, 2009). While cellulose is formed in situ at the plasma membrane by the cellulose synthase complex (McFarlane et al., 2014), hemicelluloses and pectin are produced in the Golgi apparatus and transported to the apoplast through vesicular trafficking. Besides these carbohydrate polymers, cell walls contain variable amounts of structural proteins and secondary modifications such as impregnation with lignin and suberin for physical reinforcement and increased water impermeability. While the aerial parts of the plant (leaves and stems) have an external cuticle layer as a transpiration barrier and as a protection against biotic and abiotic agents, many roots have two internal barriers, the endodermis and the hypodermis (see above; Schreiber, 2010).

While the endodermis is a universal feature of the roots of land plants, the nature of the hypodermis is very variable among the higher plants. Whereas some xerophytes have highly reinforced hypodermal cells that serve as a secondary cuticle, the hypodermis of other species like for example *Arabidopsis thaliana* represents a normal cortex layer without specialized cell wall features. *Petunia* takes an intermediate position with a distinguished hypodermis (**Figure 1A**).

A specialized cell wall feature of roots is the Casparian strip, a hydrophobically impregnated cell wall band between adjacent endodermal cells that seals the inside of the root from the outside (Geldner, 2013a). This barrier blocks the diffusion of harmful xenobiotics (exogenous toxic substances) from the soil into the vasculature and the leakage of nutrients in the reverse direction. Hence, while a large part of the nutrient and water transport pathway between the epidermis and the vasculature can proceed through the apoplast (along and across the cell walls), at the level of the endodermis, the nutrients have to pass the plasma membrane by active selective uptake, representing an effective filtering mechanism which is exemplified by the polar localization of nutrient influx and efflux carriers at the outer and inner side of endodermal cells, respectively (Ma et al., 2007; Alassimone et al., 2010; Takano et al., 2010).

While the first description of the Casparian strip dates back to 1865 (Caspary, 1865), its development has only recently been addressed at the molecular level in the non-mycorrhizal model species *Arabidopsis thaliana* (Geldner, 2013b). Thanks to mutants affected in the formation of the Casparian strip (Roppolo et al., 2011; Lee et al., 2013), the physiological significance of this specialized cell wall domain can now be addressed functionally in *Arabidopsis*. Similar mutants in AM-competent plant species will allow to address the question whether the Casparian strip could potentially contribute to the restriction of AM fungal colonization to the cortex.

Although plant cells are surrounded by cell walls, they are by no means isolated from each other. Thin cytoplasmic connections, referred to as plasmodesmata, bridge the walls between neighboring cells and allow for the exchange of signals and nutrients (Lucas et al., 2009). The plasmodesmata play an important role in root development by transporting the transcription factor SHORT ROOT from the vascular stele to the endodermal cell layer (Petricka et al., 2012). Furthermore, the plasmodesmata contribute to symplastic nutrient transport between the epidermis and the vasculature.

SECONDARY DEVELOPMENT OF THE ROOT

The fine root hairs are restricted to a stretch of a few millimeters to centimeters behind the root tip. At later stages of development, and as a result of progressive damage of the delicate epidermal cells, the entire epidermis (including the root hairs) degenerates and the hypodermis becomes the new skin of the root (Meyer and Peterson, 2013). In these older parts of the root, AM can functionally replace the root hairs by the long hyphal extensions of the fungus (Smith and Read, 2008). In cases of perennial plants, the root shows secondary lateral growth as in the aerial stem (Chaffey, 2013). Interestingly, also herbaceous plants like *Arabidopsis thaliana* exhibit secondary root growth, which involves the generation of a cambial layer in the vascular stele that will form xylem to the inside and phloem to the outside, while the outer cell layers (endodermis, cortex, hypodermis, and epidermis) are shed (Dolan et al., 1993).

Besides the developmental aspects of secondary root development, the root is highly plastic to adapt to various environmental conditions such as drought anoxia, salinity etc. A distinct developmental program is triggered by anoxia (caused by flooding), when the roots form longitudinal air channels (aerenchyma) along the root which allow the transport of oxygen from the aerial parts of the plant to the root tips (Yamauchi et al., 2013). Formation of aerenchyma is associated with programmed cell death of cortical cell files, which causes these cell tiers to collapse and give rise to distinct tubular cavities along the root (Yamauchi et al., 2013). The root system also responds to nutrient availability in the soil. If nutrients are distributed heterogeneously, the roots explore the rich patches with intensely branching lateral roots. A genetically fixed adaptation of this kind is the formation of cluster roots, which are designed to the local exploitation of nutrients in the soil (Neumann and Martinoia, 2002). At a finer scale, the formation of lateral roots is stimulated by microbial signals (Olah et al., 2005; Maillet et al., 2011), whereas the development of entire mycorrhizal root systems is rather decreased relative to the shoot, conceivably as a consequence of the highly efficient role of the AM fungus in nutrient acquisition (Nouri et al., 2014).

A ROLE FOR CELL WALLS IN SYMBIONT RECOGNITION AND HOST SPECIFICITY?

Since the cell walls are the outermost boundary of the root, they are the first interface for interactions with soil-borne microbes. Based on this notion, it has early been postulated that interactions of the cell walls between plants and microbes may play an important role in symbiotic interactions. Indeed, genetic analysis in legumes and rhizobia have led to the discovery that interactions between components of the host apoplast and extracellular determinants of the rhizobial partner can influence host range and symbiotic compatibility.

Rhizobia produce surface-bound and secreted polysaccharides such as lipopolysaccharides (LPS; consisting of carbohydrate backbones with lipid side chains; Becker et al., 2005), exopolysaccharides (EPS), and capsid polysaccharides (KPS) that influence the development of RNS. Some bacterial mutants in LPS are unable to infect their host plants, an effect that can be complemented either with co-inoculation with bacteria that provide the missing LPS, or with purified LPS (Mathis et al., 2005). Besides the function of LPS at early stages of the interaction, later phenotypes during infection indicate that LPS plays a role in suppression of defense and promotion of compatibility (Margaret et al., 2013). Since bacterial polysaccharides can affect the host range of rhizobia, they have been proposed to contribute to host specificity (Wang et al., 2012a). Similar functional evidence was obtained with mutants in EPS (Niehaus and Becker, 1998).

Bacterial polysaccharides can be bound by plant lectins with a certain degree of specificity (De Hoff et al., 2009). Lectins of legumes are localized to the tips of root hairs where the rhizobia first attach and are subsequently incorporated (Diaz et al., 1995; van Rhijn et al., 1998). Lectins were therefore proposed to play a role as determinants of host specificity (Bohlool and Schmidt, 1974). Indeed, it was shown that transformation with heterologous lectins extended the ability of the transgenic host to interact with the cognate symbiont of the gene donor, thereby increasing the host range (Diaz et al., 1989; van Rhijn et al., 1998). Although lectins can influence the host range they are currently thought to promote the progression of infection and to sustain compatibility, rather than being involved in early communication (De Hoff et al., 2009). With their ability to bind bacterial cells to the root hairs, they perhaps promote the communication with diffusible molecules and therefore could enhance signaling with flavonoid/nod factor (see below).

In the case of AM, the epidermal cell walls of the host can stimulate hyphopodium formation (see The Infection Process: Overcoming the Cell Wall), however, it is at present not clear to which degree this effect is relevant for host range determination and compatibility (see below). The fact that the development of AM is associated with the induction of lectin genes indicates that compatibility of the partners may also involve lectins in this fungal symbiosis (van Rhijn et al., 1998; Frenzel et al., 2005; Valot et al., 2005). Taken together, these examples show that cell wall interactions can modulate compatibility and therefore affect host

specificity, although symbiotic communication relies primarily on diffusible signals (see below).

DIFFUSIBLE SIGNALS IN ROOT SYMBIOSES

Due to their immobility and short range of action, cell wall bound agents such as LPS can only play a limited role in the soil, in particular at the early stages of the interaction before the partners have established a first physical contact with each other. Indeed, early experiments had shown that the growth and morphogenesis of AM fungi is influenced by diffusible factors emanating from host roots (Gianinazzi-Pearson et al., 1989; Giovannetti et al., 1994, 1996). Partial purification (Buée et al., 2000) and chemical identification (Akiyama et al., 2005) revealed that the stimulatory agent is represented by strigolactone (SL) that is exuded from host roots. SLs comprise a family of chemically related compounds (Xie et al., 2010) which have originally been identified as stimulants of seed germination in parasitic weeds such as *Striga*, and which later turned out to function as phytohormones in shoot development (Gomez-Roldan et al., 2008; Umehara et al., 2008).

Strigolactones may be the factor that causes chemotropic growth of AM fungi toward host roots (Sbrana and Giovannetti, 2005). In *Petunia*, SL is exuded by the ABC transporter pleiotropic drug resistance 1 (PDR1) from hypodermal passage cells (Kretzschmar et al., 2012), indicating that chemotropism could potentially occur at a short range on the root surface. PDR1 expression is enhanced by phosphate starvation but remains confined to hypodermal passage cells. SLs stimulate fungal metabolism resulting in increased hyphal branching (Besserer et al., 2006), conceivably increasing the chances of interaction with the host. However, the fact that SL-defective plants are still colonized to a considerable degree (Gomez-Roldan et al., 2008; Breuillin et al., 2010), and that fungal development is not affected in an obvious way Kretzschmar et al. (2012) suggests that SLs are not an absolute requirement for AM symbiosis. Conceivably, in the absence of SLs, other root-borne factors such as flavonoids may stimulate AM fungal growth and infection (Abdel-Lateif et al., 2012).

In the case of RNS, the chemical communication between the plant host and the bacterial symbiont has been particularly well studied thanks to the excellent genetic tools in both symbiotic partners (Oldroyd et al., 2011). Legume roots release flavonoids, that attract rhizobia chemotactically. The flavonoids are recognized in rhizobia by the NodD receptor proteins, which act as transcription factors that induce a suite of bacterial *nod* genes. These encode enzymes which produce specific symbiotic signals, the nod factors (NFs), chitin-related oligomers of *N*-acetylglucosamin of a length of 4–5 units with a fatty acid chain and various additional substitutions (Oldroyd, 2013). These lipochitooligosaccharides (LCO) trigger in the plant host the activation of a dedicated symbiosis signaling pathway, which is referred to as common symbiosis signaling pathway (further referred to as common SYM pathway), because it is involved in both, nodulation and AM (Oldroyd, 2013). Activating the common SYM pathway induces a rhythmic calcium signal, referred to as calcium spiking, which in turn triggers symbiosis-related gene expression and a cascade of morphogenetic events which result in the development of a new organ, the nodule, that hosts the symbiotic bacteria. The specificity in the interaction between the diffusible signals, flavonoids and NFs, and their respective receptors, NodD proteins and NF receptors (NFR), are thought to represent the basis of host specificity in RNS (Wang et al., 2012a).

Recently, AM fungal signals where discovered that are remarkably similar to NFs and are therefore further referred to as myc factors. They consist of LCO (Maillet et al., 2011), or even of bare short-chain chitin oligomers that can trigger signaling through the common SYM pathway (Genre et al., 2013). The latter finding could potentially resolve a long-standing conundrum of AM, their very low host specificity, since chitin-derived signals are unspecific. However, this mode of action would require additional signals from AM fungi that allows the host to distinguish them from other fungi with chitinous cell walls.

Two recent reports on the AM mutants *reduced arbuscular mycorrhiza1* (*ram1*), and *ram2* in *Medicago truncatula* shed new light on an interesting aspect of AM development. *RAM1* encodes a GRAS-type transcription factor that is highly induced in mycorrhizal roots (Gobbato et al., 2012). One of the functions of *RAM1* is to activate *RAM2*, which in turn encodes a glycerol-3-phosphate acyl transferase (GPAT) that is also highly induced during AM symbiosis (Wang et al., 2012b). The similarity of the phenotypes of *ram1* and *ram2* indicates that the main function of *RAM1* is to induce *RAM2*. GPATs catalyze the addition of a fatty acid moiety onto glycerol-3-phosphate, thereby generating an intermediate for lipid biosynthesis (both for storage and as membrane constituents) and for cutin/suberin biosynthesis. Whereas lipid biosynthesis is quite well characterized, the production of the extracellular cutin or suberin layer is still only partially understood (Pollard et al., 2008; Yeats and Rose, 2013). The following questions remain to be answered: Which are the intermediates secreted from the cytoplasm and how do they pass the plasma membrane? How are the intermediates linked to a 3-dimensional network in the apoplast and what is the exact structure of this network? Based on the similarity to GPATs of *Arabidopsis thaliana* it has been hypothesized that RAM2 contributes to the production of unknown cutin precursors (C16 and C18 hydroxy fatty acids) that are essential for AM (Wang et al., 2012b). Since the number of hyphopodia is reduced in *ram2* mutants, one of the potential interpretations has been that *ram2* mutants are defective in the production of a signaling compound that stimulates hyphopodium formation (Wang et al., 2012b). Surprisingly, appressorium formation by the oomycete pathogen *Phytophthora palmivora* was affected as well, suggesting that despite their very far phylogenetic distance the mycorrhizal fungus and the oomycete are stimulated by similar mechanisms (Wang et al., 2012b).

Interestingly, GPAT6 from *Arabidopsis thaliana* was able to complement the *Medicago ram2* mutant. GPAT6 is involved in cutin biosynthesis in *Arabidopsis* petals, suggesting that *ram2* function in *M. truncatula* could also be related to cutin biosynthesis. Indeed, the increase of GPAT activity by overexpression of *RAM2* in *Arabidopsis* leaves caused an increase in the cutin content of some C16 and C18 components in the leaf cutin layer. However, the fact that roots do not have a cutin layer suggests that an intermediate of cutin biosynthesis could regulate the interaction between AM fungi and plants. Indeed, roots contain several hydroxy fatty acids that normally constitute the cutin layer (Wang

et al., 2012b), and the levels of some of these are reduced in *ram2*. A resulting conundrum is the fact that the mutation in the *RAM2* gene causes the substrate of the reaction (fatty acids) to decrease rather than the product (acyl glycerol). Along the same lines, the *ram2* mutant was complemented chemically with the substrate of the GPAT reaction rather than with its product (Wang et al., 2012b). In this context, it is interesting to note that hydroxy fatty acids can stimulate hyphal branching of AM fungi (Nagahashi and Douds, 2011). Taken together, some components of the cutin biosynthetic pathway are involved in the establishment of AM, however, further research is required to establish the exact nature of the molecules involved, and their function in symbiosis.

THE INFECTION PROCESS: OVERCOMING THE CELL WALL

After legumes have established the first physical contact with the rhizobial partner, the root hairs undergo a characteristic morphogenetic change referred to as root hair curling, which results in the formation of a coil in which a bacterial micro-colony is trapped. This phenomenon represents a specific growth response of the root hair cell, brought about by reorientation of tip growth (Gage and Margolin, 2000). The orientation of root hair tip growth is thought to be controlled primarily by the direction of vesicle trafficking at the tip, which in turn is regulated by the actin cytoskeleton (Ketelaar, 2013). An active role of the plant cell in this process is evident from the fact that progression of the infection thread precedes bacterial migration (Fournier et al., 2008). Similarly, a signal from the epidermis triggers organogenesis in the cortex before the bacterial endosymbiont has reached these region of the root (Rival et al., 2012).

In the case of AM development, the fungus forms infection structures, from which penetration of the epidermis is initiated. These infection structures were initially termed appressoria in analogy to the infection structures of leaf pathogens that force their way through the cuticle and the outer cell wall by a combination of lytic enzymes and physical force (Deising et al., 2000). However, in the absence of evidence for the generation of physical force, they have recently been renamed to hyphopodia (hyphal foot). Indeed, based on the active role of the plant in infection, they function as "a foot in the door" rather than as a strong tool to force the door open. Experiments with isolated cell walls showed that the outer epidermal cell walls of the host *Daucus carota* (carrot) elicited hyphopodium formation in AM fungi, whereas cell walls of its vasculature, or epidermal cell walls of the non-host *Beta vulgaris* (sugar beet) did not (Nagahashi and Douds, 1997). Similar results were obtained by analysis of AM fungal growth patterns on intact roots of the non-host lupin (Giovannetti et al., 1993). Hence, the formation of hyphopodia requires stimulation by the host, which in this case involves specific physico-chemical stimulation. However, a purely physical (thigmotropic) stimulus as in the case of the appressoria of some pathogens (Deising et al., 2000) was excluded based on the fact that AM fungi do not form hyphopodia on artificial surfaces (Giovannetti et al., 1993).

The fact that AM fungi can form hyphopodia on isolated cell walls of host plants suggests that the fungus can recognize specific cell wall features (Nagahashi and Douds, 1997). Consistent with a central role for cell wall features in hyphopodium formation, SL-deficient plants can be colonized by AM fungi and appear morphologically normal, although the degree of colonization is significantly reduced (Gomez-Roldan et al., 2008; Breuillin et al., 2010; Kretzschmar et al., 2012). Hence, SLs are not an indispensable prerequisite for AM infection. Similarly, mutants in the common SYM pathway induce hyphopodium formation in the fungus, sometimes even at higher rates than in the wild type, although the hyphopodia often exhibit an aberrant morphology (Novero et al., 2002; Demchenko et al., 2004). In rice, which forms three root types (see Structure of Cell Walls in Roots), hyphopodium formation is restricted to the CR and large lateral roots, indicating that the SLRs lack a stimulating signal. Taken together, these observations indicate that features of the cell wall play an important role in hyphopodium formation.

Hyphopodia are often formed in the groove between neighboring epidermal cells (**Figures 1A** and **2C**), from where the fungus either invades the neighboring epidermal cells or proceeds to penetrate the hypodermis. Evidence from *in vivo* imaging with fluorescently labeled cellular markers has shown that root cells actively prepare for infection and dictate the spatio-temporal progression of AM fungi infection through a structure referred to as prepenetration apparatus (PPA; Genre et al., 2005, 2008). The PPA is formed exactly below the hyphopodium, indicating that the plant cell can precisely locate the position of the hyphopodium. In plants that have a dimorphic hypodermis (see Anatomy of Plant Roots) penetration proceeds preferentially through hypodermal passage cells (Sharda and Koide, 2008). Conceivably, this is because of their greater permeability and weaker cell wall impregnation relative to the remaining hypodermal cells, and due to their release of SL (Kretzschmar et al., 2012). The mechanisms by which the cell walls are prepared to allow microbial penetration remain largely elusive, but are likely to involve cell wall softening agents (see below).

In apparent contrast to the evidence discussed above, a recent study reported the colonization of the non-host plant *Arabidopsis thaliana* by the AM fungus *Rhizophagus irregularis* (Veiga et al., 2013). This is surprising, in view of the fact that *Arabidopsis* misses many of the genes required for symbiotic signaling, and has not been reported to be colonized by AM fungi before. However, several characteristics of the interaction suggest that it does not represent a *bona fide* functional mycorrhizal interaction (Veiga et al., 2013). In general, colonized cells of *Arabidopsis* appeared to be dead or degenerating based on electron microscopic analysis, and the growth of colonized *Arabidopsis* plants was strongly inhibited (Veiga et al., 2013). The fact that the non-host *Arabidopsis* was invaded at all is probably due to the inoculum type, namely fungal hyphae emanating from mycorrhizal clover, which represents a very strong inoculum potential. Taken together, these aspects suggest that under the conditions of this study, the AM fungus acts like an opportunistic pathogen of *Arabidopsis*, rather than like a mutualistic symbiont.

THE INTERACTION BETWEEN *Petunia hybrida* AND *Rhizophagus irregularis* – AN ANATOMICAL CASE STUDY

Petunia hybrida is a powerful experimental model system for forward and reverse genetic analysis (Gerats and Vandenbussche,

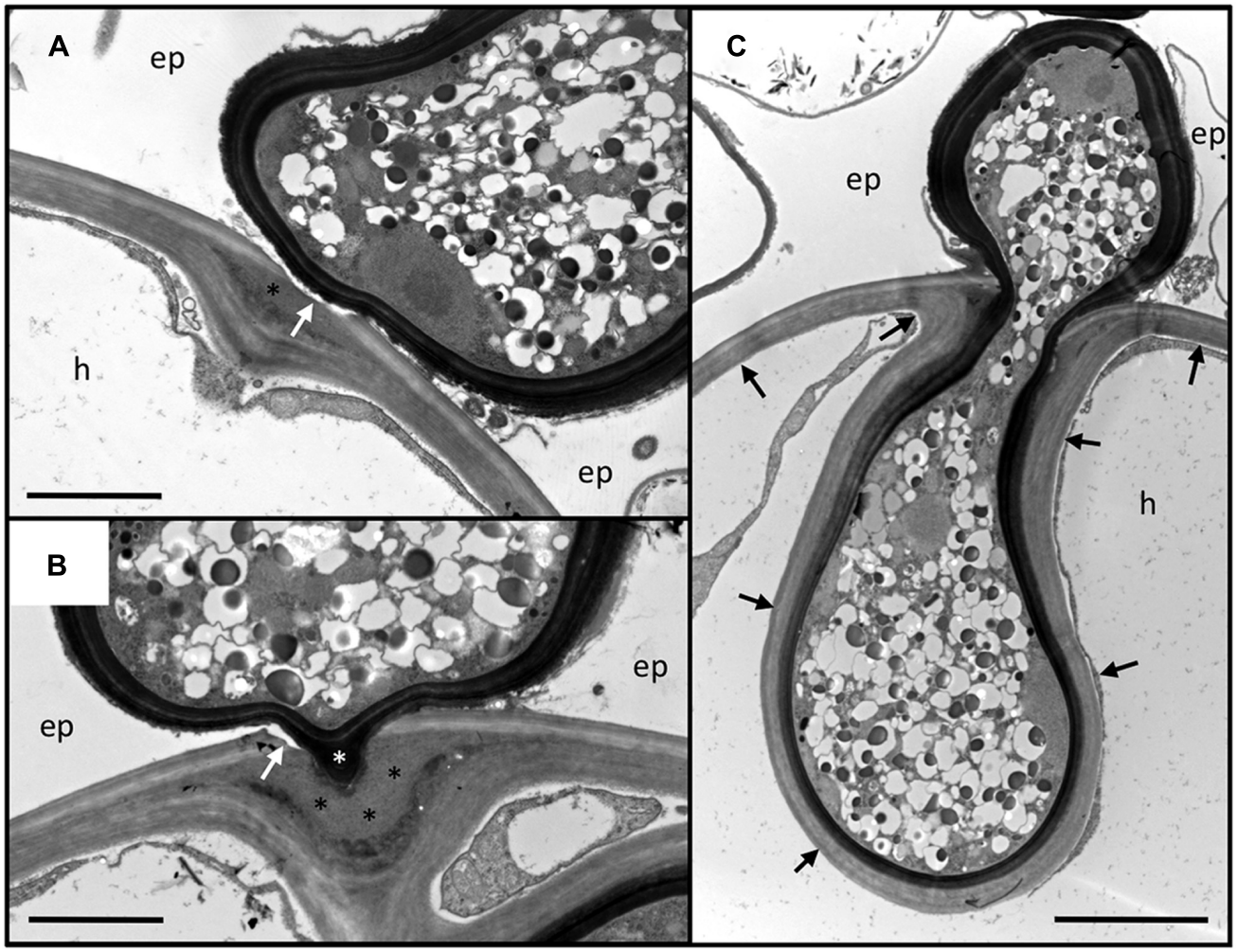


FIGURE 2 | Penetration of the outer root cell layers of *Petunia hybrida* by *Rhizophagus irregularis*. Serial sections of an infection site at the level of the hypodermis. **(A)** Section through the hyphopodium (surrounded by a dark cell wall), that has entered between two adjacent epidermal cells (ep), and attached to a hypodermal cell (h). Note the alterations of the cell wall at the penetration site. The primary wall (light staining) is perforated (white arrow), whereas the secondary wall invaginates into the cellular lumen. In between, an interstitial matrix has been formed (asterisk). **(B)** Section close to the penetration hypha. The fungus has penetrated the primary wall (white arrow), whereas the secondary (inner) layers of the wall yield to the fungal penetration hypha (white asterisk). A thick layer of interstitial matrix material (black asterisks) has accumulated between the penetrated outer wall and the invaginated inner wall. **(C)** Median section through the penetration hypha. Note the inward deformation of the primary cell wall. The secondary wall has invaginated and is continuous with the original secondary wall (black arrows). ep, epidermal cell; h, hypodermal cell. Size bar 2 μm in **(A,B)** and 5 μm in **(C)**.

2005), and has been used successfully for the analysis of genetic and nutritional regulation of AM (Breuillin et al., 2010; Feddermann et al., 2010; Nouri et al., 2014). Here, we describe the infection process of the AM fungus *Rhizophagus irregularis* (formerly *Glomus intraradices*) in *Petunia hybrida* with the focus on the events at the level of the cell wall during penetration of the hypodermis and in the established symbiosis (**Figures 2** and **3**). Since transmission electron microscopic (TEM) technology is destructive and can therefore not represent serial time course stages as in confocal microscopy (Genre et al., 2005, 2008), we provide a spatial gradient of cell wall modification at three different distances from a successful fungal entry point (**Figure 2**).

Usually, fungal hyphopodia insert between the adjacent anticlinal walls of two neighboring epidermal cells (**Figure 2**). Subsequently, the fungus invades the subtending hypodermal cell. In *Petunia*, the hypodermal cell layer is particularly thick-walled, with two clearly distinguished layers, an outer (primary) layer that is weakly stained by osmium tetroxide, and a thicker inner (secondary) layer that appears more electron-dense (**Figures 2A,B**). Penetration of this thick multilayer cell wall involves two mechanisms: firstly, the primary cell wall is dissolved locally, and secondly, the secondary cell wall invaginates to give way to the fungal penetration hypha. During hyphal growth, the secondary wall retains its original thickness around the entire hyphal coil indicating that the cell wall is continuously remodeled and extended (**Figure 2C**). The thick mass of interstitial matrix material (**Figures 2A,B**; black asterisks) may be involved in the softening of the cell wall, or may represent

remnants of the dissolved cell wall. Hence, the outer part of the wall is degraded whereas the inner part is softened, remodeled, and extended, indicating that large amounts of new cell wall material are produced. Based on the fact that *Rhizophagus irregularis* does not have any cell wall lytic enzymes (Tisserant et al., 2013), and that penetration depends on recognition by the host (Parniske, 2008), it can be assumed that the cell wall alterations associated with fungal entry are orchestrated by the plant host.

Establishment of the intracellular symbiotic interface for nutrient exchange involves the colonization of the cellular lumen by arbuscules. These finely branched hyphal structures can occupy almost the entire cellular lumen, resulting in invasion and fragmentation of the central vacuole (**Figures 3A,B**). The fungal branches are surrounded by a thin layer of matrix material (red in **Figure 3A**). The finest hyphal branches are surrounded by a very thin layer of interfacial material and are embedded in cytoplasmic pockets rich in ER and organelles, indicative of strong metabolic and biosynthetic activity (**Figure 3C**).

SYMBIOTIC INTERFACES

Nutrient exchange between the symbionts is regulated at the level of the plasma membrane of the microbe on the one side, and at the level of the host membrane that surrounds it on the other side, the peri-arbuscular membrane in AM, and the symbiosome membrane in RNS (Bapaume and Reinhardt, 2012). In between lies the periarbuscular space (in the case of AM), and the peribacteroid space (in the case of RNS), across which the nutrients are transferred, respectively. Collectively, this represents the symbiotic interface that controls communication and the exchange of nutrients between the cytoplasms of the two symbiotic partners.

In the case of RNS, the host membrane and all its contents (bacteroid and interface) are referred to as the symbiosome, hence representing functional units like the organelles with prokaryotic origin, the chloroplasts and the mitochondria (Kereszt et al., 2011). Based on the invaginated infection thread, which is related to the apoplast, the interface of the symbiosome could be compared topologically with the plant cell wall compartment, although, the symbiosomes represent rather specialized minivacuoles that harbor bacteroids and control their activities.

The feeding structures of AM fungi, the arbuscules, can be regarded as inverse haustoria that releases nutrients to the plant host. Haustoria are highly specialized feeding structures of parasitic fungi and oomycetes that serve to take up nutrients from the host (Deising et al., 2000; Panstruga, 2003; Yi and Valent, 2013). Active arbuscules are surrounded by an acidic compartment, as revealed by the accumulation of Neutral Red in the periarbuscular space (**Figure 3D**). Notably, the arbuscular trunk shows only light NR staining, whereas extracellular hypha are completely devoid of it. This indicates that the periarbuscular space may be sealed at the level of the trunk (Pumplin and Harrison, 2009), in order to avoid the loss of nutrients, and to maintain the acidic pH required to energize nutrient uptake from the interface (Gianinazzi-Pearson et al., 2000; Guttenberger, 2000). Similarly, the peribacteroid space represents an acidic compartment from which the plant host and the bacteroid can take up nutrients using the proton gradient as motive force (Pierre et al., 2013).

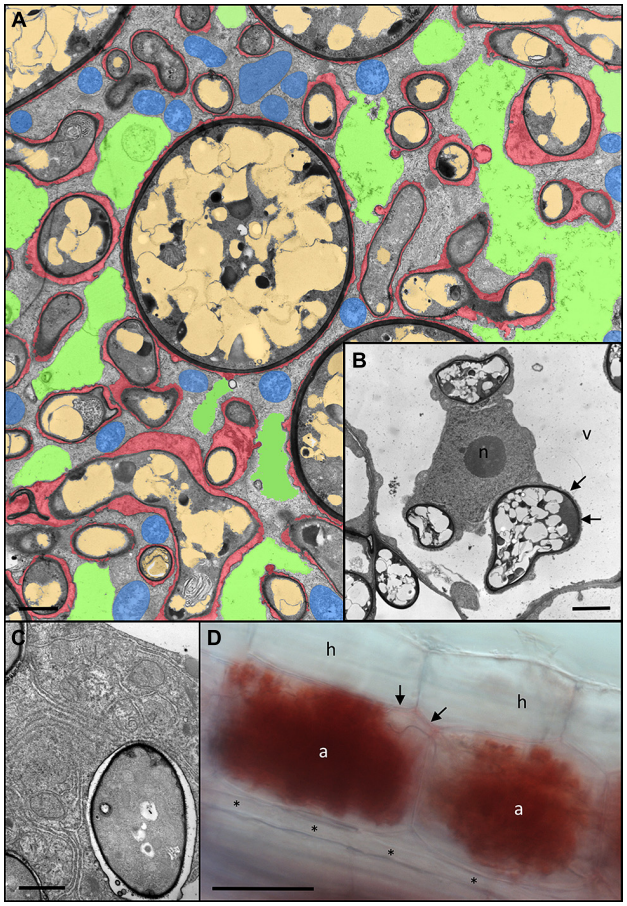


FIGURE 3 | Anatomy of *Rhizophagus irregularis* arbuscules and acidic nature of the periarbuscular space. (A) Cortex cell of *Petunia hybrida* colonized by a fully developed arbuscule with hyphal branches of variable diameters. Remnants of the fragmented central vacuole are colored green, plant mitochondria are blue, fungal vacuoles are light brown, and the periarbuscular space is red (modified from Bapaume and Reinhardt, 2012). **(B)** Host nucleus (n) of a colonized cell between three fungal hyphae. Note the close association of the fungus with the host cytoplasm resulting in no distinguishable periarbuscular space between the fungal wall (dark layer), and the thin cytoplasmic layer (arrows) between the fungus and the host vacuole (v). **(C)** Close up of an individual fungal fine branch of an arbuscule. The fine branch is embedded in organelle-rich host cytoplasm with endoplasmatic reticulum and plastids. **(D)** A pair of mature arbuscules (a) sharing a joined trunk hypha (arrows). Staining with Neutral Red reveals the acidic nature of the periarbuscular space, whereas the trunk hypha shows only weak staining, and a longitudinal intercellular hypha (asterisks) is completely devoid of staining. a, arbuscule; h, hypodermal cell; n, host nucleus; v, host vacuole. Size bar 2 μm in **(A,B)**, 1 μm in **(C)**, and 50 μm in **(D)**.

The periarbuscular space consists of loosely assembled cell wall polymers including cellulose, hemicellulose, pectin, and arabinogalactan/hydroxyprolin-rich proteins (Gianinazzi-Pearson, 1996; Balestrini and Bonfante, 2005). In addition, the symbiotic interface may contain some of the predicted extracellular proteins that are induced in AM and often restricted to cells with arbuscules (e.g., van Buuren et al., 1999; Journet et al., 2001).

Notably, cells with arbuscules express specific cell wall remodeling proteins that may help establish the interface during the complex morphogenesis of the two symbiotic partners (Balestrini et al., 2005; Dermatsev et al., 2010; see below).

In functional nodules, the rhizobia undergo fundamental reprogramming, which often results in terminal differentiation of the prokaryote, i.e., it loses vital functions and becomes entirely dedicated to its symbiotic function, namely N-fixation. At this stage, they are referred to as bacteroids, and they are confined by a host-derived membrane that controls nutrient exchange and the fate of the endosymbiont. While the nature of the arbuscular interface has been studied primarily by immunocytochemistry and gene expression analysis (Balestrini and Bonfante, 2005), the composition of the interface in symbiosomes has been addressed by proteomics, based on the fact that symbiosomes can be easily enriched and purified (Saalbach et al., 2002). However, a problem of this technique is that the interface and the symbiosome membrane tend to be contaminated from cytoplasmic bacteroid proteins. Nevertheless, this approach confirmed that the peribacteroid space in pea nodules contains a lectin that has earlier been recognized as a resident protein of the peribacteroid space (Kardailsky et al., 1996). An interesting case of secreted peptides that accumulate in the peribacteroid space are the nodule-specific cysteine-rich (NCR) peptides. NCR peptides cause terminal differentiation in bacteroids, indicating that the host uses them as a tools to manipulate the development of the endosymbiont (Van de Velde et al., 2010), in order to prevent it from overproliferation (Kereszt et al., 2011).

TRAFFICKING AT THE SYMBIOTIC INTERFACE

The primary function of the symbiotic interface is nutrient exchange. The specialized function of the interface in AM is exemplified by the expression of transporters for phosphate (Rausch et al., 2001; Harrison et al., 2002; Yang et al., 2012), and ammonium (Guether et al., 2009b; Kobae et al., 2010; Koegel et al., 2013) in colonized cells. A pair of ABC transporters in the periarbuscular membrane, STUNTED ARBUSCULE (STR), and STR2, that are functionally conserved between the legume *M. truncatula* and the cereal rice (Zhang et al., 2010; Gutjahr et al., 2012), may be involved either in the transport of a metabolically relevant substrate, or in signaling between the partners, however, until the identification of its substrate the function of STR and STR2 remains elusive. Furthermore, periarbuscular membranes carry ATPases for acidification of the symbiotic interface (Marx et al., 1982; Gianinazzi-Pearson et al., 2000).

Among the AM-related genes with high induction ratios in various plant species are proteases that are predicted to carry a secretion signal peptide (Takeda et al., 2007). These proteins are therefore expected to enter the protein trafficking pathway and to be delivered to the vacuole, the apoplast, or, potentially, to the symbiotic interface. The signal peptide of an AM-associated protease of the subtilase type has indeed been shown to confer localization to the periarbuscular space (Takeda et al., 2009). Silencing of this protease hampers AM development, indicating that it is involved in fungal growth or in symbiotic signaling (Takeda et al., 2009). However, its precise function in AM remains elusive.

Many genes induced during nodulation (nodulins) encode secreted proteins, for example the early nodulins (ENOD proteins) among which there are many prolin-rich extracellular proteins (Brewin, 2004). A detailed study of ENOD8 localization showed that it has multiple independent determinants of symbiosome localization, including the N-terminal signal peptide (Meckfessel et al., 2012). Interestingly, in non-symbiotic cells, ENOD8 is delivered to the vacuole. This suggests that either the symbiosome has vacuolar features, or that protein trafficking is rerouted to the symbiosome in symbiotic cells. Interestingly, a general rerouting of protein trafficking has been documented in AM symbiosis, where all membrane proteins expressed from the symbiosis-specific PT4 promoter were localized to the periarbuscular membrane (Pumplin et al., 2012). The surprising conclusion of these experiments is that protein trafficking in mycorrhizal cells is not based on the peptide sequence but on the timing of gene expression. Taken together, these results indicate that protein trafficking toward the symbiotic interface may be proceed by default in symbiotic cells. Components with a potential role in secretion to the symbiotic interface include the symbiosis-induced SNARE proteins VAMP72d and VAMP72e which are localized to the host membrane at the symbiotic interface (Ivanov et al., 2012).

CELL WALL REMODELING IN SYMBIOSIS

Invasion of host cells by microbes requires local softening of the cell wall at the point of penetration. Cell wall softening can be achieved either by partial degradation of the wall polymers, or by weakening of the intermolecular interactions between the cell wall polymers (Cosgrove, 2000a). Evidence from TEM analysis of the interaction between *Petunia hybrida* and *Rhizophagus irregularis* suggests that both mechanisms may be involved simultaneously (**Figure 2**).

Conceptually, the cell wall lytic or loosening agents could be encoded by genes of the host or the microbe. *Rhizobium leguminosarum* possesses a cell-wall-bound β-1,4 endoglucanase (cellulase CelC2), required for formation of infection threads and for successful bacterial infection, suggesting that the bacterial endosymbiont uses the cellulase to breach the cell wall of root hair tips (Robledo et al., 2008). However, early work has shown that local cell wall loosening can also occur in the absence of the microbial partner during the formation of pre-infection threads, indicating that in this case the loosening agent is encoded by the host (van Brussel et al., 1992; van Spronsen et al., 1994). Indeed, *Lotus japonicus* has an inducible pectate lyase gene that is required for rhizobial infection (Xie et al., 2012).

In contrast to rhizobia, the AM fungal model species *Rhizophagus irregularis* appears to lack genes with a predicted cell wall degrading, modifying or remodeling activity (Tisserant et al., 2012, 2013). The same is true for the obligate biotrophic pathogen *Blumeria graminis* (Spanu et al., 2010). Along the same lines, the genomes of the ectomycorrhizal fungi *Laccaria bicolor* and *Amanita bisporigera* have greatly reduced numbers of potential cell wall degrading genes compared to saprophytic fungi (Martin and Selosse, 2008; Martin et al., 2008; Nagendran et al., 2009). The

reduction or elimination of genes encoding cell wall-modifying proteins in biotrophic microbes may represent a selective advantage given the fact that plants have extremely sensitive detection mechanisms for such microbial activities and react with a defense response (see below). Hence, in the case of AM fungi, the wall loosening activity appears to be encoded primarily or entirely by the host.

In contrast to lytic enzymes, expansins act by loosening cell walls non-enzymatically, presumably by allowing cellulose microfibrils to creep relative to the surrounding wall matrix in a fashion analogous to a lubricant. (Cosgrove, 2000b). Expansin is induced during RNS (Giordano and Hirsch, 2004) as well as in AM (Balestrini et al., 2005; Dermatsev et al., 2010). Indeed, the silencing of tomato expansin interferes with AM, indicating that expansin activity may be required for proper arbuscule development (Dermatsev et al., 2010).

Apart from the expansins, several symbiosis-related transcripts encode host genes with a potential role in cell wall remodeling, for example xyloglucan endotransglycosidase induced in colonized cells of AM (van Buuren et al., 1999). In AM, the formation of the PPA is characterized by high vesicular activity (Genre et al., 2008) and by induction of cell wall modifying proteins such as cellulose synthase and expansin-like proteins (Siciliano et al., 2007). Conceivably, these proteins are targeted to the PPA and released to prepare the cell wall for penetration.

DEFENSE RESPONSES IN SYMBIOSIS

Plants have very sensitive perception mechanisms to detect microbial infection and to react with a defense response to inhibit microbial growth (Jones and Dangl, 2006). The signal molecules derived from pathogens are referred to as pathogen-associated molecular patterns (PAMPs) and in cases where rather unspecific signals from a wide range of microbes (e.g., chitin) are concerned, the signals are referred to as microbe-associated molecular patterns (MAMPs; Boller and Felix, 2009). Often, the microbe is detected via its direct or indirect effects on the host cell wall. Many pathogens release hydrolytic enzymes that weaken the cell wall by degrading the polymers in the cuticle (cutinase) or the cell wall (xylanase, glucanase etc.), thereby releasing monomers that are perceived by plants at concentrations in the nanomolar range (Boller and Felix, 2009). In addition, plants can detect metabolites that emanate from damaged tissues and therefore serve as indicators of damage [damage-associated molecular patterns (DAMPs)]. Some MAMPs can be produced as a result of defense-related hydrolytic enzymes of the host (chitinase, glucanase) that release oligomers from the microbial cell wall (Boller and Felix, 2009).

It has long been suggested that mutualistic microbes also release or induce MAMPs and DAMPs, and indeed, symbiosis is often associated with the induction of defense markers (Blee and Anderson, 2000; Lambais, 2000; Shaul et al., 2000; Gianinazzi-Pearson and Smith, 2003; Lopez-Gomez et al., 2012; Zamioudis and Pieterse, 2012). Although it has often been observed that defense responses in AM are only transient (Gianinazzi-Pearson et al., 1996), some homolog of defense marker genes remain strongly induced during symbiosis raising the question whether they can be regarded as defense markers (see below). Cellular and biochemical defense responses, such as the production of reactive oxygen species or the hypersensitive response, could potentially interfere with symbiosis. A possible mechanisms to counteract such defense responses and to promote compatibility in symbiosis would be to remove the signals that trigger them. Such a function has been proposed for host-derived chitinase that could potentially degrade fungal chitin oligomers in mycorrhizal associations (Salzer and Boller, 2000), or even transform them into short chain chitin oligosaccharides that serve as symbiotic signals (Genre et al., 2013).

Pathogens have evolved efficient tools, collectively referred to as effectors, to suppress defense responses. Effectors are delivered to the host to inhibit defense at various levels from signal perception to the induction of the defense response (Stergiopoulos and de Wit, 2009; Deslandes and Rivas, 2012). Several recent studies have revealed similar mechanisms in mycorrhizal fungi and rhizobia (Kloppholz et al., 2011; Plett et al., 2011; Zhang et al., 2011; Xin et al., 2012; Okazaki et al., 2013). The large number of symbiosis-induced predicted secreted proteins in the genomes of the ectomycorrhizal fungus *Laccaria bicolor* (Martin and Selosse, 2008; Martin et al., 2008) and the AM fungus *Rhizophagus irregularis* (Tisserant et al., 2012, 2013) indicates that still many mycorrhizal effectors remain to be discovered. Interestingly, NF-defective rhizobia can establish symbiosis using effectors delivered by the type-three secretion system (Okazaki et al., 2013). This suggests that NFs and myc factors could be regarded as specialized effector-like tools to promote compatibility during nodulation and AM development, respectively, a concept that has received recent support from *Arabidopsis thaliana* (Liang et al., 2013). In this context, it is interesting to note that legume mutants with a defect in the common SYM pathway, which is required for NF/myc factor signaling, mount a distinct defense response upon inoculation with AM fungi (Gollotte et al., 1993, 1994). Hence, one function of the common SYM pathway is to suppress defense in symbiotic interactions.

ROLE OF PR PROTEINS IN SYMBIOSIS

Plant defense is often accompanied by the induction of pathogenesis-related (PR) proteins that are thought to have antimicrobial activity for instance by hydrolyzing components of microbial cell walls (van Loon et al., 2006). Many PR proteins are secreted and reside in the apoplast (van Loon et al., 2006). Interestingly, homolog of PR proteins are also induced during AM (Liu et al., 2003; Manthey et al., 2004; Güimil et al., 2005; Hohnjec et al., 2005; Grunwald et al., 2009; Guether et al., 2009a; Breuillin et al., 2010). Transcriptomic analysis in *Petunia hybrida* revealed that numerous PR protein homolog were undetectable in non-mycorrhizal roots, whereas others showed strong induction ratios over their constitutive basal expression levels (Breuillin et al., 2010). Notably, they were induced not just transiently at the onset of the interaction, but in fully developed mycorrhizal roots, indicating that they are more than markers of a transient initial defense response.

Based on the observation that AM fungi appear to be generally insensitive to high constitutive PR gene expression in plants (Vierheilig et al., 1993; Gianinazzi-Pearson et al., 1996), it could be

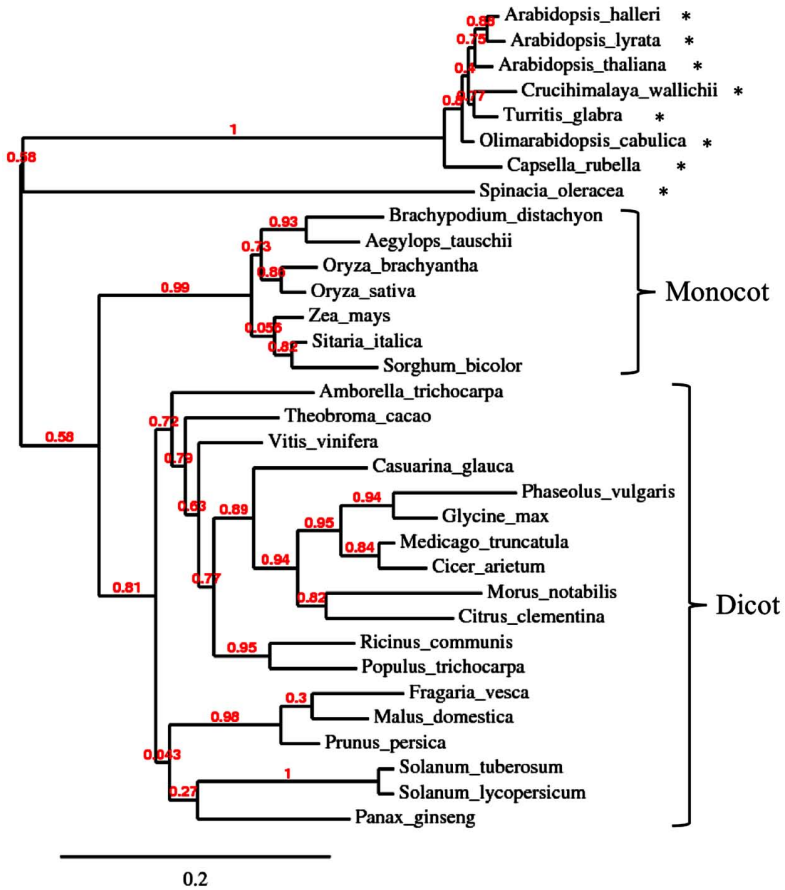


FIGURE 4 | Phylogenetic analysis of type III chitinase in higher plants. Unrooted phylogenetic tree of chitinase type III of various angiosperm species. Protein sequences were retrieved by pblast from NCBI using *Medicago truncatula* chitinase III-3 (AY238969.1) as query. For each species, the first hit was taken for phylogenetic analysis. Non-mycorrhizal species are marked with an asterisk. Note that the non-mycorrhizal *Brassicacea* and spinach (*Spinacia oleracea*) form an outgroup relative to the more conserved chitinases of the mycorrhizal monocot and dicot species.

argued that the induction of PR genes contributes to a generally induced disease resistance status in mycorrhizal plants (Pozo and Azcon-Aguilar, 2007). Alternatively, AM-specific PR gene isoforms could play specific roles in symbiosis. Indeed, it has been shown for the example of Chitinase III in *M. truncatula* that individual isoforms are expressed specifically during defense and mutualism, respectively (Salzer et al., 2000). Although a role for AM-related chitinase III has been suggested based on its stimulatory effect on spore germination (Elfstrand et al., 2005), its role in established mycorrhizal roots remains to be explained.

A symbiosis-specific role of symbiosis-related PR protein homolog is conceivable in cases, where they are encoded by genes that are conserved to a higher degree in AM-competent plant taxa than in non-symbiotic species. For example, the AM-specific chitinase III isoform of *M. truncatula* has close homolog in mycorrhizal dicots as well as monocots, whereas the AM-incompetent *Brassicaceae* and the *Chenopodiacae* species spinach have more distant homolog (**Figure 4**). The fact that this chitinase isoform is particularly well conserved among AM-competent taxa indicates that it is under selective pressure for a function in AM. For example, chitinase could act by generating chitin oligomers that are involved in AM signaling (Genre et al., 2013). In this context, it is interesting to note that nodulation also involves chitinases that are thought to influence NF signaling (Goormachtig et al., 1998; Tian et al., 2013).

FOOD FOR THOUGHT: THE INVOLVEMENT OF THE CELL WALL COMPARTMENT IN NUTRITION OF SYMBIOTIC MICROBES

Arbuscular mycorrhiza fungi are obligate biotrophs, i.e., they rely on living root tissues for survival, implying that they depend on the supply of some plant metabolites that cannot be provided in the form of plant extracts. Whether this concerns the acquisition of reduced carbon or of some other vital factor is unknown. Also, it is not clear whether AM fungi acquire their nutrients over the arbuscules or along their intercellular hyphae. The fact that ectomycorrhizal fungi can live without intracellular feeding structures indicates that the apoplast of roots can provide sufficient extracellular resources to sustain a mycorrhizal network, although the formation of the dense Hartig net indicates that the efficient acquisition of these resources requires an extended hyphal surface.

Photoassimilates are transported through the phloem in the form of sucrose. In order to become available to fungal symbionts

in the root cortex, the sucrose (or a derived carbohydrate) has to cross the endodermis symplastically and to be released to the apoplast or the symbiotic interface in the cortex. Based on circumstantial evidence, it has been suggested that AM fungi use hexoses as carbon source (Solaiman and Saito, 1997; Pfeffer et al., 1999; Douds et al., 2000). The fact that the genome of the AM fungal model species *Rhizophagus irregularis* does not contain any secreted invertases or sucrose transporters is compatible with the idea that this fungus relies entirely on the supply of hexoses from the plant (Tisserant et al., 2013), and the recent discovery of a versatile monosaccharide transporter (MST2) from *Rhizophagus irregularis* support this view (Helber et al., 2011). In the absence of efficient transformation protocols for AM fungi (Helber and Requena, 2008), the function of MST2 was addressed by expression of a silencing construct against the fungal gene in the host. The intimate interaction of the symbiotic partners over the symbiotic interface allows for efficient silencing of the fungal target gene, a method referred to as host-induced gene silencing (HIGS; Helber et al., 2011). The fact that MST2 is expressed both in arbuscules and in intercellular hypha suggests that hexose uptake could potentially proceed anywhere along the extended intraradical fungal network.

While the induction of an invertase in mycorrhizal cells of tomato is consistent with the model discussed above (Schaarschmidt et al., 2006), AM colonization could not be stimulated by increased invertase activity in transgenic tobacco, suggesting that under the conditions examined, hexose supply to the fungus is not limiting (Schaarschmidt et al., 2007). On the other hand, silencing of a cytoplasmic sucrose synthase (a sucrose cleaving enzyme) of *M. truncatula* interferred with both AM colonization and nodulation (Baier et al., 2007, 2010), pointing to a connection of sink strength and symbiosis. However, due to the pleiotropic phenotype of *SucS1* silencing, these results have to be interpreted with caution. Taken together, the nutrition of AM fungi remains far from clear, whereas it is commonly accepted that in RNS the prokaryotic endosymbiont is fed primarily by dicarboxylic acids (Yurgel and Kahn, 2004).

CONCLUSION

As the outermost border of the plant body, the cell wall mediates many interactions with the environment of plants. Besides the multiple roles of the cell wall in basic plant life (growth, nutrient exchange etc.), the cell wall plays a central role in interactions with the biotic environment. While it represents the primary barrier against pathogens, it has to yield to permit the invasion of mutualistic microbes such as rhizobia and AM fungi. The cumulative evidence suggests that the plant itself plays a central role in the softening and remodeling of the cell wall during symbioses. Future work should address the molecular components involved in cell wall remodeling during endosymbiont infection, and further characterize the nature of the symbiotic interface during the different stages of the interaction.

REFERENCES

Abdel-Lateif, K., Bogusz, D., and Hocher, V. (2012). The role of flavonoids in the establishment of plant roots endosymbioses with arbuscular mycorrhiza fungi, rhizobia and *Frankia* bacteria. *Plant Signal. Behav.* 7, 636–641. doi: 10.4161/psb.20039

Akiyama, K., Matsuzaki, K., and Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435, 824–827. doi: 10.1038/nature03608

Alassimone, J., Naseer, S., and Geldner, N. (2010). A developmental framework for endodermal differentiation and polarity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5214–5219. doi: 10.1073/pnas.0910772107

Baier, M. C., Barsch, A., Küster, H., and Hohnjec, N. (2007). Antisense repression of the *Medicago truncatula* nodule-enhanced sucrose synthase leads to a handicapped nitrogen fixation mirrored by specific alterations in the symbiotic transcriptome and metabolome. *Plant Physiol.* 145, 1600–1618. doi: 10.1104/pp.107.106955

Baier, M. C., Keck, M., Godde, V., Niehaus, K., Küster, H., and Hohnjec, N. (2010). Knockdown of the symbiotic sucrose synthase *MtSucS1* affects arbuscule maturation and maintenance in mycorrhizal roots of *Medicago truncatula*. *Plant Physiol.* 152, 1000–1014. doi: 10.1104/pp.109.149898

Balestrini, R., and Bonfante, P. (2005). The interface compartment in arbuscular mycorrhizae: a special type of plant cell wall? *Plant Biosyst.* 139, 8–15. doi: 10.1080/11263500500056799

Balestrini, R., Cosgrove, D. J., and Bonfante, P. (2005). Differential location of alpha-expansin proteins during the accommodation of root cells to an arbuscular mycorrhizal fungus. *Planta* 220, 889–899. doi: 10.1007/s00425-004-1431-2

Bapaume, L., and Reinhardt, D. (2012). How membranes shape symbioses: signaling and transport in nodulation and arbuscular mycorrhiza. *Front. Plant Sci.* 3:223. doi: 10.3389/fpls.2012.00223

Becker, A., Fraysse, N., and Sharypova, L. (2005). Recent advances in studies on structure and symbiosis-related function of rhizobial K-antigens and lipopolysaccharides. *Mol. Plant Microbe Interact.* 18, 899–905. doi: 10.1094/MPMI-18-0899

Besserer, A., Puech-Pagès, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., et al. (2006). Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. *PLoS Biol.* 4:e226. doi: 10.1371/journal.pbio.0040226

Blee, K. A., and Anderson, A. J. (2000). "Defense responses in plants to arbuscular mycorrhizal fungi," in *Current Advances in Mycorrhizae Research*, eds G. K. Podila and D. D. Douds (St. Paul: APS Press), 27–44.

Bohlool, B. B., and Schmidt, E. L. (1974). Lectins – possible basis for specificity in *Rhizobium*-legume root nodule symbiosis. *Science* 185, 269–271. doi: 10.1126/science.185.4147.269

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Breuillin, F., Schramm, J., Hajirezaei, M., Ahkami, A., Favre, P., Druege, U., et al. (2010). Phosphate systemically inhibits development of arbuscular mycorrhiza in *Petunia hybrida* and represses genes involved in mycorrhizal functioning. *Plant J.* 64, 1002–1017. doi: 10.1111/j.1365-313X.2010.04385.x

Brewin, N. J. (2004). Plant cell wall remodelling in the *Rhizobium*-legume symbiosis. *Crit. Rev. Plant Sci.* 23, 293–316. doi: 10.1080/07352680490480734

Brundrett, M. C. (2002). Coevolution of roots and mycorrhizas of land plants. *New Phytol.* 154, 275–304. doi: 10.1046/j.1469-8137.2002.00397.x

Buée, M., Rossignol, M., Jauneau, A., Ranjeva, R., and Bécard, G. (2000). The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. *Mol. Plant Microbe Interact.* 13, 693–698. doi: 10.1094/MPMI.2000.13.6.693

Caffall, K. H., and Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr. Res.* 344, 1879–1900. doi: 10.1016/j.carres.2009.05.021

Caspary, R. (1865). Bemerkungen über die Schutzscheide und die Bildung des Stammes und der Wurzel. *Jahrb. Wiss. Bot.* 4, 101–124.

Chaffey, N. (2013). "Secondary growth of tree roots," in *Plant Roots – The Hidden Half*, Chap. 8, eds A. Eshel and T. Beeckman (Boca Raton: CRC Press), 1–24.

Cosgrove, D. J. (2000a). Expansive growth of plant cell walls. *Plant Physiol. Biochem.* 38, 109–124. doi: 10.1016/S0981-9428(00)00164-9

Cosgrove, D. J. (2000b). Loosening of plant cell walls by expansins. *Nature* 407, 321–326. doi: 10.1038/35030000

De Hoff, P. L., Brill, L. M., and Hirsch, A. M. (2009). Plant lectins: the ties that bind in root symbiosis and plant defense. *Mol. Genet. Genomics* 282, 1–15. doi: 10.1007/s00438-009-0460-8

Deising, H. B., Werner, S., and Wernitz, M. (2000). The role of fungal appressoria in plant infection. *Microbes Infect.* 2, 1631–1641. doi: 10.1016/S1286-4579(00)01319-8

Demchenko, K., Winzer, T., Stougaard, J., Parniske, M., and Pawlowski, K. (2004). Distinct roles of *Lotus japonicus SYMRK* and *SYM15* in root colonization and arbuscule formation. *New Phytol.* 163, 381–392. doi: 10.1111/j.1469-8137.2004.01123.x

Dermatsev, V., Weingarten-Baror, C., Resnick, N., Gadkar, V., Wininger, S., Kolotilin, I., et al. (2010). Microarray analysis and functional tests suggest the involvement of expansins in the early stages of symbiosis of the arbuscular mycorrhizal fungus *Glomus intraradices* on tomato (*Solanum lycopersicum*). *Mol. Plant Pathol.* 11, 121–135. doi: 10.1111/j.1364-3703.2009.00581.x

Deslandes, L., and Rivas, S. (2012). Catch me if you can: bacterial effectors and plant targets. *Trends Plant Sci.* 17, 644–655. doi: 10.1016/j.tplants.2012.06.011

Diaz, C. L., Logman, T. J. J., Stam, H. C., and Kijne, J. W. (1995). Sugar-binding activity of pea lectin expressed in white clover hairy roots. *Plant Physiol.* 109, 1167–1177.

Diaz, C. L., Melchers, L. S., Hooykaas, P. J. J., Lugtenberg, B. J. J., and Kijne, J. W. (1989). Root lectin as a determinant of host plant specificity in the *Rhizobium*-legume symbiosis. *Nature* 338, 579–581. doi: 10.1038/338579a0

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., et al. (1993). Cellular organization of the *Arabidopsis thaliana* root. *Development* 119, 71–84.

Douds, D. D., Pfeffer, P. E., and Shachar-Hill, Y. (2000). "Carbon partitioning, cost, and metabolism of arbuscular mycorrhizas," in *Arbuscular Mycorrhizas: Physiology and Function*, eds Y. Kapulnik and D. D. Douds (Dordrecht: Kluwer Academic Publishers).

Elfstrand, M., Feddermann, N., Ineichen, K., Nagaraj, V. J., Wiemken, A., Boller, T., et al. (2005). Ectopic expression of the mycorrhiza-specific chitinase gene *Mtchit 3–3* in *Medicago truncatula* root-organ cultures stimulates spore germination of glomalean fungi. *New Phytol.* 167, 557–570. doi: 10.1111/j.1469-8137.2005.01397.x

Enstone, D. E., Peterson, C. A., and Ma, F. S. (2002). Root endodermis and exodermis: structure, function, and responses to the environment. *J. Plant Growth Regul.* 21, 335–351. doi: 10.1007/s00344-003-0002-2

Feddermann, N., Duvvuru Muni, R. R., Zeier, T., Stuurman, J., Ercolin, F., Schorderet, M., et al. (2010). The *PAM1* gene of petunia, required for intracellular accommodation and morphogenesis of arbuscular mycorrhizal fungi, encodes a homologue of VAPYRIN. *Plant J.* 64, 470–481. doi: 10.1111/j.1365-313X.2010.04341.x

Fournier, J., Timmers, A. C. J., Sieberer, B. J., Jauneau, A., Chabaud, M., and Barker, D. G. (2008). Mechanism of infection thread elongation in root hairs of *Medicago truncatula* and dynamic interplay with associated rhizobial colonization. *Plant Physiol.* 148, 1985–1995. doi: 10.1104/pp.108.125674

Frenzel, A., Manthey, K., Perlick, A. M., Meyer, F., Puhler, A., Küster, H., et al. (2005). Combined transcriptome profiling reveals a novel family of arbuscular mycorrhizal-specific *Medicago truncatula* lectin genes. *Mol. Plant Microbe Interact.* 18, 771–782. doi: 10.1094/MPMI-18-0771

Gage, D. J., and Margolin, W. (2000). Hanging by a thread: invasion of legume plants by rhizobia. *Curr. Opin. Microbiol.* 3, 613–617. doi: 10.1016/S1369-5274(00)00149-1

Geldner, N. (2013a). Casparian strips. *Curr. Biol.* 23, R1025–R1026. doi: 10.1016/j.cub.2013.08.052

Geldner, N. (2013b). The endodermis. *Annu. Rev. Plant Biol.* 64, 531–558. doi: 10.1146/annurev-arplant-050312-120050

Genre, A., Chabaud, M., Balzergue, C., Puech-Pagès, V., Novero, M., Rey, T., et al. (2013). Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. *New Phytol.* 198, 179–189. doi: 10.1111/nph.12146

Genre, A., Chabaud, M., Faccio, A., Barker, D. G., and Bonfante, P. (2008). Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both *Medicago truncatula* and *Daucus carota*. *Plant Cell* 20, 1407–1420. doi: 10.1105/tpc.108.059014

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., and Barker, D. G. (2005). Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. *Plant Cell* 17, 3489–3499. doi: 10.1105/tpc.105.035410

Gerats, T., and Vandenbussche, M. (2005). A model system for comparative research: *Petunia*. *Trends Plant Sci.* 10, 251–256. doi: 10.1016/j.tplants.2005.03.005

Gianinazzi-Pearson, V. (1996). Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. *Plant Cell* 8, 1871–1883. doi: 10.1105/tpc.8.10.1871

Gianinazzi-Pearson, V., Arnould, C., Oufattole, M., Arango, M., and Gianinazzi, S. (2000). Differential activation of H^+-ATPase genes by an arbuscular mycorrhizal fungus in root cells of transgenic tobacco. *Planta* 211, 609–613. doi: 10.1007/s004250000323

Gianinazzi-Pearson, V., Branzanti, B., and Gianinazzi, S. (1989). *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. *Symbiosis* 7, 243–255.

Gianinazzi-Pearson, V., Dumas-Gaudot, E., Gollotte, A., Tahiri-Alaoui, A., and Gianinazzi, S. (1996). Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. *New Phytol.* 133, 45–57. doi: 10.1111/j.1469-8137.1996.tb04340.x

Gianinazzi-Pearson, V., and Smith, F. A. (2003). Mycorrhiza 2002 – a productive year. *Mycorrhiza* 13, 1. doi: 10.1007/s00572-003-0230-0

Giordano, W., and Hirsch, A. M. (2004). The expression of *MaEXP1*, a *Melilotus alba* expansin gene, is upregulated during the sweetclover-*Sinorhizobium meliloti* interaction. *Mol. Plant Microbe Interact.* 17, 613–622. doi: 10.1094/MPMI.2004.17.6.613

Giovannetti, M., Avio, L., Sbrana, C., and Citernesi, A. S. (1993). Factors affecting appressorium development in the vesicular arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. *New Phytol.* 123, 115–122. doi: 10.1111/j.1469-8137.1993.tb04537.x

Giovannetti, M., Sbrana, C., Citernesi, A. S., and Avio, L. (1996). Analysis of factors involved in fungal recognition responses to host derived signals by arbuscular mycorrhizal fungi. *New Phytol.* 133, 65–71. doi: 10.1111/j.1469-8137.1996.tb04342.x

Giovannetti, M., Sbrana, C., and Logi, C. (1994). Early processes involved in host recognition by arbuscular mycorrhizal fungi. *New Phytol.* 127, 703–709. doi: 10.1111/j.1469-8137.1994.tb02973.x

Gobbato, E., Marsh, J. F., Vernie, T., Wang, E., Maillet, F., Kim, J., et al. (2012). A GRAS-type transcription factor with a specific function in mycorrhizal signaling. *Curr. Biol.* 22, 2236–2241. doi: 10.1016/j.cub.2012.09.044

Gollotte, A., Gianinazzi-Pearson, V., and Gianinazzi, S. (1994). Immunocytochemical study of plant-arbuscular mycorrhizal fungus interfaces in isogenic peas myc^+ or mycorrhiza resistant (myc^-). *Acta Bot. Gall.* 141, 449–454. doi: 10.1080/12538078.1994.10515182

Gollotte, A., Gianinazzi-Pearson, V., Giovannetti, M., Sbrana, C., Avio, L., and Gianinazzi, S. (1993). Cellular localization and cytochemical probing of resistance reactions to arbuscular mycorrhizal fungi in a locus a myc-mutant of *Pisum sativum* L. *Planta* 191, 112–122. doi: 10.1007/BF00240902

Gomez-Roldan, V., Fermas, S., Brewer, P. B., Puech-Pagès, V., Dun, E. A., Pillot, J. P., et al. (2008). Strigolactone inhibition of shoot branching. *Nature* 455, 189–194. doi: 10.1038/nature07271

Goormachtig, S., Lievens, S., Van de Velde, W., Van Montagu, M., and Holsters, M. (1998). Srchi13, a novel early nodulin from *Sesbania rostrata*, is related to acidic class III chitinases. *Plant Cell* 10, 905–915. doi: 10.1105/tpc.10.6.905

Grunwald, U., Guo, W. B., Fischer, K., Isayenkov, S., Ludwig-Müller, J., Hause, B., et al. (2009). Overlapping expression patterns and differential transcript levels of phosphate transporter genes in arbuscular mycorrhizal, P_i-fertilised and phytohormone-treated *Medicgo truncatula* roots. *Planta* 229, 1023–1034. doi: 10.1007/s00425-008-0877-z

Guether, M., Balestrini, R., Hannah, M., He, J., Udvardi, M., and Bonfante, P. (2009a). Genome-wide reprogramming of regulatory networks, cell wall and membrane biogenesis during arbuscular-mycorrhizal symbiosis in *Lotus japonicus*. *New Phytol.* 182, 200–212. doi: 10.1111/j.1469-8137.2008.02725.x

Guether, M., Neuhauser, B., Balestrini, R., Dynowski, M., Ludewig, U., and Bonfante, P. (2009b). A mycorrhizal-specific ammonium transporter from *Lotus japonicus* acquires nitrogen released by arbuscular mycorrhizal fungi. *Plant Physiol.* 150, 73–83. doi: 10.1104/pp.109.136390

Güimil, S., Chang, H. S., Zhu, T., Sesma, A., Osbourn, A., Roux, C., et al. (2005). Comparative transcriptomics of rice reveals an ancient pattern of response to

microbial colonization. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8066–8070. doi: 10.1073/pnas.0502999102

Gutjahr, C., and Parniske, M. (2013). Cell and developmental biology of arbuscular mycorrhiza symbiosis. *Annu. Rev. Cell Dev. Biol.* 29, 593–617. doi: 10.1146/annurev-cellbio-101512-122413

Gutjahr, C., and Paszkowski, U. (2013). Multiple control levels of root system remodeling in arbuscular mycorrhizal symbiosis. *Front. Plant Sci.* 4:204. doi: 10.3389/fpls.2013.00204

Gutjahr, C., Radovanovic, D., Geoffroy, J., Zhang, Q., Siegler, H., Chiapello, M., et al. (2012). The half-size ABC transporters STR1 and STR2 are indispensable for mycorrhizal arbuscule formation in rice. *Plant J.* 69, 906–920. doi: 10.1111/j.1365-313X.2011.04842.x

Guttenberger, M. (2000). Arbuscules of vesicular-arbuscular mycorrhizal fungi inhabit an acidic compartment within plant roots. *Planta* 211, 299–304. doi: 10.1007/s004250000324

Harrison, M. J., Dewbre, G. R., and Liu, J. Y. (2002). A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. *Plant Cell* 14, 2413–2429. doi: 10.1105/tpc.004861

Helber, N., and Requena, N. (2008). Expression of the fluorescence markers DsRed and GFP fused to a nuclear localization signal in the arbuscular mycorrhizal fungus *Glomus intraradices*. *New Phytol.* 177, 537–548. doi: 10.1111/j.1469-8137.2007.02257.x

Helber, N., Wippel, K., Sauer, N., Schaarschmidt, S., Hause, B., and Requena, N. (2011). A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus *Glomus* sp is crucial for the symbiotic relationship with plants. *Plant Cell* 23, 3812–3823. doi: 10.1105/tpc.111.089813

Hohnjec, N., Vieweg, M. E., Puhler, A., Becker, A., and Küster, H. (2005). Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different *Glomus* fungi provide insights into the genetic program activated during arbuscular mycorrhiza. *Plant Physiol.* 137, 1283–1301. doi: 10.1104/pp.104.056572

Ivanov, S., Fedorova, E. E., Limpens, E., De Mita, S., Genre, A., Bonfante, P., et al. (2012). *Rhizobium*-legume symbiosis shares an exocytotic pathway required for arbuscule formation. *Proc. Natl. Acad. Sci. U.S.A.* 109, 8316–8321. doi: 10.1073/pnas.1200407109

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Journet, E. P., El-Gachtouli, N., Vernoud, V., de Billy, F., Pichon, M., Dedieu, A., et al. (2001). *Medicago truncatula* ENOD11: a novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. *Mol. Plant Microbe Interact.* 14, 737–748. doi: 10.1094/MPMI.2001.14.6.737

Kardailsky, I. V., Sherrier, D. J., and Brewin, N. J. (1996). Identification of a new pea gene, *PsNlec1*, encoding a lectin-like glycoprotein isolated from the symbiosomes of root nodules. *Plant Physiol.* 111, 49–60. doi: 10.1104/pp.111.1.49

Kereszt, A., Mergaert, P., and Kondorosi, E. (2011). Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims? *Mol. Plant Microbe Interact.* 24, 1300–1309. doi: 10.1094/MPMI-06-11-0152

Ketelaar, T. (2013). The actin cytoskeleton in root hairs: all is fine at the tip. *Curr. Opin. Plant Biol.* 16, 749–756. doi: 10.1016/j.pbi.2013.10.003

Kim, H. K., Park, J., and Hwang, I. (2014). Investigating water transport through the xylem network in vascular plants. *J. Exp. Bot.* 65, 1895–1904. doi: 10.1093/jxb/eru075

Kloppholz, S., Kuhn, H., and Requena, N. (2011). A secreted fungal effector of *Glomus intraradices* promotes symbiotic biotrophy. *Curr. Biol.* 21, 1204–1209. doi: 10.1016/j.cub.2011.06.044

Kobae, Y., Tamura, Y., Takai, S., Banba, M., and Hata, S. (2010). Localized expression of arbuscular mycorrhiza-inducible ammonium transporters in soybean. *Plant Cell Physiol.* 51, 1411–1415. doi: 10.1093/pcp/pcq099

Koegel, S., Lahmidi, N. A., Arnould, C., Chatagnier, O., Walder, F., Ineichen, K., et al. (2013). The family of ammonium transporters (AMT) in *Sorghum bicolor*: two AMT members are induced locally, but not systemically in roots colonized by arbuscular mycorrhizal fungi. *New Phytol.* 198, 853–865. doi: 10.1111/nph.12199

Kretzschmar, T., Kohlen, W., Sasse, J., Borghi, L., Schlegel, M., Bachelier, J. B., et al. (2012). A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. *Nature* 483, 341–346. doi: 10.1038/nature10873

Lambais, M. R. (2000). "Regulation of plant defense-related genes in arbuscular mycorrhizae," in *Current Advances in Mycorrhizae Research*, eds G. K. Podila and D. D. Douds (St. Paul: APS Press), 45–60.

Lee, Y., Rubio, M. C., Alassimone, J., and Geldner, N. (2013). A mechanism for localized lignin deposition in the endodermis. *Cell* 153, 402–412. doi: 10.1016/j.cell.2013.02.045

Liang, Y., Cao, Y. R., Tanaka, K., Thibivilliers, S., Wan, J. R., Choi, J., et al. (2013). Nonlegumes respond to rhizobial Nod factors by suppressing the innate immune response. *Science* 341, 1384–1387. doi: 10.1126/science.1242736

Liu, J. Y., Blaylock, L. A., Endre, G., Cho, J., Town, C. D., VandenBosch, K. A., et al. (2003). Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. *Plant Cell* 15, 2106–2123. doi: 10.1105/tpc.014183

Lopez-Gomez, M., Sandal, N., Stougaard, J., and Boller, T. (2012). Interplay of flg22-induced defence responses and nodulation in *Lotus japonicus*. *J. Exp. Bot.* 63, 393–401. doi: 10.1093/jxb/err291

Lucas, W. J., Ham, L. K., and Kim, J. Y. (2009). Plasmodesmata – bridging the gap between neighboring plant cells. *Trends Cell Biol.* 19, 495–503. doi: 10.1016/j.tcb.2009.07.003

Ma, J. F., Yamaji, N., Mitani, N., Tamai, K., Konishi, S., Fujiwara, T., et al. (2007). An efflux transporter of silicon in rice. *Nature* 448, 209–212. doi: 10.1038/nature05964

Maillet, F., Poinsot, V., André, O., Puech-Pagès, V., Haouy, A., Gueunier, M., et al. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469, 58–64. doi: 10.1038/nature09622

Manthey, K., Krajinski, F., Hohnjec, N., Firnhaber, C., Puhler, A., Perlick, A. M., et al. (2004). Transcriptome profiling in root nodules and arbuscular mycorrhiza identifies a collection of novel genes induced during *Medicago truncatula* root endosymbioses. *Mol. Plant Microbe Interact.* 17, 1063–1077. doi: 10.1094/MPMI.2004.17.10.1063

Margaret, I., Lucas, M. M., Acosta-Jurado, S., Buendia-Claveria, A. M., Fedorova, E., Hidalgo, A., et al. (2013). The *Sinorhizobium fredii* HH103 lipopolysaccharide is not only relevant at early soybean nodulation stages but also for symbiosome stability in mature nodules. *PLoS ONE* 8:e74717. doi: 10.1371/journal.pone.0074717

Martin, F., Aerts, A., Ahren, D., Brun, A., Danchin, E. G. J., Duchaussoy, F., et al. (2008). The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature* 452, 88–92. doi: 10.1038/nature06556

Martin, F., and Selosse, M. A. (2008). The *Laccaria* genome: a symbiont blueprint decoded. *New Phytol.* 180, 296–310. doi: 10.1111/j.1469-8137.2008.02613.x

Marx, C., Dexheimer, J., Gianinazzi-Pearson, V., and Gianinazzi, S. (1982). Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhizas. 4. Ultracytoenzymological evidence (ATPase) for active transfer processes in the host-arbuscule interface. *New Phytol.* 90, 37–43. doi: 10.1111/j.1469-8137.1982.tb03238.x

Mathis, R., Van Gijsegem, F., De Rycke, R., D'Haeze, W., Van Maelsaeke, E., Anthonio, E., et al. (2005). Lipopolysaccharides as a communication signal for progression of legume endosymbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2655–2660. doi: 10.1073/pnas.0409816102

McFarlane, H., Döring, A., and Persson, S. (2014). The cell biology of cellulose synthesis. *Annu. Rev. Plant Biol.* 65, 69–94. doi: 10.1146/annurev-arplant-050213-040240

Meckfessel, M. H., Blancaflor, E. B., Plunkett, M., Dong, Q. F., and Dickstein, R. (2012). Multiple domains in MtENOD8 protein including the signal peptide target it to the symbiosome. *Plant Physiol.* 159, 299–310. doi: 10.1104/pp.111.191403

Meyer, C. J., and Peterson, C. A. (2013). "Structure and function of three suberized cell layers: epidermis, exodermis, and endodermis," in *Plant Roots – The Hidden Half*, Chap. 5, eds A. Eshel and T. Beeckman (Boca Raton, FL: CRC Press), 1–20.

Nagahashi, G., and Douds, D. D. (1997). Appressorium formation by AM fungi on isolated cell walls of carrot roots. *New Phytol.* 136, 299–304. doi: 10.1046/j.1469-8137.1997.00739.x

Nagahashi, G., and Douds, D. D. (2011). The effects of hydroxy fatty acids on the hyphal branching of germinated spores of AM fungi. *Fungal Biol.* 115, 351–358. doi: 10.1016/j.funbio.2011.01.006

Nagendran, S., Hallen-Adams, H. E., Paper, J. M., Aslam, N., and Walton, J. D. (2009). Reduced genomic potential for secreted plant cell-wall-degrading enzymes in the ectomycorrhizal fungus *Amanita bisporigera*, based on the secretome of

Trichoderma reesei. *Fungal Genet. Biol.* 46, 427–435. doi: 10.1016/j.fgb.2009.02.001

Neumann, G., and Martinoia, E. (2002). Cluster roots – an underground adaptation for survival in extreme environments. *Trends Plant Sci.* 7, 162–167. doi: 10.1016/S1360-1385(02)02241-0

Niehaus, K., and Becker, A. (1998). The role of microbial surface polysaccharides in the *Rhizobium*–legume interaction. *Subcell. Biochem.* 29, 73–116. doi: 10.1007/978-1-4899-1707-2_3

Nouri, E., Breuillin-Sessoms, F., Feller, U., and Reinhardt, D. (2014). Phosphorus and nitrogen regulate arbuscular mycorrhizal symbiosis in *Petunia hybrida*. *PLoS ONE* 9:e90841. doi: 10.1371/journal.pone.0090841

Novero, M., Faccio, A., Genre, A., Stougaard, J., Webb, K. J., Mulder, L., et al. (2002). Dual requirement of the *LjSym4* gene for mycorrhizal development in epidermal and cortical cells of *Lotus japonicus* roots. *New Phytol.* 154, 741–749. doi: 10.1046/j.1469-8137.2002.00424.x

Okazaki, S., Kaneko, T., Sato, S., and Saeki, K. (2013). Hijacking of leguminous nodulation signaling by the rhizobial type III secretion system. *Proc. Natl. Acad. Sci. U.S.A.* 110, 17131–17136. doi: 10.1073/pnas.1302360110

Olah, B., Briere, C., Bécard, G., Dénarié, J., and Gough, C. (2005). Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the DMI1/DMI2 signalling pathway. *Plant J.* 44, 195–207. doi: 10.1111/j.1365-313X.2005.02522.x

Oldroyd, G. E. D. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nat. Rev. Microbiol.* 11, 252–263. doi: 10.1038/nrmicro2990

Oldroyd, G. E. D., Murray, J. D., Poole, P. S., and Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet.* 45, 119–144. doi: 10.1146/annurev-genet-110410-132549

Panstruga, R. (2003). Establishing compatibility between plants and obligate biotrophic pathogens. *Curr. Opin. Plant Biol.* 6, 320–326. doi: 10.1016/S1369-5266(03)00043-8

Parniske, M. (2000). Intracellular accommodation of microbes by plants: a common developmental program for symbiosis and disease? *Curr. Opin. Plant Biol.* 3, 320–328. doi: 10.1016/S1369-5266(00)00088-1

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nat. Rev. Microbiol.* 6, 763–775. doi: 10.1038/nrmicro1987

Petricka, J. J., Winter, C. M., and Benfey, P. N. (2012). Control of *Arabidopsis* root development. *Annu. Rev. Plant Biol.* 63, 563–590. doi: 10.1146/annurev-arplant-042811-105501

Pfeffer, P. E., Douds, D. D., Becard, G., and Shachar-Hill, Y. (1999). Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. *Plant Physiol.* 120, 587–598. doi: 10.1104/pp.120.2.587

Pierre, O., Engler, G., Hopkins, J., Brau, F., Boncompagni, E., and Hérouart, D. (2013). Peribacteroid space acidification: a marker of mature bacteroid functioning in *Medicago truncatula* nodules. *Plant Cell Environ.* 36, 2059–2070. doi: 10.1111/pce.12116

Plett, J. M., Kemppainen, M., Kale, S. D., Kohler, A., Legue, V., Brun, A., et al. (2011). A secreted effector protein of *Laccaria bicolor* is required for symbiosis development. *Curr. Biol.* 21, 1197–1203. doi: 10.1016/j.cub.2011.05.033

Pollard, M., Beisson, F., Li, Y. H., and Ohlrogge, J. B. (2008). Building lipid barriers: biosynthesis of cutin and suberin. *Trends Plant Sci.* 13, 236–246. doi: 10.1016/j.tplants.2008.03.003

Pozo, M. J., and Azcon-Aguilar, C. (2007). Unraveling mycorrhiza-induced resistance. *Curr. Opin. Plant Biol.* 10, 393–398. doi: 10.1016/j.pbi.2007.05.004

Pumplin, N., and Harrison, M. J. (2009). Live-cell imaging reveals periarbuscular membrane domains and organelle location in *Medicago truncatula* roots during arbuscular mycorrhizal symbiosis. *Plant Physiol.* 151, 809–819. doi: 10.1104/pp.109.141879

Pumplin, N., Zhang, X., Noar, R. D., and Harrison, M. J. (2012). Polar localization of a symbiosis-specific phosphate transporter is mediated by a transient reorientation of secretion. *Proc. Natl. Acad. Sci. U.S.A.* 109, E665–E672. doi: 10.1073/pnas.1110215109

Rausch, C., Daram, P., Brunner, S., Jansa, J., Laloi, M., Leggewie, G., et al. (2001). A phosphate transporter expressed in arbuscule-containing cells in potato. *Nature* 414, 462–466. doi: 10.1038/35106601

Rival, P., de Billy, F., Bono, J. J., Gough, C., Rosenberg, C., and Bensmihen, S. (2012). Epidermal and cortical roles of NFP and DMI3 in coordinating early steps of nodulation in *Medicago truncatula*. *Development* 139, 3383–3391. doi: 10.1242/dev.081620

Robledo, M., Jimenez-Zurdo, J. I., Velazquez, E., Trujillo, M. E., Zurdo-Pineiro, J. L., Ramirez-Bahena, M. H., et al. (2008). *Rhizobium* cellulase CelC2 is essential for primary symbiotic infection of legume host roots. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7064–7069. doi: 10.1073/pnas.0802547105

Roppolo, D., De Rybel, B., Tendon, V. D., Pfister, A., Alassimone, J., Vermeer, J. E. M., et al. (2011). A novel protein family mediates Casparian strip formation in the endodermis. *Nature* 473, 380–383. doi: 10.1038/nature10070

Saalbach, G., Erik, P., and Wienkoop, S. (2002). Characterisation by proteomics of peribacteroid space and peribacteroid membrane preparations from pea (*Pisum sativum*) symbiosomes. *Proteomics* 2, 325–337. doi: 10.1002/1615-9861(200203)2:3<325::AID-PROT325>3.0.CO;2-W

Salzer, P., and Boller, T. (2000). "Elicitor-induced reactions in mycorrhizae and their suppression," in *Current Advances in Mycorrhizae Research*, eds G. K. Podila and D. D. Douds (St. Paul: APS Press), 1–10.

Salzer, P., Bonanomi, A., Beyer, K., Vögeli-Lange, R., Aeschbacher, R. A., Lange, J., et al. (2000). Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. *Mol. Plant Microbe Interact.* 13, 763–777. doi: 10.1094/MPMI.2000.13.7.763

Sbrana, C., and Giovannetti, M. (2005). Chemotropism in the arbuscular mycorrhizal fungus *Glomus mosseae*. *Mycorrhiza* 15, 539–545. doi: 10.1007/s00572-005-0362-5

Schaarschmidt, S., Gonzalez, M. C., Roitsch, T., Strack, D., Sonnewald, U., and Hause, B. (2007). Regulation of arbuscular mycorrhization by carbon. The symbiotic interaction cannot be improved by increased carbon availability accomplished by root-specifically enhanced invertase activity. *Plant Physiol.* 143, 1827–1840. doi: 10.1104/pp.107.096446

Schaarschmidt, S., Roitsch, T., and Hause, B. (2006). Arbuscular mycorrhiza induces gene expression of the apoplastic invertase *LIN6* in tomato (*Lycopersicon esculentum*) roots. *J. Exp. Bot.* 57, 4015–4023. doi: 10.1093/jxb/erl172

Scheller, H. V., and Ulvskov, P. (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289. doi: 10.1146/annurev-arplant-042809-112315

Schreiber, L. (2010). Transport barriers made of cutin, suberin and associated waxes. *Trends Plant Sci.* 15, 546–553. doi: 10.1016/j.tplants.2010.06.004

Sharda, J. N., and Koide, R. T. (2008). Can hypodermal passage cell distribution limit root penetration by mycorrhizal fungi? *New Phytol.* 180, 696–701. doi: 10.1111/j.1469-8137.2008.02600.x

Shaul, O., David, R., Sinvani, G., Ginzberg, I., Ganon, D., Wininger, S., et al. (2000). "Plant defense responses during arbuscular mycorrhizal symbiosis," in *Current Advances in Mycorrhizae Research*, eds G. K. Podila and D. D. Douds (St. Paul: APS Press), 61–68.

Shishkoff, N. (1987). Distribution of the dimorphic hypodermis of roots in angiosperm families. *Ann. Bot.* 60, 1–15.

Siciliano, V., Genre, A., Balestrini, R., Cappellazzo, G., deWit, P., and Bonfante, P. (2007). Transcriptome analysis of arbuscular mycorrhizal roots during development of the prepenetration apparatus. *Plant Physiol.* 144, 1455–1466. doi: 10.1104/pp.107.097980

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis.* New York: Academic Press.

Solaiman, M. D. Z., and Saito, M. (1997). Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. *New Phytol.* 136, 533–538. doi: 10.1046/j.1469-8137.1997.00757.x

Spanu, P. D., Abbott, J. C., Amselem, J., Burgis, T. A., Soanes, D. M., Stuber, K., et al. (2010). Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* 330, 1543–1546. doi: 10.1126/science.1194573

Stergiopoulos, I., and de Wit, P. J. G. M. (2009). Fungal effector proteins, *Annu. Rev. Phytopathol.* 47, 233–263. doi: 10.1146/annurev.phyto.112408.132637

Takano, J., Tanaka, M., Toyoda, A., Miwa, K., Kasai, K., Fuji, K., et al. (2010). Polar localization and degradation of *Arabidopsis* boron transporters through distinct trafficking pathways. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5220–5225. doi: 10.1073/pnas.0910744107

Takeda, N., Kistner, C., Kosuta, S., Winzer, T., Pitzschke, A., Groth, M., et al. (2007). Proteases in plant root symbiosis. *Phytochemistry* 68, 111–121. doi: 10.1016/j.phytochem.2006.09.022

Takeda, N., Sato, S., Asamizu, E., Tabata, S., and Parniske, M. (2009). Apoplastic plant subtilases support arbuscular mycorrhiza development in *Lotus japonicus*. *Plant J.* 58, 766–777. doi: 10.1111/j.1365-313X.2009.03824.x

Tian, Y., Liu, W., Cai, J., Zhang, L. Y., Wong, K. B., Feddermann, N., et al. (2013). The nodulation factor hydrolase of *Medicago truncatula*: characterization of an enzyme specifically cleaving rhizobial nodulation signals. *Plant Physiol.* 163, 1179–1190. doi: 10.1104/pp.113.223966

Tisserant, E., Kohler, A., Dozolme-Seddas, P., Balestrini, R., Benabdellah, K., Colard, A., et al. (2012). The transcriptome of the arbuscular mycorrhizal fungus *Glomus intraradices* (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. *New Phytol.* 193, 755–769. doi: 10.1111/j.1469-8137.2011.03948.x

Tisserant, E., Malbreil, M., Kuo, A., Kohler, A., Symeonidi, A., Balestrini, R., et al. (2013). Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 110, 20117–20122. doi: 10.1073/pnas.1313452110

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., et al. (2008). Inhibition of shoot branching by new terpenoid plant hormones. *Nature* 455, 195–200. doi: 10.1038/nature07272

Valot, B., Dieu, M., Recorbet, G., Raes, M., Gianinazzi, S., and Dumas-Gaudot, E. (2005). Identification of membrane-associated proteins regulated by the arbuscular mycorrhizal symbiosis. *Plant Mol. Biol.* 59, 565–580. doi: 10.1007/s11103-005-8269-2

van Brussel, A. A. N., Bakhuizen, R., Vanspronsen, P. C., Spaink, H. P., Tak, T., Lugtenberg, B. J. J., et al. (1992). Induction of preinfection thread structures in the leguminous host plant by mitogenic lipooligosaccharides of *Rhizobium*. *Science* 257, 70–72. doi: 10.1126/science.257.5066.70

van Buuren, M. L., Maldonado-Mendoza, I. E., Trieu, A. T., Blaylock, L. A., and Harrison, M. J. (1999). Novel genes induced during an arbuscular mycorrhizal (AM) symbiosis formed between *Medicago truncatula* and *Glomus versiforme*. *Mol. Plant Microbe Interact.* 12, 171–181. doi: 10.1094/MPMI.1999.12.3.171

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., et al. (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* 327, 1122–1126. doi: 10.1126/science.1184057

van Loon, L. C., Rep, M., and Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162. doi: 10.1146/annurev.phyto.44.070505.143425

van Rhijn, P., Goldberg, R. B., and Hirsch, A. M. (1998). *Lotus* corniculatus nodulation specificity is changed by the presence of a soybean lectin gene. *Plant Cell* 10, 1233–1249. doi: 10.1105/tpc.10.8.1233

van Spronsen, P. C., Bakhuizen, R., van Brussel, A. A., and Kijne, J. W. (1994). Cell wall degradation during infection thread formation by the root nodule bacterium *Rhizobium leguminosarum* is a two-step process. *Eur. J. Cell Biol.* 64, 88–94.

Veiga, R. S. L., Faccio, A., Genre, A., Pieterse, C. M. J., Bonfante, P., and van der Heijden, M. G. A. (2013). Arbuscular mycorrhizal fungi reduce growth and infect roots of the non-host plant *Arabidopsis thaliana*. *Plant Cell Environ.* 36, 1926–1937. doi: 10.1111/pce.12102

Vierheilig, H., Alt, M., Neuhaus, J. M., Boller, T., and Wiemken, A. (1993). Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. *Mol. Plant Microbe Interact.* 6, 261–264. doi: 10.1094/MPMI-6-261

Wang, D., Yang, S. M., Tang, F., and Zhu, H. Y. (2012a). Symbiosis specificity in the legume – rhizobial mutualism. *Cell. Microbiol.* 14, 334–342. doi: 10.1111/j.1462-5822.2011.01736.x

Wang, E. T., Schornack, S., Marsh, J. F., Gobbato, E., Schwessinger, B., Eastmond, P., et al. (2012b). A common signaling process that promotes mycorrhizal and oomycete colonization of plants. *Curr. Biol.* 22, 2242–2246. doi: 10.1016/j.cub.2012.09.043

Xie, F., Murray, J. D., Kim, J., Heckmann, A. B., Edwards, A. Oldroyd, G. E. D., et al. (2012). Legume pectate lyase required for root infection by rhizobia. *Proc. Natl. Acad. Sci. U.S.A.* 109, 633–638. doi: 10.1073/pnas.1113992109

Xie, X. N., Yoneyama, K., and Yoneyama, K. (2010). The strigolactone story. *Annu. Rev. Phytopathol.* 48, 93–117. doi: 10.1146/annurev-phyto-073009-114453

Xin, D. W., Liao, S., Xie, Z. P., Hann, D. R., Steinle, L., Boller, T., et al. (2012). Functional analysis of NopM, a novel E3 ubiquitin ligase (NEL) domain effector of *Rhizobium* sp strain NGR234. *PLoS Pathog.* 8:e1002707. doi: 10.1371/journal.ppat.1002707

Yamauchi, T., Shimamura, S., Nakazono, M., and Mochizuki, T. (2013). Aerenchyma formation in crop species: a review. *Field Crops Res.* 152, 8–16. doi: 10.1016/j.fcr.2012.12.008

Yang, S. Y., Gronlund, M., Jakobsen, I., Grotemeyer, M. S., Rentsch, D., Miyao, A., et al. (2012). Nonredundant regulation of rice arbuscular mycorrhizal symbiosis by two members of the *PHOSPHATE TRANSPORTER1* gene family. *Plant Cell* 24, 4236–4251. doi: 10.1105/tpc.112.104901

Yeats, T. H., and Rose, J. K. C. (2013). The formation and function of plant cuticles. *Plant Physiol.* 163, 5–20. doi: 10.1104/pp.113.222737

Yi, M., and Valent, B. (2013). Communication between filamentous pathogens and plants at the biotrophic interface. *Annu. Rev. Phytopathol.* 51, 587–611. doi: 10.1146/annurev-phyto-081211-172916

Yurgel, S. N., and Kahn, M. L. (2004). Dicarboxylate transport by *Rhizobia*. *FEMS Microbiol. Rev.* 28, 489–501. doi: 10.1016/j.femsre.2004.04.002

Zamioudis, C., and Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant Microbe Interact.* 25, 139–150. doi: 10.1094/MPMI-06-11-0179

Zhang, L., Chen, X. J., Lu, H. B., Xie, Z. P., and Staehelin, C. (2011). Functional analysis of the type 3 effector nodulation outer protein L (NopL) from *Rhizobium* sp NGR_{234}. *J. Biol. Chem.* 286, 32178–32187. doi: 10.1074/jbc.M111.265942

Zhang, Q., Blaylock, L. A., and Harrison, M. J. (2010). Two *Medicago truncatula* half-ABC transporters are essential for arbuscule development in arbuscular mycorrhizal symbiosis. *Plant Cell* 22, 1483–1497. doi: 10.1105/tpc.110.074955

Zonia, L., and Munnik, T. (2007). Life under pressure: hydrostatic pressure in cell growth and function. *Trends Plant Sci.* 12, 90–97. doi: 10.1016/j.tplants.2007.01.006

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 March 2014; accepted: 12 May 2014; published online: 02 June 2014.
Citation: Rich MK, Schorderet M and Reinhardt D (2014) The role of the cell wall compartment in mutualistic symbioses of plants. Front. Plant Sci. ***5****:238. doi: 10.3389/fpls.2014.00238*
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 04 June 2014
doi: 10.3389/fpls.2014.00237

Cell wall remodeling in mycorrhizal symbiosis: a way towards biotrophism

***Raffaella Balestrini*[1]* and *Paola Bonfante*[2]**

[1] *Institute for Sustainable Plant Protection, National Research Council, Torino, Italy*
[2] *Department of Life Science and Systems Biology, University of Torino, Torino, Italy*

Edited by:
Jean-Pierre Metraux, Université de Fribourg, Switzerland

Reviewed by:
Maria J. Pozo, Consejo Superior de Investigaciones Científicas, Spain
Clare Gough, Centre National de la Recherche Scientifique–Institut National de la Recherche Agronomique, France

***Correspondence:**
Raffaella Balestrini, Institute for Sustainable Plant Protection, National Research Council, Viale Mattioli 25, Turin 10125, Italy
e-mail: r.balestrini@ipp.cnr.it

Cell walls are deeply involved in the molecular talk between partners during plant and microbe interactions, and their role in mycorrhizae, i.e., the widespread symbiotic associations established between plant roots and soil fungi, has been investigated extensively. All mycorrhizal interactions achieve full symbiotic functionality through the development of an extensive contact surface between the plant and fungal cells, where signals and nutrients are exchanged. The exchange of molecules between the fungal and the plant cytoplasm takes place both through their plasma membranes and their cell walls; a functional compartment, known as the symbiotic interface, is thus defined. Among all the symbiotic interfaces, the complex intracellular interface of arbuscular mycorrhizal (AM) symbiosis has received a great deal of attention since its first description. Here, in fact, the host plasma membrane invaginates and proliferates around all the developing intracellular fungal structures, and cell wall material is laid down between this membrane and the fungal cell surface. By contrast, in ectomycorrhizae (ECM), where the fungus grows outside and between the root cells, plant and fungal cell walls are always in direct contact and form the interface between the two partners. The organization and composition of cell walls within the interface compartment is a topic that has attracted widespread attention, both in ecto- and endomycorrhizae. The aim of this review is to provide a general overview of the current knowledge on this topic by integrating morphological observations, which have illustrated cell wall features during mycorrhizal interactions, with the current data produced by genomic and transcriptomic approaches.

Keywords: cell wall, mycorrhizal interactions, fungal genomes, interface, gene expression

INTRODUCTION

Plant cell walls form a dynamic extracellular matrix that actively controls growth and development, and are essential for the functionality of plants (Keegstra, 2010). Cell walls provide shape to the many different cell types needed to form tissues and organs and, forming the interface between neighboring cells, they control intercellular communication. Therefore, cell walls mediate most plant–microbe interactions. However, cell walls are not exclusive to photosynthetic organisms: fungi also have walls that determine hyphal growth, shape and responses (Durán and Nombela, 2004).

Unlike pathogenic interactions, where the fungal pathogen may be effective even with a limited presence in the plant tissues, in mycorrhizae, i.e., the widespread symbiotic associations established between plant roots and soil fungi (Bonfante and Genre, 2010), fungal colonization may involve as much as 80% of the secondary roots (Smith and Read, 2008). Irrespectively of their typology and their partner's identity, mycorrhizal interactions achieve their functionality through the development of an extensive contact surface between plant and fungal cells, allowing signals and nutrients to be exchanged. In other words, the transfer of molecules from the fungus to the plant cytoplasm and *viceversa* takes place through both partners' plasma membranes and cell walls, defining an apoplastic compartment known as the symbiotic interface on the basis of the first ultra-structural morphological observations (Scannerini and Bonfante-Fasolo, 1983).

In spite of the impressive biodiversity that is hidden behind the word "mycorrhiza" (Smith and Read, 2008), the interface has been considered a useful unifying concept to describe these plant-fungal interactions and to deal with both morphological (Bonfante, 2001; Peterson and Massicotte, 2004; Balestrini and Bonfante, 2005; Genre and Bonfante, 2012), molecular and genetic aspects (Harrison, 1999; Bücking et al., 2007; Reinhardt, 2007; Parniske, 2008; Gutjahr and Parniske, 2013).

The aim of this review is to provide an overview of the current knowledge on the dynamics of plant and fungal walls in mycorrhizae, as well as on their symbiotic interfaces, which – not surprisingly – have attracted a great deal of attention from the scientific community. Attention will mostly be focused on ectomycorrhizae (ECM) and arbuscular-mycorrhizae (AM). In ECMs the fungus covers the root tips, forming a mantle, and grows between the root cells, while in AM symbiosis the fungus develops inter- and intra-cellularly all along the root. Once the cortical layers are reached, fungal hyphae branch, leading to unique structures called arbuscules (Bonfante and Genre, 2010). The structural issues that result from morphological observations, and the biosynthetic aspects that stem from genomic and transcriptomic approaches, will be considered in this review.

THE SYMBIOTIC INTERFACE: HOW TO INCREASE THE PARTNERS' CONTACTS WHILE MAINTAINING BIOTROPHY

Among all the mycorrhizal interfaces, the complex intracellular interface of AM symbiosis has received considerable attention since its first descriptions in the seventies. Following the findings on fungal pathogens (Bracker and Littlefield, 1973; Scannerini and Bonfante, 1976) observed that the AM fungus is always surrounded by a plant-derived membrane, which leads to an interfacial zone consisting of a fungal plasma membrane, a specialized interfacial matrix, and a plant membrane, which was called the periarbuscular membrane (**Figure 1**). At that time, observations were mostly made on the cortical cells that host branched fungal arbuscules. The presence of this interface compartment is a typical feature of all endomycorrhizae (Scannerini and Bonfante-Fasolo, 1983; Peterson and Massicotte, 2004). In orchid, ericoid and arbutoid interactions, the intracellular fungus resulted to be confined within this compartment, that provides the structural basis of biotrophic interactions, since both partners maintain their individuality and remain alive. In the meantime, it causes a huge increase in the contact surface between the two partners, and the plant membrane increases in length several-fold during arbuscule development (Cox and Sanders, 1974).

The improved knowledge on the AM colonization process has allowed to demonstrate that the symbiotic interface is not limited to the arbusculated stage (Gutjahr and Parniske, 2013). When the AM germ-tube comes into contact with the epidermal cells, forming hyphopodia (Genre and Bonfante, 2007), these cells generate a colonization structure, the prepenetration apparatus (PPA), which is a transient formation that comprises cytoskeletal and endoplasmic reticulum (ER) components (Genre et al., 2005, 2008). Following this event, the hypha enters and crosses the epidermal cell, but prevents direct contact with the host cytoplasm thanks to a newly synthesized membrane of host origin. The biosynthesis of this novel perifungal membrane is the result of a process which involves exocytosis of the Golgi vesicles at

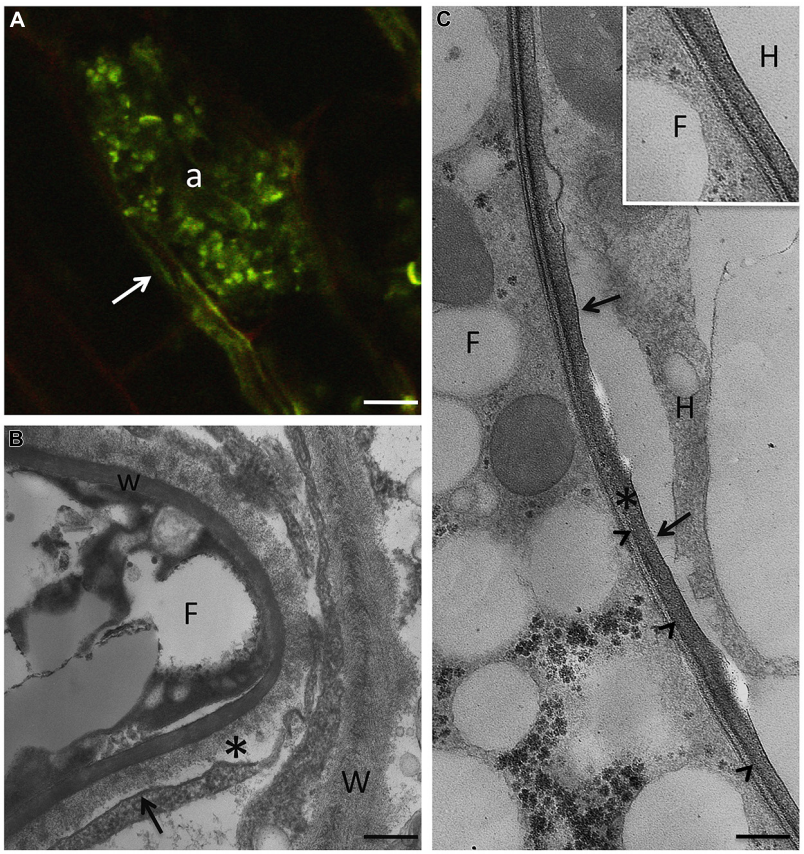


FIGURE 1 | In AM symbiosis, once the fungus overcomes the epidermal layer, it grows inter- and intracellularly all along the root in order to spread fungal structures. Only when the fungus reaches the cortical layers, does a peculiar branching process that leads to the highly branched structures, called arbuscules, which are the main site for nutrient exchanges. **(A)** *R. irregularis* arbuscule (a) after staining with wheat germ agglutinin-FITC, on paraffin section of *M. truncatula* root, to detect chitin in the fungal cell wall. Arrow points to an intercellular hypha. Bar, 7 μm. **(B)** At the electron microscope level, a new apoplastic space, based on membrane proliferation (arrow), is evident around the intracellular hyphae (F). The picture shows the morphology of the interface material (asterisk), with respect to the plant cell walls (W), where neatly arranged fibers are evident. w, fungal cell wall. Bar, 0.17 μm. **(C)** The host membrane surrounding the fungus (F) is smooth (arrows) in a clover root prepared through high pressure/freeze substitution. The interface material is electron-dense after PATAg treatment, and the fungal wall (arrowheads) is very thin. Bar, 0.3 μm. Inset: High magnification of the interface compartment. F, fungus; H, host cell. Bar, 0.25 μm.

the growing tips of the AM fungus. Using different GFP *Medicago* contructs, Genre et al. (2012) and Ivanov et al. (2012) have demonstrated that two VAMPs (vesicle-associated membrane proteins), belonging to R-SNAREs, are required to assemble the perifungal membrane. Interestingly, these proteins have also been detected at the cell plate of dividing meristematic root cells (Ivanov et al., 2012), thus offering the first molecular demonstration of the similarities between cell division leading to the construction of a new cell wall and development of the symbiotic interface.

Detailed electron microscope observations had already shown that the interfacial compartment contains cell-wall like material (Scannerini and Bonfante-Fasolo, 1983; Balestrini and Bonfante, 2005), and this has led to the question about whether the perifungal membrane maintains the capacity to synthesize and deliver cell wall-related molecules, like the peripheral membrane, (i.e., the plasma membrane of the host cell), but directed towards the interface space. The development of many *in situ* techniques (enzymes, lectins, and antibodies) has made it possible to validate this hypothesis: β-1,4-glucans, nonesterified homogalacturonans, xyloglucans, proteins rich in hydroxyproline (HRGPs), and arabinogalactan proteins (AGPs) have been located at the interface in many different plant/AM fungus combinations, as on peripheral cell wall, i.e., the cell wall of host cells containing the fungal structures (Bonfante et al., 1990a; Bonfante-Fasolo et al., 1991; Balestrini et al., 1994, 1996b; Gollotte et al., 1995; Balestrini and Bonfante, 2005). Expansins, which are extracellular proteins involved in cell wall-loosening and in the growth of plant cells (Cosgrove et al., 2002), have also been located in AM roots: they are present both in the cell walls of the host cells and in the interface, suggesting that this class of proteins, involved in cell wall loosening, may be crucial in the accommodation process of the fungus inside the cortical cells (Balestrini et al., 2005).

Although the molecular content of the interface reflects the composition of the host cell wall, the morphology of the interface material indicates that its components are assembled differently, and this leads to a more amorphous structure (**Figures 2** and **3**). In addition, this cell wall-like material changes in morphology during the arbuscule's life cycle: it is very thick and compact around the arbuscule trunk, it becomes very thin around the fungal branches, and it again becomes thicker when the arbuscule collapses. Such dynamics probably mirror the uneven distribution of plant membrane proteins along the perifungal membrane (see Gutjahr and Parniske, 2013 for a review). Expansins and/or secreted proteins of fungal origin (see below) could also play a role in keeping the interfacial material loose.

In liverworts, the disappearance of cell wall autofluorescence, which is a normal feature of non-colonized parenchyma cells, has been observed in the fungus-colonized areas, suggesting changes in cell wall composition upon fungal colonization, and a localized decrease in cell wall-bound phenolic compounds (Ligrone et al., 2007).

Compared to AM symbiosis, the symbiotic interface in ECM appears much simpler, at least on a morphological level: the plant and fungal cell walls are always in direct contact, since the ECM fungus remains apoplastic (Balestrini et al., 1996a; Peterson et al., 2004; **Figures 2** and **3**). When the hyphae penetrate between the root cells, only subtle alterations can be observed in the PCW, although a localized loosening has been reported in several ECMs (Balestrini et al., 1996a). Ultra-structural observations of ECMs formed by *Hebeloma cylindrosporum* IAA-overproducing mutants suggest that fungal IAA may play a role in Hartig net development by affecting PCW loosening (Gea et al., 1994). Components specific to both plant (e.g., cellulose and pectins) and fungal (e.g., SRAPs and hydrophobins) cell walls have been identified in the cell walls at the symbiotic interface (Balestrini et al., 1996a; Laurent et al., 1999; Tagu et al., 2001; **Figure 3**).

In conclusion, functioning mycorrhizal symbiosis requires both partners to be alive in order to exchange nutrients in a balanced way (Kiers et al., 2011). From a structural point of view, this requires the partners to maintain their individuality: this is guaranteed by a simple wall-to-wall contact during intercellular interactions and by a more sophisticated interface when the fungus becomes intracellular. Interestingly, the development of such a complex interface is also characteristic of biotrophic pathogenic interactions (Yi and Valent, 2013), suggesting that some plant responses reflect ancient mechanisms which are independent of the outcome of the interaction. Quoting Yi and Valent (2013) "…the interface is the site of active secretion by both players. This cross-talk at the interface determines the winner in adversarial relationships and establishes the partnership in mutualistic relationships."

FROM STRUCTURE TO BIOSYNTHESIS: PLANT CELL WALL RELATED GENES RESPOND TO SYMBIOSIS

Starting from *in situ* localization data, attention has been focused on the genesis of the interfacial material, and in particular on the plant genes involved in the cell wall metabolism. Using targeted approaches, it was found that genes encoding a putative AGP and an HRGP were induced in mycorrhizal roots of *Medicago truncatula* and maize, respectively, and the transcripts were specifically localized in arbusculated cells (Balestrini et al., 1997; van Buuren et al., 1999). An endotransglucosylase/hydrolase gene, *Mt-XHT1*, induced in *M. truncatula* roots during AM symbiosis, was identified (Maldonado-Mendoza et al., 2005). The analysis of transgenic roots expressing an *Mt-XHT1* fusion promoter has shown that expression is enhanced not only in the root regions colonized by the fungus, but also at distal sites. On the basis of this expression pattern, it was suggested that Mt-XHT might be involved in the systemic modification of a cell wall structure in order to enable fungal penetration.

Coming to the availability of sequenced genomes for several plants that are AM hosts, and the possibility of obtaining global transcriptional profiling of mycorrhizal roots, the regulation of cell-wall related genes has been studied at a larger scale. On the basis of localization data, an expansin gene has been found among the most up-regulated genes in *M. truncatula* mycorrhizal roots (Liu et al., 2003). Similarly, expansin/expansin-like genes were up-regulated during the early symbiotic stages in both *M. truncatula* (Weidmann et al., 2004; Siciliano et al., 2007) and tomato roots (*EXLB1*; Dermatsev et al., 2009), suggesting that an increase in PCW plasticity is a prerequisite to the accommodation of the

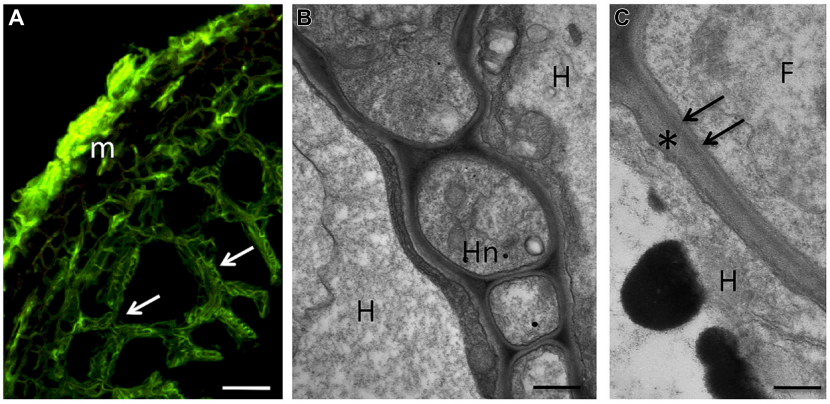


FIGURE 2 | During the symbiotic phase, ECM fungi form a fungal sheath (the mantle), which consists of aggregated hyphae that surround the root surface. This mycelium is linked to extramatrical hyphae that explore the substrate and are responsible for the mineral nutrition and water uptake of the symbiotic tissues. Some hyphae from the inner zone of the mantle penetrate between the root cells to form the Hartig net, an intercellular hyphal network inside the root tissues where metabolites are exchanged between the symbiotic partners. The hyphae always remain apoplastic and can colonize epidermal and cortical cell layers. **(A)** Confocal micrograph showing a section of hazelnut – *Tuber melanosporum* ectomycorrhizal root. The mantle (m), formed by packed hyphae, and the Hartig net (arrows), which surrounds the epidermal and outer cortical cells, show a green signal after treatment with WGA-FITC. Bar, 15 μm. **(B)** Hartig net (Hn) in a fully truffle developed mycorrhiza. Hyphae develop among plant cells, and their cell walls are in direct contact with the plant cell walls, showing a simple interface structure. H, host cell; Hn, Hartig net. Bar, 0.6 μm. **(C)** Magnification of the contact zone between plant (asterisk) and fungal cell wall (arrows). F, fungus; H, host cell. Bar, 0.4 μm.

fungus in the plant. *In situ* hybridization experiments on *Medicago* roots have in fact revealed that an expansin-like protein was preferentially expressed in epidermal cells in contact with the hyphopodium (Siciliano et al., 2007). A cellulose synthase-like gene was also shown to be up-regulated at the same stage (Siciliano et al., 2007). In agreement with the proposed role played by the PPA, the up-regulation of genes involved in cell wall synthesis/remodeling, during hyphopodium development, suggests that the building of the interface starts prior to fungal colonization. The symbiosis-dependent expression of an early nodulation gene (*ENOD11),* induced during the early stages of root nodulation and coding for a putative proline-rich protein (PRP), has also been detected in epidermal and cortical cells during root colonization by AM fungi (Journet et al., 2001; Chabaud et al., 2002). However, the role of these PRP proteins remains purely hypothetical. Thanks to a cDNA microarray experiment on *Lotus japonicus*, Guether et al. (2009) have demonstrated that a large number of genes related to membrane dynamics and cell wall metabolism are induced in mycorrhizal roots. These data support the hypothesis of plant cells having an active role in fungus accommodation *via* membrane proliferation and cell wall construction. Among the up-regulated genes related to cell wall metabolism, transcripts encoding for an endo-β-1,4-beta-glucanase (*LjCel1*) and a putative cellulose synthase (*LjCesA*) were detected exclusively in arbusculated cells, using a microdissection approach. These data are in agreement with those previously observed for *M. truncatula,* where the homologous gene *MtCel1* was found to be specifically expressed during symbiosis and, more specifically, within arbusculated cells (Liu et al., 2003). Considering the membrane domain, it was suggested that MtCel1 was located in the periarbuscular membrane and that it was directly involved in the assembly of the cellulose/hemicellulose matrix detected at the interface through *in situ* methods. Similarly, transcripts of a putative cellulose synthase, *LjCesA*, were also found to accumulate in arbusculated cells. CesA proteins are part of the cellulose synthase complex in higher plants (Taylor, 2008), which is a membrane-located enzymatic system that is responsible for cellulose synthesis.

Morphometric analyses have suggested an increase in the size of cells containing arbuscules (Balestrini et al., 2005). The authors hypothesized that *LjCesA* plays a role in cell expansion during arbuscule development, in conjunction with other proteins involved in cell wall remodeling (Balestrini and Bonfante, 2005). The role of two apoplastic plant proteases of the subtilase family (LjSbtM1 and LjSbtM3) during AM fungal colonization was also reported (Takeda et al., 2009). The members inside this protease family, as extracellular enzymes, are expressed during organ development and may be involved in the modification of cell wall structure, thus contributing to cell wall dynamics (Schaller et al., 2012). It has been proposed that these two *Lotus* genes, which are specifically expressed during the symbiosis, could play a role in cell wall modifications: they could facilitate fungal growth, or communication between the symbiotic partners, e.g., through the generation of peptides with a signaling role. Localization data have also shown that LjSbtM1, which is targeted for secretion, is localized in the apoplastic compartments, including the PCWs of the colonized cells, the intercellular spaces and the periarbuscular space.

Considering these data, it is tempting to suggest that the AM fungus not only leads to the construction of the interface compartment, but may also have the peripheral cell wall as an additional target. This has also been suggested for an ascorbate oxidase gene (*LjAO1*), which is up-regulated during AM symbiosis, where it shows a double location: in the apoplast and in the interface. This suggests that AO is possibly involved in the accommodation

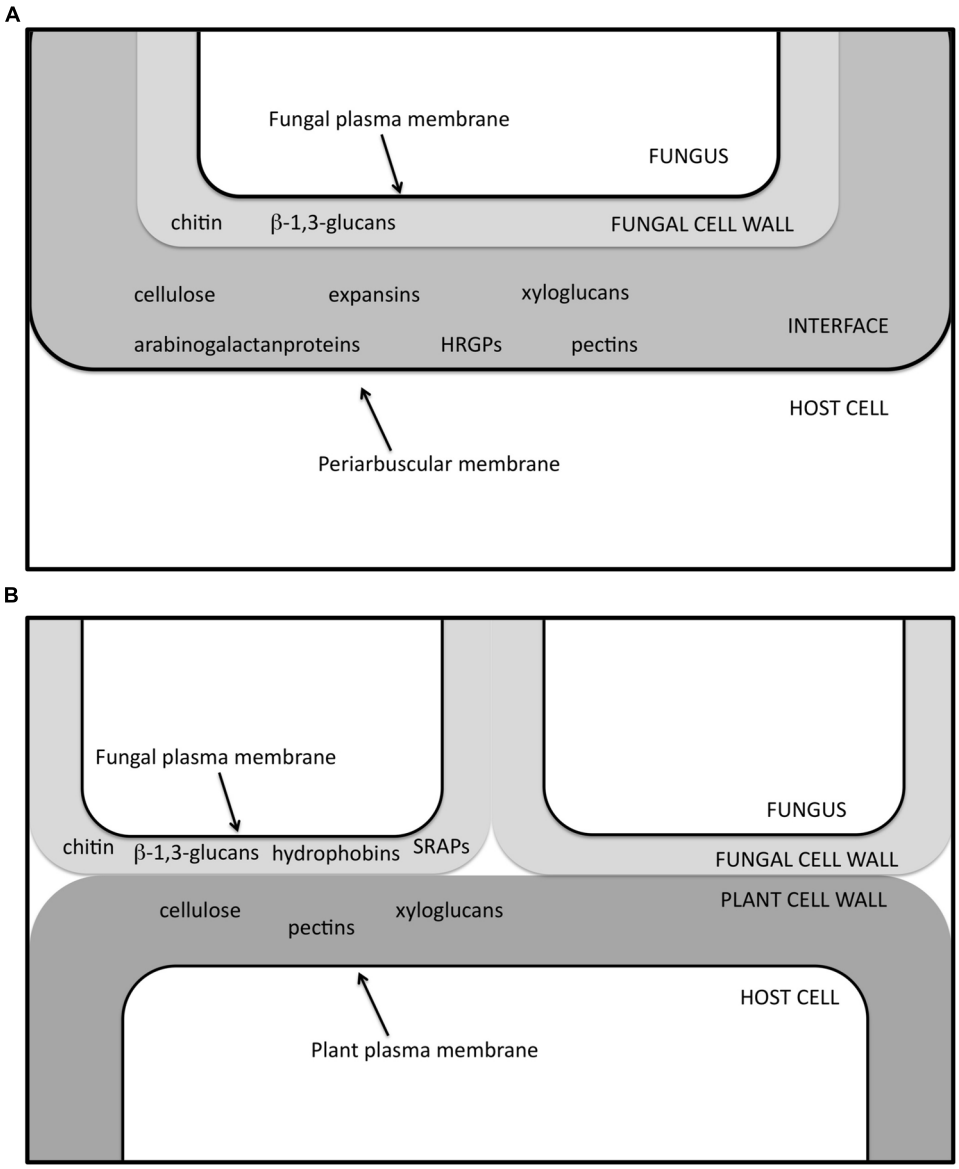


FIGURE 3 | Schematic view of the interface zone in AM (A) and ECM (B) symbiosis, in which several of the molecules so far determined through *in situ* labeling experiments (Balestrini et al., 1996a,b; Laurent et al., 1999; Tagu et al., 2001; Balestrini and Bonfante, 2005) are listed. HRGPs, hydroxyproline-rich glycoprotein; SRAPs, symbiosis-regulated acidic polypeptides.

of fungal structures, with the role of maintaining the structure of the interface compartment, as well as in cell wall loosening and extension, as observed in arbusculated cells (Balestrini et al., 2012a).

The activation of a β-xylosidase α-I-arabinosidase (which can contribute to the turnover of cell wall xylose and arabinose) has also been shown to be induced in tomato mycorrhizal roots (Fiorilli et al., 2009); the corresponding transcripts were localized exclusively in the arbuscule-containing cells, suggesting its involvement during the formation of the periarbuscular matrix, although involvement in the remodeling of the peripheral wall cannot be excluded. A monosaccharide transporter, MST2, which has been identified in an AM fungus (Helber et al., 2011), has been shown to transport not only glucose, but also cell wall monosaccharides, i.e., xylose. This result is in agreement with the fact that the plant-fungal interface in AM symbiosis contains primary PCW components (Balestrini and Bonfante, 2005). The authors have suggested that a versatile sugar transporter – capable of transporting monosaccharides from the apoplast – might be an optimal adaptation to the biotrophic life style of AMF (Helber et al., 2011).

Changes in the plant transcriptomic profiles of ECM roots have been well documented (Voiblet et al., 2001; Johansson et al., 2004; Duplessis et al., 2005; Le Quéré et al., 2005; Tuskan et al., 2006;

Heller et al., 2008), although poplar is the only ECM-forming plant whose genome has been sequenced so far. As a consequence, the regulation of PCW genes has only been investigated poorly, while more information is available on the fungal side (see next section).

The regulation of some PCW-related genes, e.g., the down-regulation of a pectin methylesterase gene and the up-regulation of genes coding for an expansin and a cellulose-synthase-like, has been reported in *Paxillus involutus*-poplar ECMs (Luo et al., 2009). The peripheral root cells of these ECM plants are swollen in comparison to non-ECM root cells, suggesting a role of these genes in cell wall expansion that mirrors cell enlargement. Genes coding for PCW proteins, mainly PRP, have been found to be up-regulated during the ECM association between oak and *Piloderma croceum* (Tarkka et al., 2013). This result is in agreement with previous observations where one PRP transcript was over-expressed in pre-mycorrhizal and mature roots (Frettinger et al., 2007). Using an oak contig assembly, Tarkka et al. (2013) also revealed that two extensins and several peroxidase genes were down-regulated, suggesting a reduced potential for cross-linking of the cell wall components in ECMs, while an expansin contig, with a putative role in cell wall relaxation also resulted to be up-regulated.

In conclusion, the development of genomics and transcriptomics tools has made it possible to demonstrate that symbiotic interface construction leads to a consistent variation in the expression profile of many cell-wall related genes. In AMs, the up-regulation of genes linked to the synthesis of cellulose and other polysaccharides is in line with the presence of cell wall polysaccharides in the interfacial matrix. However, it is interesting to note that relevant transcriptomic changes are also detectable in the ECMs, where no conspicuous morphological modifications have been described. Finally, the impact of mycorrhizal fungi on the cell wall metabolism does not seem to be limited to the symbiotic interfaces, since a still not fully acknowledged aspect is the increase in size of the colonized cells, which likely results from cell wall relaxation. Interestingly, it has recently been demonstrated that a loss in function mutation in a mycorrhiza-specific maize *Pi* transporter gene (*Pht1;6*) leads to a down-regulation of cell wall-related genes in AM roots (Willmann et al., 2013), although the mechanisms at the base of this response still have to be investigated.

DECIPHERING THE GENOMES OF MYCORRHIZAL FUNGI SHEDS LIGHT ON CELL WALL DYNAMICS

The sequenced genomes of mycorrhizal fungi are crucial tools to obtain a deeper understanding of the molecular mechanisms that underlie the symbiotic lifestyle. Focusing on cell wall genes, the so far sequenced fungi have allowed new information to be obtained on the fungal cell wall machinery, including genes that potentially code for fungal cell wall proteins involved in the interaction with plants, and those that may code for enzymes which act on PCW. This is a great step forward, since unlike for pathogenic fungi, a biochemical characterization of the main cell wall components, (i.e., polysaccharides and proteins) is not yet available for mycorrhizal fungi.

The involvement of fungal cell wall proteins, mainly small secreted proteins, (e.g., SRAPs and hydrophobins), has been well studied during the development of the symbiotic interface in ECM associations (Martin et al., 1999; Laurent et al., 1999; Tagu et al., 2001). Hydrophobins are morphogenetic small-secreted and moderately hydrophobic proteins that are typically present in fungi and which are involved in several aspects of fungal biology (Wösten, 2001). They are also thought to play important roles during ECM establishment (Tagu et al., 2001; Voiblet et al., 2001; Plett et al., 2012). They have been localized on the fungal cell wall in the symbiotic structures formed by *Pisolithus tinctorius* and *Eucalyptus globulus* (Tagu et al., 2001), and several hydrophobins are currently considered to belong to apoplastic MiSSPs (Mycorrhizal-induced Small Secreted Proteins; Plett et al., 2012). A genome-wide inventory of hydrophobin genes from two different genomes of the ECM fungus *L. bicolor* (Martin et al., 2008) has recently been obtained. This inventory shows a complex diversity and a range of expression profiles inside this multi-gene family (Plett et al., 2012). The authors suggest that, during evolution, some hydrophobin proteins might have acquired new roles that are specific of the mutualistic lifestyle. Hydrophobin genes have been identified in the *Tuber melanosporum* genome (Martin et al., 2010; Balestrini et al., 2012b), and, among these genes, *TmelHYD3,* seems to be slightly up-regulated in ECMs *vs* the free-living mycelium. However, these proteins seem to be absent in *Rhizophagus irregularis,* as in mildews where hydrophobins are lacking (Spanu et al., 2010).

The main carbohydrate components of fungal cell walls are chitin and glucans, and these have been localized in the cell walls of different mycorrhizal fungi (Bonfante et al., 1990b; Balestrini et al., 1994, 1996a, 2012b; Lemoine et al., 1995; **Figures 1** and **2**).

Taking advantage of the genome sequence, a genome-wide inventory of the proteins involved in cell wall synthesis and remodeling has been obtained for the *T. melanosporum* black truffle, while expression results have revealed that cell wall-related genes can be involved in the morphogenetic transition from mycelium growth to the ectomycorrhizal branched hyphae (Balestrini et al., 2012b).

In addition to their structural role, chitin-derived molecules are widespread microbial signals that trigger various defense responses in plant cells (Shimizu et al., 2010; Hayafune et al., 2014). In *Rhizobium*-legume symbiosis, the nitrogen-fixing bacteria produce lipochitooligosaccharides (LCOs), which are termed Nod factors (Dénarié and Cullimore, 1993). It has long been proposed that AM fungi also release signal molecules (Myc factors), which are essential for the recognition of the fungal partner (Catoira et al., 2000; Parniske, 2008). These molecular signals have recently been characterized as a mixture of sulfated and non-sulfated LCOs (Maillet et al., 2011). Myc-LCOs, which are present at very low concentrations in the exudates of mycorrhizal carrot roots and *R. irregularis* germinated spores, stimulate AM symbiosis formation, and increase root branching in *M. truncatula* (Maillet et al., 2011). Fungal orthologs of bacterial genes coding for enzymes involved in symbiotic LCO factors synthesis have not been identified so far in the genome of *R. irregularis*, the first AM fungus to be sequenced (Tisserant et al., 2013). Additionally, it has recently been observed

that short-chain chitin oligomers (COs) of AM fungus origin activate a response (Ca^{2+} spiking) in root epidermal cells, with the maximum activity being observed for CO4 and CO5 (Genre et al., 2013). Short-chain COs are therefore part of the molecular dialog with the host plant, that leads to the activation of the common symbiosis signaling pathway: these data reveal that AM fungi may produce different chitin-based signals (Genre et al., 2013). The *R. irregularis* genome (Tisserant et al., 2013; Lin et al., 2014) has revealed many genes involved in the chitin metabolism. In addition, transcriptomic data have shown that several of these genes, including, e.g., chitin synthases and putative chitin deacetylases, are expressed during the different stages of the fungal life cycle, including the pre-symbiotic stage (Tisserant et al., 2012). Further investigation on the regulation of specific members inside these gene families, will provide new information on the relationships between the generation of chitin-derived signals by AM fungi and their life cycle. It is interesting to note that, until now, the production of chitin-related molecules with a signaling meaning has not been described in ECM fungi.

Fungal cell wall-related signals therefore seem to play an important role in pre-symbiotic communication between the AM symbiosis partners. This also seems to be true for the plant side: cutin monomers, which are hydrophobic components of the cell walls that are mostly present in epidermal cells, have been identified as signal molecules that induce hyphopodium differentiation (Wang et al., 2012; Murray et al., 2013).

Several fungal genes encoding enzymes involved in cell wall metabolism are expressed during AM fungal colonization, suggesting a role in the remodeling of the fungal cell wall during intracellular colonization (Lanfranco et al., 1999; Tisserant et al., 2012, 2013). AM fungal cell walls undergo a conspicuous change in their organization during their life cycle: the spore wall is thick and layered with a highly fibrillar chitin, while the hyphal wall becomes progressively thinner during the intracellular phase, reaching a thin amorphous structure in the thinner arbuscular branches (Bonfante et al., 1990b). This would seem to suggest that fungal growth inside the root cells requires a strict regulation of the genes related to both cell wall synthesis and degradation. Additionally, the expression of plant chitinase genes has been reported in arbuscule-containing cells, suggesting a putative role in fungal cell wall modification, during the development of the arbuscule branches, as well as in reducing the amount of chitin-derived elicitors, during the intracellular colonization and the development of the symbiotic interface (Bonanomi et al., 2001; Hogekamp et al., 2011).

As far as the *R. irregularis* genome is concerned, a surprising observation is the loss of glycosyl hydrolase (GH) genes and – among them – genes known to be involved in degrading PCW polysaccharides (Tisserant et al., 2013). PCW degradation requires the production of different enzymes that are regulated by the type and complexity of the plant material, and fungi can produce these enzymes (Tian et al., 2009). A decreased repertoire of PCW degrading enzymes, compared to saprotrophic and pathogenic fungi, has also been reported for the ECM fungi *L. bicolor* and *T. melanosporum* (Martin et al., 2008, 2010). Although both genomes have shown a reduced set of enzymes that target PCW components, subtle differences have been observed in the enzyme repertoire between the two fungi and in their expression (Martin et al., 2010; Plett and Martin, 2011). Despite the similarity of the symbiotic structures that they form, the expression results suggest differences in the mode of interaction with the respective host plants. For example, a limited number of PCW degrading enzymes are expressed in truffle symbiotic tissues, while *L. bicolor* expresses very few PCW degrading enzymes, and mainly secretes expansin-like proteins that may play a role in cell wall remodeling during hyphal penetration (Plett and Martin, 2011). Similarly, the ECM fungus *Amanita bisporigena* (Nagendran et al., 2009) lacks genes that code for extracellular enzymes which are active on cell wall linkages. A reduced set of PCW-degrading enzymes has also been found in some obligate biotrophic pathogens, i.e., *Blumeria graminis* (Spanu et al., 2010). Unlike the enzymatic arsenal observed in other pathogenic fungi, the great reduction in PCW-degrading enzymes in fungi that are phylogenetically and functionally diverse suggests that this feature is related to their biotrophic life style. This could be one of the strategies that biotrophic fungi adopt to lower the defense reactions of their hosts.

In conclusion, genome sequence projects have, for the first time, allowed hypotheses to be formulated on the meaning of biotrophy in mycorrhizal fungi. They may use chitin-related molecules to dialog with their plant partners, but in spite of their deep intra-root habit, they do not possess PCW-degrading enzymes that can act on the PCW. It can be hypothesized that the signal molecules released by mycorrhizal fungi are perceived by the plant cells, which – in turn – elicit the activation of their own PCW-degrading enzymes. This indirect mechanism should prevent the activation of strong defense reactions.

CONCLUSION

Genome/transcriptome approaches applied to mycorrhizal fungi, and not only to the green partners, have had a profound effect on our knowledge of the biology of mycorrhizal symbiosis. As expected, the formation of the complex intracellular interface present in AMs is accompanied by a profound modulation of the PCW related genes (as well as the membrane-related ones). Surprisingly, important transcriptional changes have been detected in ECMs, where cell wall remodeling does not lead to evident morphological changes. The production of signaling molecules (Maillet et al., 2011; Genre et al., 2013), whose composition is related to the fungal cell wall, is probably a key element in the understanding of biotrophism. The mechanisms thanks to which the plant releases PCW enzymes, which allow fungal colonization, merit further detailed investigation. New genome data sets from several mycorrhizal fungi (ECM and AM), which are currently being sequenced (see http://genome.jgi.doe.gov/, http://mycor.nancy.inra.fr/genomeResources.html web sites), and from other non-AM endomycorrhizal fungi, e.g., *Oidiodendron maius* (ericoid symbiont) and *Tulasnella calospora* (orchid symbiont), will increase the knowledge on how different mycorrhizal fungi have an impact on PCW remodeling, and, consequently, on how the different mycorrhizal strategies have developed during the evolution.

ACKNOWLEDGMENTS

The Authors would like to thank Francis Martin for his support during the annotation of fungal cell wall-related genes and Marco Incarbone for his help in the English revision. The work has in part been funded by IPP-CNR, and MIUR projects Fungen™ PRIN 2007 and PRO-ROOT PRIN 2012.

REFERENCES

Balestrini, R., Hahn, M. G., and Bonfante, P. (1996a). Location of cell-wall components in ectomycorrhizae of *Corylus avellana* and *Tuber magnatum*. *Protoplasma* 191, 55–69. doi: 10.1007/BF01280825

Balestrini, R., Hahn, M. G., Faccio, A., Mendgen, K., and Bonfante, P. (1996b). Differential localization of carbohydrate epitopes in plant cell walls in the presence and absence of arbuscular mycorrhizal fungi. *Plant Physiol.* 111, 203–213. doi: 10.1104/pp.111.1.203

Balestrini, R., Romera, C., Puigdomenech, P., and Bonfante, P. (1994). Location of a cell-wall hydroxyproline-rich glycoprotein, cellulose and β-1,3-glucans in apical and differentiated regions of maize mycorrhizal roots. *Planta* 195, 201–209. doi: 10.1007/BF00199680

Balestrini, R., Jose-Estanyol, M., Puigdomenech, P., and Bonfante, P. (1997). Hydroxyproline-rich glycoprotein mRNA accumulation in maize root cells colonized by an arbuscular mycorrhizal fungus as revealed by *in situ* hybridization. *Protoplasma* 198, 36–42. doi: 10.1007/BF01282129

Balestrini, R., and Bonfante, P. (2005). The interface compartment in arbuscular mycorrhizae: a special type of plant cell wall? *Plant Biosyst.* 139, 8–15. doi: 10.1080/11263500500056799

Balestrini, R., Cosgrove, D. J., and Bonfante, P. (2005). Differential location of α-expansin proteins during the accommodation of root cells to an arbuscular mycorrhizal fungus. *Planta* 220, 889–899. doi: 10.1007/s00425-004-1431–1432

Balestrini, R., Ott, T., Guether, M., Bonfante, P., Udvardi, M. K., and De Tullio, M. C. (2012a). Ascorbate oxidase: the unexpected involvement of a 'wasteful enzyme' in the symbioses with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi. *Plant Physiol. Biochem.* 59, 71–79. doi: 10.1016/j.plaphy.2012.07.006

Balestrini, R., Sillo, F., Kohler, A., Schneider, G., Faccio, A., Tisserant, E., et al. (2012b). Genome-wide analysis of cell wall-related genes in *Tuber melanosporum*. *Curr. Genet.* 58, 165–177. doi: 10.1007/s00294-012-0374-6

Bonanomi, A., Wiemken, A., Boller, T., and Salzer, P. (2001). Local induction of a mycorrhiza-specific class III chitinase gene in cortical root cells of *Medicago truncatula* containing developing or mature arbuscules. *Plant Biol.* 3, 194–200. doi: 10.1055/s-2001-12902

Bonfante, P., Vian, B., Perotto, S., Faccio, A., and Knox, J. P. (1990a). Cellulose and pectin localization in roots of mycorrhizal *Allium porrum*: labeling continuity between host cell wall and interfacial material. *Planta* 180, 537–547. doi: 10.1007/BF02411452

Bonfante, P., Faccio, A., Perotto, S., and Schubert, A. (1990b). Correlation between chitin distribution and cell wall morphology in the mycorrhizal fungus *Glomus versiforme*. *Mycol. Res.* 94, 157–165. doi: 10.1016/S0953-7562(09)80607-2

Bonfante-Fasolo, P., Tamagnone, L., Peretto, R., Esquerré-Tugayé, M. T., Mazau, D., Mosiniak, M., et al. (1991). Immunocytochemical location of hydroxyproline rich glycoproteins at the interface between a mycorrhizal fungus and its host plants. *Protoplasma* 165, 127–138. doi: 10.1007/BF01322283

Bonfante, P. (2001). "At the interface between mycorrhizal fungi and plants: the structural organization of cell wall, plasma membrane and cytoskeleton," in *The Mycota, IX: Fungal Associations*, ed. B. Hock (Berlin: Springer), 45–61.

Bonfante, P., and Genre, A. (2010). Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nat. Commun.* 1, 48. doi: 10.1038/ncomms1046

Bracker, C. E., and Littlefield, L. J. (1973). "Structural concepts of host-pathogen interfaces," in *Fungal Pathogenicity and the Plant's Response*, eds R. J. W. Byrde and C. V. Cutting (London, NY: Academic Press), 159–317.

Bücking, H., Hans, R., and Heyser, W. (2007). "The apoplast of ectomycorrhizal roots – site of nutrient uptake and nutrient exchange between the symbiotic partners," in *The Apoplast of Higher Plants: Compartment of Storage, Transport and Reactions*, eds B. Sattelmacher and W. J. Horst (Dordrecht: Springer-Verlag), 97–108. doi: 10.1007/978-1-4020-5843-1_7

Catoira, R., Galera, C., de Billy, F., Penmetsa, R. V., Journet, E. P., Maillet, F., et al. (2000). Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. *Plant Cell* 12, 1647–1666 doi: 10.1105/tpc.12.9.1647

Chabaud, M., Venard, C., Defaux-Petras, A., Bécard, G., and Barker, D. G. (2002). Targeted inoculation of *Medicago truncatula in vitro* root cultures reveals *MtENOD11* expression during early stages of infection by arbuscular mycorrhizal fungi. *New Phytol.* 156, 265–273. doi: 10.1046/j.1469-8137.2002.00508.x

Cosgrove, D. J., Li, L. C., Cho, H.-T., Hoffmann-Benning, S., Moore, R. C., and Blecker, D. (2002). The growing world of expansins. *Plant Cell Physiol.* 43, 1436–1444. doi: 10.1093/pcp/pcf180

Cox, G., and Sanders, F. (1974). Ultrastructure of the host-fungus interface in a vesicular-arbuscular mycorrhiza. *New Phytol.* 73, 901–912. doi: 10.1111/j.1469-8137.1974.tb01319.x

Dermatsev, V., Weingarten-Baror, C., Resnick, N., Gadkar, V., Wininger, S., Kolotilin, I., et al. (2009). Microarray analysis and functional tests suggest the involvement of expansins in the early stages of symbiosis of the arbuscular mycorrhizal fungus *Glomus intraradices* on tomato (*Solanum lycopersicum*). *Mol. Plant Pathol.* 11, 121–135. doi: 10.1111/j.1364-3703.2009.00581.x

Dénarié, J., and Cullimore, J. (1993). Lipo-oligosaccharide nodulation factors: a new class of signalling molecules mediating recognition and morphogenesis. *Cell* 74, 951–954. doi: 10.1016/0092-8674(93)90717-5

Duplessis, S., Courty, P. E., Tagu, D., and Martin, F. (2005). Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globulus* and *Pisolithus microcarpus*. *New Phytol.* 165, 599–611. doi: 10.1111/j.1469-8137.2004.01248.x

Durán, A., and Nombela, C. (2004). Fungal cell wall biogenesis: building a dynamic interface with the environment. *Microbiology* 150, 3099–3103. doi: 10.1099/mic.0.27551-0

Fiorilli, V., Catoni, M., Miozzi, L., Novero, M., Accotto, G. P., and Lanfranco, L. (2009). Global and cell-type gene expression profiles in tomato plants colonized by an arbuscular mycorrhizal fungus. *New Phytol.* 184, 975–987. doi: 10.1111/j.1469-8137.2009.03031.x

Frettinger, P., Derory, J., Herrmann, S., Plomion, C., Lapeyrie, F., Oelmüller, R., et al. (2007). Transcriptional changes in two types of pre-mycorrhizal roots and in ectomycorrhizas of oak microcuttings inoculated with *Piloderma croceum*. *Planta* 225, 331–40. doi: 10.1007/s00425-006-0355-4

Gea, L., Normand, L., Vian, B., and Gay, G. (1994). Structural aspects of ectomycorrhizas of Pinus pinaster (Ait.) Sol. formed by an IAA overproducer mutant of the fungus *Hebeloma cylindrosporum Romagnesi*. *New Phytol.* 128, 659–670. doi: 10.1111/j.1469-8137.1994.tb04030.x

Genre, A., and Bonfante, P. (2007). Check-in procedures for plant cell entry by biotrophic microbes. *Mol. Plant Microbe Interact.* 20, 1023–1030. doi: 10.1094/MPMI-20-9-1023

Genre, A., and Bonfante, P. (2012). "The interface between plants and mycorrhizal fungi: nutrient exchange, signaling and cell organization," in *The Mycota, IX: Fungal Associations,* 2nd Edn, ed. B. Hock (Berlin: Springer), 39–49. doi: 10.1007/978-3-642-30826-0_3

Genre, A., Chabaud, M., Balzergue, C., Puech-Pagès, V., Novero, M., Rey, T., et al. (2013). Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. *New Phytol.* 198, 190–202. doi: 10.1111/nph.12146

Genre, A., Chabaud, M., Faccio, A., Barker, D. G., and Bonfante, P. (2008). Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both *Medicago truncatula* and *Daucus carota*. *Plant Cell* 20, 1407–1420. doi: 10.1105/tpc.108.059014

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., and Barker, D. G. (2005). Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. *Plant Cell* 17, 3489–3499. doi: 10.1105/tpc.105.035410

Genre, A., Ivanov, S., Fendrych, M., Faccio, A., Žárský, V., Bisseling, T., et al. (2012). Multiple exocytotic markers accumulate at the sites of perifungal membrane biogenesis in arbuscular mycorrhizas. *Plant Cell Physiol.* 53, 244–255. doi: 10.1093/pcp/pcr170

Gollotte, A., Gianinazzi-Pearson, V., and Gianinazzi, S. (1995). Immunodetection of infection thread glycoprotein and arabinogalactan protein in wild type *Pisum sativum* (L.) or an isogenic mycorrhiza-resistant mutant interacting with *Glomus mosseae*. *Symbiosis* 18, 69–85.

Guether, M., Balestrini, R., Hannah, M., He, J., Udvardi, M. K., and Bonfante, P. (2009). Genome-wide reprogramming of regulatory networks, transport, cell wall and membrane biogenesis during arbuscular mycorrhizal symbiosis in *Lotus japonicus*. *New Phytol.* 182, 200–212. doi: 10.1111/j.1469-8137.2008.02725.x

Gutjahr, C., and Parniske, M. (2013). Cell and developmental biology of arbuscular mycorrhiza symbiosis. *Annu. Rev. Cell Dev. Biol.* 29, 593–617. doi: 10.1146/annurev-cellbio-101512-122413

Harrison, M. J. (1999). Biotrophic interfaces and nutrient transport in plant fungal symbioses. *J. Exp. Bot.* 50, 1013–1022. doi: 10.1093/jexbot/50.suppl_1.1013

Hayafune, M., Berisio, R., Marchetti, R., Silipo, A., Kayama, M., Desaki, Y., et al. (2014). Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. *Proc. Natl. Acad. Sci. U.S.A.* 111, E404–E413. doi: 10.1073/pnas.1312099111

Helber, N., Wippel, K., Sauer N., Saarschmidt, S., Hause, B., and Requena, N. (2011). A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus *Glomus* sp. is crucial for the symbiotic relationship with plants. *Plant Cell* 23, 3812–3823. doi: 10.1105/tpc.111.089813

Heller, G., Adomas, A., Li, G., Osborne, J., van Zyl, L., Sederoff, R., et al. (2008). Transcriptional analysis of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus *Laccaria bicolor*. *BMC Plant Biol.* 8:19. doi: 10.1186/1471-2229-8-19

Hogekamp, C., Arndt, D., Pereira, P. A., Becker, J. D., Hohnjec, N., and Küster, H. (2011). Laser microdissection unravels cell-type-specific transcription in arbuscular mycorrhizal roots, including caat-box transcription factor gene expression correlating with fungal contact and spread. *Plant Physiol.* 157, 2023–2043. doi: 10.1104/pp.111

Keegstra, K. (2010). Plant cell walls. *Plant Physiol.* 154, 483–486. doi: 10.1104/pp.110.161240

Kiers, E. T., Duhamel, M., Beesetty, Y., Mensah, J. A., Franken, O., Verbruggen, E., et al. (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* 333, 880–882. doi: 10.1126/science.1208473

Ivanov, S., Fedorova, E. E., Limpens, E., De Mita, S., Genre, A., Bonfante, P., et al. (2012). Rhizobium–legume symbiosis shares an exocytotic pathway required for arbuscule formation. *Proc. Natl. Acad. Sci. U.S.A.* 109, 8316–8321. doi: 10.1073/pnas.1200407109

Johansson, T., Le Quéré, A., Ahren, D., Söderström, B., Erlandsson, R., Lundeberg, J., et al. (2004). Transcriptional responses of *Paxillus involutus* and *Betula pendula* during formation of ectomycorrhizal root tissue. *Mol. Plant Microbe Interact.* 17, 202–215. doi: 10.1094/MPMI.2004.17.2.202

Journet, E. P., El-Gachtouli, N., Vernoud, V., de Billy, F., Pichon, M., Dedieu, A., et al. (2001). *Medicago truncatula* ENOD11: a novel RPRPencoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. *Mol. Plant Microbe Interact.* 14, 737–748. doi: 10.1094/MPMI.2001.14.6.737

Lanfranco, L., Vallino, M., and Bonfante, P. (1999). Expression of chitin synthase genes in the arbuscular mycorrhizal fungus *Gigaspora margarita*. *New Phytol.* 142, 347–354. doi: 10.1046/j.1469-8137.1999.00393.x

Laurent, P., Voiblet, C., Tagu, D., de Carvalho, D., Nehls, U., De Bellis, R., et al. (1999). A novel class of ectomycorrhiza-regulated cell wall polypeptides in *Pisolithus tinctorius*. *Mol. Plant Microbe Interact.* 12, 862–871. doi: 10.1094/MPMI.1999.12.10.862

Lemoine, M. C., Gollotte, A., and Gianinazzi-Pearson, V. (1995). Localization of ß(1-3) glucan in walls of the endomycorrhizal fungi *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe and *Acaulospora laevis* Gerd. & Trappe during colonization of host roots. *New Phytol.* 129, 97–105. doi: 10.1111/j.1469-8137.1995.tb03013.x

Le Quéré, A., Wright, D. P., Soderstrom, B., Tunlid, A., and Johansson, T. (2005). Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) fr. *Mol. Plant Microbe Interact.* 18, 659–673. doi: 10.1094/MPMI-18-0659

Ligrone, R., Carafa, A., Lumini, E., Bianciotto, V., Bonfante, P., and Duckett, J. G. (2007). Glomeromycotean associations in liverworts: A molecular cellular and taxonomic analysis. *Am. J. Bot.* 94, 1756–1777. doi: 10.3732/ajb.94.11.1756

Lin, K., Limpens, E., Zhang, Z., Ivanov, S., Saunders, D. G. O., Mu, D., et al. (2014). Single nucleus genome sequencing reveals high similarity among nuclei of an endomycorrhizal fungus. *PLoS Genet.* 10:e1004078. doi: 10.1371/journal.pgen.1004078

Liu, J., Blaylock, L., Endre, G., Cho, J., Town, C. D., VandenBosch, K., et al. (2003). Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of the arbuscular mycorrhizal symbiosis. *Plant Cell* 15, 2106–2123. doi: 10.1105/tpc.014183

Luo, Z.-B., Janz, D., Jiang, X., Göbel, C., Wildhagen, H., Tan, Y., et al. (2009). Upgrading root physiology for stress tolerance by ectomycorrhizas: Insights from metabolite and transcriptional profiling into reprogramming for stress anticipation. *Plant Physiol.* 151, 1902–1917. doi: 10.1104/pp.109.143735

Maillet, F., Poinsot, V., André, O., Puech-Pagès, V., Haouy, A., Gueunier, M., et al. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469, 58–63. doi: 10.1038/nature09622

Maldonado-Mendoza, I. E., Dewbre, G. R., Blaylock, L., and Harrison, M. J. (2005). Expression of a xyloglucan endotransglucosylase/hydrolase gene, Mt-XTH1, from *Medicago truncatula* is induced systemically in mycorrhizal roots. *Gene* 345, 191197. doi: 10.1016/j.gene.2004.10.028

Martin, F., Aerts, A., Ahren, D., Brun, A., Danchin, E. G. J., Duchaussoy, F., et al. (2008). Symbiosis insights from the genome of the mycorrhizal basidiomycete *Laccaria bicolor*. *Nature* 452, 88–92. doi: 10.1038/nature06556

Martin, F., Kohler, A., Murat, C., Balestrini, R., Coutinho, P. M., Jaillon, O., et al. (2010). Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. *Nature* 464, 1033–1038. doi: 10.1038/nature08867

Martin, F., Laurent, P., de Carvalho, D., Voiblet, C., Balestrini, R., Bonfante, P., et al. (1999). Cell wall proteins of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*: Identification, function, and expression in symbiosis. *Fungal Genet. Biol.* 27, 161–174. doi: 10.1006/fgbi.1999.1138

Murray, J. D., Cousins, D. R., Jackson, K. J., and Liu, C. (2013). Signaling at the root surface: The role of cutin monomers in mycorrhization. *Mol. Plant* 6, 1381–1383. doi: 10.1093/mp/sst090

Nagendran, S., Hallen-Adams, H. E., Paper, J. M., Aslama, N., and Walton, J. D. (2009). Reduced genomic potential for secreted plant cell-wall-degrading enzymes in the ectomycorrhizal fungus *Amanita bisporigera*, based on the secretome of *Trichoderma reesei*. *Fungal Genet. Biol.* 46, 427–435. doi: 10.1016/j.fgb.2009.02.001

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nat. Rev. Microb.* 6, 763–775. doi: 10.1038/nrmicro1987

Peterson, R. L., and Massicotte, H. B. (2004). Exploring structural definitions of mycorrhizas with emphasis on nutrient exchange interfaces. *Can. J. Bot.* 82, 1074–1088. doi: 10.1139/b04-071

Peterson, R. L., Massicotte, H. B., and Melville, L. H. (2004). *Mycorrhizas: Anatomy and Cell Biology*. Toronto: CABI.

Plett, J. M., Gibon, J., Kohler, A., Duffy, K., Hoegger, P. J., Velagapudi, R., et al. (2012). Phylogenetic, genomic organization and expression analysis of hydrophobin genes in the ectomycorrhizal basidiomycete *Laccaria bicolor*. *Fungal Genet. Biol.* 49,199–209. doi: 10.1016/j.fgb.2012.01.002

Plett, J. M., and Martin, F. (2011). Blurred boundaries: lifestyle lessons from ectomycorrhizal fungal genomes. *Trends Genet.* 27, 14–22. doi: 10.1016/j.tig.2010.10.005

Reinhardt, D. (2007). Programming good relations – development of the arbuscular mycorrhizal symbiosis. *Curr. Opin. Plant Biol.* 10, 98–105. doi: 10.1016/j.pbi.2006.11.001

Scannerini, S., and Bonfante, P. (1976). "Ultrastructural features of a vesicular-arbuscular mycorrhiza," in *Sixth European Congress on Electron Microscopy*, Vol. 2, ed. Y. Ben-Shaul (Jerusalem: TAL Ramat Gan.), 492–494.

Scannerini, S., and Bonfante-Fasolo, P. (1983). Comparative ultrastructural analysis of mycorrhizal associations. *Can. J. Bot.* 61, 917–943. doi: 10.1139/b83-104

Schaller, A., Stintzi, A., and Graff, L. (2012). Subtilases – versatile tools for protein turnover, plant development, and interactions with the environment. *Physiol. Plant.* 145, 52–66. doi: 10.1111/j.1399-3054.2011.01529.x

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., et al. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* 64, 204–214. doi: 10.1111/j.1365-313X.2010.04324.x

Siciliano, V., Genre, A., Balestrini, R., Cappellazzo, G., deWit, P. J. G. M., and Bonfante, P. (2007). Transcriptome analysis of arbuscular mycorrhizal roots during development of the prepenetration apparatus. *Plant Physiol.* 144, 1455–1466. doi: 10.1104/pp.107

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis*, 3rd Edn. London: Academic Press.

Spanu, P. D., Abbott, J. C., Amselem, J., Burgis, T. A., Soanes, D. M., Stüber, K., et al. (2010). Genome expansion and gene loss in powdery mildew fungi reveal functional tradeoffs in extreme parasitism. *Science* 330, 1543–1546. doi: 10.1126/science.1194573

Tagu, D., De Bellis, R., Balestrini, R., De Vries, O. M. H., Piccoli, G., Stocchi, V., et al. (2001). Immunolocalization of hydrophobin HYDPt-1 from the ectomycorrhizal basidiomycete *Pisolithus tinctorius* during colonization of *Eucalyptus globulus* roots. *New Phytol.* 149, 127–135. doi: 10.1046/j.1469-8137.2001.00009.x

Takeda, N., Sato, S., Asamizu, E., Tabata, S., and Parniske, M. (2009). Apoplastic plant subtilases support arbuscular mycorrhiza development in *Lotus japonicus*. *Plant J.* 58, 766–777. doi: 10.1111/j.1365-313X.2009.03824.x

Tarkka, M. T., Herrmann, S., Wubet, T., Feldhahn, L., Recht, S., Kurth, F., et al. (2013). OakContigDF159.1, A reference library for studying differential gene expression in *Quercus robur* during controlled biotic interactions: use for quantitative transcriptomic profiling of oak roots in ectomycorrhizal symbiosis. *New Phytol.* 199, 529–540. doi: 10.1111/nph.12317

Taylor, N. G. (2008). Cellulose biosynthesis and deposition in higher plants. *New Phytol.* 178, 239–252. doi: 10.1111/j.1469-8137.2008.02385.x

Tian, C., Beeson, W. T., Iavarone, A. T., Sun, J., Marletta, M. A., Cate, J. H. D., et al. (2009). Systems analysis of plant cell wall degradation by the model filamentous fungus *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22157–22162. doi: 10.1073/pnas.0906810106

Tisserant, E., Kohler, A., Dozolme-Seddas, P., Balestrini, R., Benabdellah, K., Colard, A., et al. (2012). The transcriptome of the arbuscular mycorrhizal fungus *Glomus intraradices* (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. *New Phytol.* 193, 755–769. doi: 10.1111/j.1469-8137.2011.03948.x

Tisserant, E., Malbreil, M., Kuo, A., Kohler, A., Symeonidi, A., Balestrini, R., et al. (2013). Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 110, 20117–20122. doi: 10.1073/pnas.1313452110

Tuskan, G. A., Difazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., et al. (2006). The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313, 1596–604. doi: 10.1126/science.1128691

van Buuren, M. L., Maldonado-Mendosa, I. E., Trieu, A. T., Blaylock, L. A., and Harrison, M. J. (1999). Novel genes induced during an arbuscular mycorrhizal symbiosis formed between *Medicago truncatula* and *Glomus versiforme*. *Mol. Plant Microbe Interact.* 12, 171–181. doi: 10.1094/MPMI.1999.12.3.171

Voiblet, C., Duplessis, S., Encelot, N., and Martin, F. (2001). Identification of symbiosis-regulated genes in *Eucalyptus globulus–Pisolithus tinctorius ectomycorrhiza* by differential hybridization of arrayed cDNAs. *Plant J.* 25, 181–191. doi: 10.1111/j.1365-313X.2001.00953.x

Wang, E., Schornack, S., Marsh, J. F., Gobbato, E., Schwessinger, B., Eastmond, P., et al. (2012). A common signaling process that promotes mycorrhizal and oomycete colonization of plants. *Curr. Biol.* 22, 2242–2246. doi: 10.1016/j.cub.2012.09.043

Weidmann, S., Sanchez, L., Descombin, J., Chatagnier, O., Gianinazzi, S., and Gianinazzi-Pearson, V. (2004). Fungal elicitation of signal transduction related plant genes precedes mycorrhiza establishment and requires the dmi3 gene in *Medicago truncatula*. *Mol. Plant Microbe Interact.*17, 1385–1393. doi: 10.1094/MPMI.2004.17.12.1385

Willmann, M., Gerlach, N., Buer, B., Polatajko, A., Nagy, R., Koebke, E., et al. (2013). Mycorrhizal phosphate uptake pathway in maize: vital for growth and cob development on nutrient poor agricultural and greenhouse soils. *Front. Plant Sci.* 4:533. doi: 10.3389/fpls.2013.00533

Wösten, H. (2001). Hydrophobins: multipurpose proteins. *Annu. Rev. Microbiol.* 55, 625–646. doi: 10.1146/annurev.micro.55.1.625

Yi, M., and Valent, B. (2013). Communication between filamentous pathogens and plants at the biotrophic interface. *Annu. Rev. Phytopathol.* 51, 587–611. doi: 10.1146/annurev-phyto-081211-172916

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 February 2014; paper pending published: 17 March 2014; accepted: 12 May 2014; published online: 04 June 2014.

*Citation: Balestrini R and Bonfante P (2014) Cell wall remodeling in mycorrhizal symbiosis: a way towards biotrophism. Front. Plant Sci. **5**:237. doi: 10.3389/fpls.2014.00237*

This article was submitted to Plant–Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 14 October 2014
doi: 10.3389/fpls.2014.00540

Experimental approaches to study plant cell walls during plant-microbe interactions

Ye Xia, Carloalberto Petti, Mark A. Williams and Seth DeBolt*

Department of Horticulture, University of Kentucky, Lexington, KY, USA

Edited by:
Vincenzo Lionetti, Sapienza "Università di Roma," Italy

Reviewed by:
Yumiko Sakuragi, University of Copenhagen, Denmark
Charles T. Anderson, The Pennsylvania State University, USA

Correspondence:
Seth Debolt, Department of Horticulture, University of Kentucky, Plant Science Building, 1405 Veterans Drive, Lexington, KY 40546-0312, USA
e-mail: sdebo2@uky.edu

Plant cell walls provide physical strength, regulate the passage of bio-molecules, and act as the first barrier of defense against biotic and abiotic stress. In addition to providing structural integrity, plant cell walls serve an important function in connecting cells to their extracellular environment by sensing and transducing signals to activate cellular responses, such as those that occur during pathogen infection. This mini review will summarize current experimental approaches used to study cell wall functions during plant-pathogen interactions. Focus will be paid to cell imaging, spectroscopic analyses, and metabolic profiling techniques.

Keywords: cell imaging, metabolic profiling, phytobiome, plant-microbe interaction, cell wall

INTRODUCTION

The plant cell wall is a complex network consisting of diverse polysaccharides, lignin, and proteins (Mutwil et al., 2008). It provides physical strength, maintains cell shape, resists internal turgor pressure, regulates cell differentiation and growth, mediates bio-molecule transit, and serves as the first barrier of defense against biotic and abiotic stress (Knox, 2008; Collinge, 2009; Endler and Persson, 2011). Cell walls are highly dynamic and are capable of modifying their structural and chemical compositions to maintain functionality during developmental growth (Brewin, 2004; Somerville et al., 2004; Gorshkova et al., 2013; Bellincampi et al., 2014). In addition to its structural roles, plant cell walls serve an important function in connecting extracellular and intracellular environments by sensing and transducing signals, and activating cellular responses (Pogorelko et al., 2013) to environmental change and pathogen attack (Aziz et al., 2004; Vorwerk et al., 2004; Hématy et al., 2009). At pathogen infection sites plants generally accumulate callose, phenolic compounds, and lignin (Underwood, 2012), and in some cases metabolites and proteins that can directly inhibit the growth of pathogens (Vorwerk et al., 2004; Haas et al., 2009). The importance of plant cell wall integrity and cell wall-mediated resistance during plant-microbial interaction has been demonstrated, but the related components and signaling pathways have not been fully elucidated (Mellersh and Heath, 2001; Collinge, 2009; Hématy et al., 2009).

This mini review will summarize current experimental approaches that may be used as tools to study the cell wall with a focus on techniques that could be applied during the interaction between a plant and an interacting microbe. In particular, focus will be given to techniques for assessing changes in metabolites during plant-microbe interaction as well as techniques for imaging the cell wall. We are particularly interested in how the phytobiome, including mutualistic endophytes, pathogens and symbionts alike interact with the plants central architectural framework, the cell wall, and how this information could be harnessed for isolation of new herbicides (Xia et al., 2014) and/or plant defense systems.

METABOLIC PROFILING FOCUSED ON INTERACTIONS BETWEEN PLANT AND MICROBE

Metabolic profiling is the characterization and quantification of low-molecular weight metabolites and their intermediates in biological systems (Roessner and Bowne, 2009). This profiling aims to capture metabolites involved in the dynamic plant response to genetic modification, growth and developmental manipulation, and biotic/abiotic stresses (Clarke and Haselden, 2008). During plant-pathogen interaction, pathogens attempt to utilize the metabolism of host plants to suppress plant defense and to obtain nutrients (Dangl and Jones, 2001; Chisholm et al., 2006; Collinge, 2009). Metabolites that are synthesized by a host plant during a plant-microbe interaction can serve as signals, sedatives, or toxins to either aid the association with the microbe, or to attempt to limit the proliferation of the microbe (Thomma et al., 2002; Krishnan et al., 2005; Allwood et al., 2010, 2011; Schwessinger et al., 2012). Ultimately, most metabolic profiling will aim to capture *in situ* changes in cellular output in a spatially or temporal discrete region (Sumner, 2006; Timischl et al., 2008; Sumner et al., 2011; Khakimov et al., 2012). In the case of plant-pathogen interactions, profiling generally focuses on the plants metabolic response. Assaying microbial metabolites that are involved in plant-microbe interaction remains challenging. Assigning signals produced by a microbe requires separating them from those of the host plant and when grown in isolation, their metabolic output may not reflect a pathogenic state. When considering the plant cell wall, the relative predictability of metabolites in specific tissues provides an excellent starting point for looking at metabolic shifts associated with microbial ingress.

ANALYSIS OF PLANT CELL WALL POLYSACCHARIDES THROUGH METABOLIC PROFILING

At a broad scale, measurement of cell wall metabolites has been well-defined for decades. Neutral and acidic polysaccharides (Blakeney et al., 1983), acid insoluble and soluble glucose (Updegraff, 1969), soluble and insoluble lignin fractions (NREL, 2000) and the linkages between glycosyl units (Tong and Gross, 1988) can be examined with spectrophotometric, high performance liquid chromatography (HPLC), or gas chromatography (GC) coupled to mass spectroscopy (GC MS) to obtain a snapshot of the cell wall composition (Kopka, 2006). Similarly, at a much higher resolution, the structure of cell wall polysaccharides can be examined by uniformly feeding the plant with a isotope trace (^{13}C-glucose and ^{15}N-ammonia) and then employing 13C-magic angle spinning solid state nuclear magnetic resonance spectroscopy (SS-NMR) (Dick-Pérez et al., 2011; Fernandes et al., 2011; Harris et al., 2012). However, the capacity to look at spatially discrete regions of cell wall composition, which are linked to microbial association, can be difficult due to the relatively large amount of material needed for many of these techniques. To get around this limitation, combining systematic metabolite profiling with immunological approaches can be effective. For example, immunological approaches have been used to investigate the glycome profiling of wide array of plant cell wall polysaccharides (Pattathil et al., 2010, 2012; DeMartini et al., 2011; Fangel et al., 2012). Currently, around 150 antibodies that can recognize diverse epitopes present on each of the major classes of plant polysaccharides exist and are continuing to be developed. These antibodies have been used for *in situ* localization of epitopes to further our understanding of cell wall composition (Pattathil et al., 2012). Carbohydrate Microarray Polymer Profiling (CoMPP) has been streamlined as a screening platform to analyze cell wall polysaccharides by combining the specificity of monoclonal antibodies with a high-throughput microarray system (Alonso-Simón et al., 2009; Moller et al., 2012). In the context of microbial ingress, antibody based polysaccharide visualization has been utilized to observe altered xyloglucan arising from infection by the fungal pathogen *Botrytis cinerea* (Nguema-Ona et al., 2012, 2013). While difficulties arise in assigning quantitative data for localized metabolite profiles via immunological techniques, the capacity to gain unparalleled qualitative data is emerging. Additionally, a versatile high-resolution oligosaccharide microarray has been developed for cell wall analysis, which aids in the validation and characterization of target oligosaccharides produced via hydrolysis of polysaccharides or *de novo* synthesis (Pedersen et al., 2012). This library of cell wall oligosaccharides has been created by coupling target oligosaccharides with cognate proteins to form neoglycoconjugates, which in turn can be printed onto a microarray format (Pedersen et al., 2012). One can imagine the importance of such techniques to identify and characterize oligosaccharides identified during metabolic profiling.

Other techniques for assessing metabolites on a screening scale include Oligosaccharide Mass Profiling (OLIMP) coupled with Matrix-Assisted Laser Desorption/Ionization Time Of Flight (MALDI-TOF)-MS (Obel et al., 2009), or using of a suite of 74 polysaccharide degrading enzymes (Bauer et al., 2006). Both techniques were developed for the small-scale assessment of plant cell wall polysaccharides and to examine the oligosaccharides formed from polysaccharides that are digested by specific degrading enzymes (Bauer et al., 2006; Obel et al., 2009). OLIMP has particularly high sensitivity, thus making it ideal for small samples. It needs short preparation time and is suitable for *in situ* wall analysis at the cellular level. Importantly, OLIMP enables the comparative analysis of the wall polymers in a Golgi-enriched fraction vs. the apoplast fraction based on matrix polysaccharides, which may extend information about cellular functions during plant-pathogen interaction. OLIMP has been used to examine microbial alterations of the cell wall (Lionetti et al., 2007; Manabe et al., 2011), and allowed researchers to pinpoint that the alteration in esterification of pectin and xylan influenced the outcome of *B. cinerea* infection.

CELL IMAGING AND SPETROSCOPIC TECHNIQUES

Advanced cellular imaging can be useful to investigate phenotypes linked to plant-microbe associations. Cellular imaging can be particularly important when applying a quantitative methodology to imaging techniques. Many microscopic techniques are available, including light (Wilt et al., 2009), fluorescence (Lichtman and Conchello, 2005), and confocal microscopy (Nwaneshiudu et al., 2012). However, the outcomes of cell imaging can be influenced by many factors, such as microscope resolution, the rate at which images can be acquired, cell type being examined and the abundance/size of the tagged protein or structure being observed (Stephens and Allan, 2003; Shaw, 2006; **Table 1** for more details). Live cell imaging techniques (**Table 1**) have facilitated our understanding of plant cell wall dynamics in several different applications (Lee et al., 2011; Sappl and Heisler, 2013), and have been broadly applied when studying specific aspects of cell wall alteration during the interaction between a host plant and microbe.

ANALYSIS OF CELL WALL STRUCTURE AND FUNCTION WITH CELL IMAGING TECHNIQUES

There are several techniques that may be used to investigate structural and functional changes of plant cell walls during plant-microbe interactions. Aside from examining the phenotype, actually pinpointing defects in the cell wall often requires the merger to two or more techniques, including profiling cell wall structure as described above. Electron microscopy, both scanning (SEM) and transmission (TEM), along with fluorescence microscopy (FM) in the form of laser scanning or spinning disk confocal microscopy are of particularly interest. These techniques have been used together to examine plant-microbe interaction through alterations in the cell wall. Here, FM and TEM (**Table 1**) revealed that multi-vesicular bodies participated in cell wall-associated defense to powdery mildew in barley (An et al., 2006). As individual techniques, neither could ascertain mechanistic association, but together these techniques allowed a snapshot of inter and intracellular occurrences. Further, the relevance of plasma membrane–cell wall adhesion for cowpea resistance to rust fungi penetration was pinpointed by an integrated use of light and confocal microscopy (Mellersh and Heath, 2001). Laser scanning confocal microscopy can be used to track both plant and microbial proteins in live tissue. For example, confocal microscopy was used to track the dynamics of a *Xanthomonas* outer protein J (XopJ) in tobacco plants (**Table 1**), and revealed

Table 1 | Comparison of different cell imaging/spectroscopy methods for plant cell wall study.

Technique	Acquisition speed	Cell damage	Labeling (Fluorescence/ Coating/Staining)	Live cells	Single cell detection	Spatial/ Temporal resolution	Sample preparation	Chemical composition analysis	Measured parameter/information provided/limitation or possible problem caused	References
Bright-field microscopy (BFM)	Slow	Yes	No	Yes	Yes	Low ~2–3 μm	Often complex	Not available	Particle shape and size, cell-wall surfaces, and multilamellar architecture	Lacayo et al., 2010; Moran-Mirabal, 2013
Fluorescence microscopy (FM)	Fast	No	Yes	Yes	Yes	High ~10–255 nm	Easier	Not available	3D-cell wall structure, relative amount of cell wall polymers among different cells, localization and interactions of different wall components; photobleaching	Shaw, 2006; Frigault et al., 2009; Lacayo et al., 2010
Confocal laser scanning microscopy (CLSM)	Slow	No	Yes	Yes	Yes	High ~0.2–0.8 μm	Easier	Not available	3D-cell wall structure, localization and interactions of different wall components, multiple labels usage, focus to small regions; Scanning speed limits	McCann et al., 2001; Stephens and Allan, 2003; Ma et al., 2013
Spinning disk confocal microscopy (SDCM)	Very fast for single color acquisition	No	Yes	Yes	Yes	High ~0.2–0.8 μm	Easier	Not available	3D-cell wall structure, broad laser focus, quantitative analysis of polymer dynamics; switching between laser lines limit the acquisition speed	Stephens and Allan, 2003; Paredez et al., 2006; Bischoff et al., 2009
Transmission electron microscopy (TEM)	Fast	Yes	Yes	No	Yes	High ~0.2–10 nm	Time and skill demanding	Not available	Cell-wall surfaces and multilamellar architecture, cell wall ultrastructural organization; Small sample areas, high resolution	Kristensen et al., 2008; Sant'Anna et al., 2013
Scanning electron microscopy (SEM)	Fast	Yes	Yes	No	Yes	High ~1–4 nm	Time and skill demanding	Not available	Cell-wall surfaces and multilamellar architecture, uses atom-coated surfaces to determine topologies	Sarkar et al., 2009; Donohoe et al., 2011
Localization microscopy (LM)	Fast	Yes	Yes	Yes	Yes	High ~2–25 nm	Easier	Not available	3D-cell wall structure, single-molecule localization, super resolution techniques, nanoscale glucan polymer analysis	Betzig et al., 2006; Eggert et al., 2014
Fourier transform infra-red (FTIR) microspec-troscopy/Raman microspec-troscopy	Slow	No	No	Yes	Yes	High ~250 nm	Time and skill demanding	Available	Multiple components chemical analysis, and orientation of the cellulose microfibrils. The results are significantly influenced by the environment and water. Spectra are difficult to analyze and interpret	Chen et al., 1997b; Agarwal et al., 2010; Gierlinger et al., 2012
Atomic force microscopy (AFM)	Fast	No	No	Yes	Yes	High ~0.1–30 nm	Easier	Available	3D-cell wall structure, topology of the cell wall surface; poor chemical resolution	Kirby et al., 1996; Zhang et al., 2012

(Continued)

Table 1 | Continued

Technique	Acquisition speed	Cell damage	Labeling (Fluorescence/ Coating/Staining)	Live cells	Single cell detection	Spatial/ Temporal resolution	Sample preparation	Chemical composition analysis	Measured parameter/information provided/limitation or possible problem caused	References
X-RAY diffraction /neutron diffractometry	Fast	No	No	Yes	Yes	High ~0.05–0.4 nm	Time and skill demanding	Available	3D cell wall structure, degree of crystallinity, crystal size, chain orientation; only use for oriented crystal polymers	Burgert, 2006; Lacayo et al., 2010; Park et al., 2014
Nuclear magnetic resonance (NMR) spectroscopy	Fast	No	No	Yes	Yes	High ~10–90 nm	Time demanding	Available	Molecular dynamics, crystal structure, cellulose orientation; Interference could be caused by high molecular weight polymers, range of temperatures and frequencies are limited	Chylla et al., 2013; Wang et al., 2013

its interference (alteration of intracellular vesicle trafficking and polarized protein secretion) with cell wall-associated defense responses (Bartetzko et al., 2009). Similarly, but for a plant protein, the application of spinning disk confocal microscopy allowed the visualization of the CELLULOSE SYNTHASE (CeSA) complexes after exposure to the dinitrite-peptide Thaxtomin-A, which is a phytotoxin produced by *Streptomyces scabies* and *S. eubacteria* (Bischoff et al., 2009). Confocal microscopy allows the user to observe the microbial effector while it influences the target plant protein or cellular process. We recently utilized a screen of microbial endophytes (Xia et al., 2013) to identify microbial factors that induce cellulose inhibition and identified the compound acetobixan from a *Bacillus* sp. (Xia et al., 2014). Confocal microscopy allowed us to validate that the target process that the microbe was altering in the host plant was cellulose biosynthesis, which revealed a specific mechanism for this association.

The mechanisms of plant cell wall organization and dynamics have been extensively studied, and the use of suitable chemical probes to examine cell wall polysaccharide organization is expanding (Vorwerk et al., 2004; Lee et al., 2011). Recently, small molecule probes that bind to polysaccharides with high resolution and sensitivity have been developed (Knox, 2008; Pattathil et al., 2010; Lee et al., 2011), particularly in the form of click chemistry (Wallace and Anderson, 2012). An example of this approach was the utilization of an alkynylated fucose analog (FucAl) incorporated into the cell wall pectin fraction to elucidate pectin delivery, architecture, and dynamics in Arabidopsis (Anderson et al., 2012). Ultimately, the development of small molecule probes compatible with live-cell imaging can further enhance the understanding of fundamental biological questions pertinent to the cell wall during plant-microbe interaction, and can even be targeted to specific events.

ADDITIONAL IMAGING TECHNIQUES OF NOTE

Atomic force microscopy (AFM) is a technique with expanding use and potential. The extremely high resolution of AFM can allow the examination of events occurring within the nm scale. AFM has recently been used to detect the interaction of a synthetic carbohydrate-binding module with plant cellulose, and the structural changes of crystalline cellulose at a cell-wall surface (Zhang et al., 2012, 2013). In terms of plant microbe interactions, AFM recently provided nanoscale imaging of cell surfaces in their native state and revealed cell wall dynamics and modification during Arabidopsis and *Fusarium oxysporum* interaction (Adams et al., 2012). These selected studies underline the necessity to utilize the ever-expanding technological advances in imaging systems, often in concert with metabolic profiling, to maximize the detail of the investigation.

Localization microscopy, which is a form of super-resolution microscopy, focuses on the localization of single fluorescent molecule. Such super-resolution microscopy has been used to analyze the infection site of the fungal pathogen powdery mildew on Arabidopsis plants at a nanoscale level (Eggert et al., 2014). The technique was sensitive enough to show that the microbial pathogen induced the synthesis of the (1,3)-β-glucan cell wall polymer callose, which interacted with the (1,4)-β-glucan cellulose to form a three-dimensional network for preventing

pathogen infection. The formation of callose associated with pathogen ingress has been well-studied, but such inter-polymer associations could not have been proven without technical breakthroughs. It will be interesting to see whether such techniques are combined with click-chemistry to observe an increasing number of interactions simultaneously.

The combined approaches of microscopy with spectroscopy can also facilitate the investigation of wall associated ultrastructure modifications, and the chemical compositions of the plant cell wall during plant-pathogen interaction (**Table 1** for more details). Fourier Transform Infrared (FT-IR) spectromicroscopy has been used to determine the presence and orientation of functional groups of cellulose and pectin in plant cell walls for well over a decade (Chen et al., 1997a,b; Kaèuráková et al., 2000; Wilson et al., 2000). This technique was used to show that a mutation in Arabidopsis *PMR6,* which encodes a pectate lyase-like protein and is required for the growth and reproduction of plant fungal powdery mildew pathogen-*Erysiphe cichoracearum*, altered plant cell wall composition by increasing pectin accumulation. Both absorbance peaks attributed to cellulose and xyloglucan shifted down in energy and broadened in the spectra of *pmr6-1* cell walls, which indicated that either the –CH2OH group or the hydrogen bond of cellulose in *pmr6-1* had been changed (Vogel et al., 2002).

Raman microscopy (Inelastic scattering with a photon from a laser light source) combined with FT-IR spectromicroscopy (Photon absorption) can facilitate the observation of ultrastructure, such as cellulosic crystals on the micro-scale ($<0.5\,\mu m$) level (Agarwal et al., 2010), as well as the alignment and orientation of cellulose microfibrils with respect to the fiber axis between different cell wall layers (Gierlinger et al., 2012). This combined approach also improves our ability to visualize and analyze the chemical composition of plant cell walls. For instance, the spectra of the two wall-matrix polymers: lignin and pectin display discernable marker bands, which do not overlap with the cellulose signature, so their distribution in the plant cell wall can be easily visualized, imaged, and analyzed using these techniques (Gierlinger and Schwanninger, 2006; Richter et al., 2011; Gierlinger et al., 2012).

PROSPECTS

Recent technical breakthroughs in combining higher resolution imaging and metabolic profiling techniques have yielded numerous discoveries in how plant cell wall function is modulated during microbial interaction. Although much effort was spent to be inclusive in this mini-review, due to space constraints we apologize for excluding numerous developing techniques not limited to but including those associated with biochemical pull downs, protein-protein interaction arrays and more. Although advanced cell imaging and spectroscopic techniques have facilitated such studies, the recent identification of the enormously complex phytobiome (Bulgarelli et al., 2012; Lundberg et al., 2012, 2013) reveals an outstanding question of the function of the phytobiome in plant-microbe associations. The use of next generation sequencing has revealed that many more microbes are present within plant tissue than those previously identified as obligate endophytes. It remains unclear how these microbial mutualists are associating with (or avoiding) the plant cell wall and associated defense pathways, and whether under pathogen interaction, they matter?

ACKNOWLEDGMENT

Funding support was provided by NSF-IOS and USDA (Hatch/FAPRU) to Seth DeBolt.

REFERENCES

Adams, E., Emerson, D., Croker, S., Kim, H. S., Modla, S., Kang, S., et al. (2012). "Atomic force microscopy: a tool for studying biophysical surface properties underpinning fungal interactions with plants and substrates," in *Plant Fungal Pathogens*, Vol. 835, eds M. D. Bolton and B. P. H. J. Thomma (New York, NY: Humana Press), 151–164.

Agarwal, U., Reiner, R., and Ralph, S. (2010). Cellulose I crystallinity determination using FT-Raman spectroscopy: univariate and multivariate methods. *Cellulose* 17, 721–733. doi: 10.1007/s10570-010-9420-z

Allwood, J. W., Clarke, A., Goodacre, R., and Mur, L. A. J. (2010). Dual metabolomics: a novel approach to understanding plant–pathogen interactions. *Phytochemistry* 71, 590–597. doi: 10.1016/j.phytochem.2010.01.006

Allwood, J. W., De, V., Ric, C. H., Moing, A., Deborde, C., Erban, A., et al. (2011). "Chapter sixteen–plant metabolomics and its potential for systems biology research: background concepts, technology, and methodology," in *Methods in Enzymology*, Vol. 500, eds M. V. Daniel Jameson and V. W. Hans (Waltham, MA: Academic Press), 299–336.

Alonso-Simón, A., Kristensen, J. B., Øbro, J., Felby, C., Willats, W. G. T., and Jørgensen, H. (2009). High-throughput microarray profiling of cell wall polymers during hydrothermal pre-treatment of wheat straw. *Biotechnol. Bioeng.* 105, 509–514. doi: 10.1002/bit.22546

An, Q., Hückelhoven, R., Kogel, K. H., Van, B., and Aart, J. E. (2006). Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. *Cell Microbiol.* 8, 1009–1019. doi: 10.1111/j.1462-5822.2006.00683.x

Anderson, C. T., Wallace, I. S., and Somerville, C. (2012). Metabolic click-labeling with a fucose analog reveals pectin delivery, architecture, and dynamics in Arabidopsis cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1329–1334. doi: 10.1073/pnas.1120429109

Aziz, A., Heyraud, A., and Lambert, B. (2004). Oligogalacturonide signal transduction, induction of defense-related responses and protection of grapevine against *Botrytis cinerea*. *Planta* 218, 767–774. doi: 10.1007/s00425-003-1153-x

Bartetzko, V., Sonnewald, S., Vogel, F., Hartner, K., Stadler, R., Hammes, U. Z., et al. (2009). The *Xanthomonas campestris* pv. vesicatoria type III effector protein XopJ inhibits protein secretion: evidence for interference with cell wall-associated defense responses. *Mol. Plant Microbe Interact.* 22, 655–664. doi: 10.1094/MPMI-22-6-0655

Bauer, S., Vasu, P., Persson, S., Mort, A. J., and Somerville, C. R. (2006). Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11417–11422. doi: 10.1073/pnas.0604632103

Bellincampi, D., Cervone, F., and Lionetti, V. (2014). Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. *Front. Plant Sci.* 5:228. doi: 10.3389/fpls.2014.00228

Betzig, E., Patterson, G. H., Sougrat, R., Lindwasser, O. W., Olenych, S., Bonifacino, J. S., et al. (2006). Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313, 1642–1645. doi: 10.1126/science.1127344

Bischoff, V., Cookson, S. J., Wu, S., and Scheible, W. R. (2009). Thaxtomin A affects CESA-complex density, expression of cell wall genes, cell wall composition, and causes ectopic lignification in *Arabidopsis thaliana* seedlings. *J. Exp. Bot.* 60, 955–965. doi: 10.1093/jxb/ern344

Blakeney, A. B., Harris, P. J., Henry, R. J., and Stone, B. A. (1983). A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* 113, 291–299. doi: 10.1016/0008-6215(83)88244-5

Brewin, N. J. (2004). Plant cell wall remodeling in the *Rhizobium*–legume symbiosis. *Crit. Rev. Plant Sci.* 23, 293–316. doi: 10.1080/07352680490480734

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van, T. E., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Burgert, I. (2006). Exploring the micromechanical design of plant cell walls. *Am. J. Bot.* 93, 1391–1401. doi: 10.3732/ajb.93.10.1391

Chen, L. M., Wilson, R. H., and McCann, M. C. (1997a). Infra-red microspectroscopy of hydrated biological systems: design and construction of a new cell with atmospheric control for the study of plant cell walls. *J. Microsc.* 188, 62–71. doi: 10.1046/j.1365-2818.1997.2470805.x

Chen, L. M., Wilson, R. H., and McCann, M. C. (1997b). Investigation of macromolecule orientation in dry and hydrated walls of single onion epidermal cells by FT-IR microspectroscopy. *J. Mol. Struct.* 408, 257–260. doi: 10.1016/S0022-2860(96)09539-7

Chisholm, S., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124, 803–814. doi: 10.1016/j.cell.2006.02.008

Chylla, R., Van Acker, R., Kim, H., Azapira, A., Mukerjee, P., Markley, J. L., et al. (2013). Plant cell wall profiling by fast maximum likelihood reconstruction (FMLR) and region-of-interest (ROI) segmentation of solution-state 2D 1H-13C NMR spectra. *Biotechnol. Biofuels* 6:45. doi: 10.1186/1754-6834-6-45

Clarke, C. J., and Haselden, J. N. (2008). Metabolic profiling as a tool for understanding mechanisms of toxicity. *Toxicol. Pathol.* 36, 140–147. doi: 10.1177/0192623307310947

Collinge, D. B. (2009). Cell wall appositions: the first line of defense. *J. Exp. Bot.* 60, 351–352. doi: 10.1093/jxb/erp001

Dangl, J. L., and Jones, J. D. G. (2001). Plant pathogens and integrated defense responses to infection. *Nature* 411, 826–833. doi: 10.1038/35081161

DeMartini, J. D., Pattathil, S., Avci, U., Szekalski, K., Mazumder, K., Hahn, M. G., et al. (2011). Application of monoclonal antibodies to investigate plant cell wall deconstruction for biofuels production. *Energy Environ. Sci.* 4, 4332–4339. doi: 10.1038/nature11336

Dick-Pérez, M., Zhang, Y., Hayes, J., Salazar, A., Zabotina, O. A., and Hong, M. (2011). Structure and interactions of plant cell-wall polysaccharides by two- and three-dimensional magic-angle-spinning solid-state NMR. *Biochemistry* 50, 989–1000. doi: 10.1021/bi101795q

Donohoe, B. S., Vinzant, T. B., Elander, R. T., Pallapolu, V. R., Lee, Y. Y., Garlock, R. J., et al. (2011). Surface and ultrastructural characterization of raw and pretreated switchgrass. *Bioresour. Technol.* 102, 11097–11104. doi: 10.1016/j.biortech.2011.03.092

Eggert, D., Naumann, M., Reimer, R., and Voigt, C. A. (2014). Nanoscale glucan polymer network causes pathogen resistance. *Sci. Rep.* 4, 1–6. doi: 10.1038/srep04159

Endler, A., and Persson, S. (2011). Cellulose synthases and synthesis in Arabidopsis. *Mol. Plant* 4, 199–211. doi: 10.1093/mp/ssq079

Fangel, J. H., Pedersen, S., Vidal-Melgosa, L., Ahl, A., Salmean, J., Egelund, M., et al. (2012). "Carbohydrate microarrays in plant science," in *High-Throughput Phenotyping in Plants*, Vol. 918, ed J. Normanly (New York, NY: Humana Press), 351–362.

Fernandes, A. N., Thomas, L. H., Altaner, C. M., Callow, P., Forsyth, V. T., Apperley, D. C., et al. (2011). Nanostructure of cellulose microfibrils in spruce wood. *Proc. Natl. Acad. Sci. U.S.A.* 108, 1195–1203. doi: 10.1073/pnas.1108942108

Frigault, M. M., Lacoste, J., Swift, J. L., and Brown, C. M. (2009). Live-cell microscopy–tips and tools. *J. Cell Sci.* 122, 753–767. doi: 10.1242/jcs.033837

Gierlinger, N., Keplinger, T., and Harrington, M. (2012). Imaging of plant cell walls by confocal Raman microscopy. *Nat. Protoc.* 7, 1694–1708. doi: 10.1038/nprot.2012.092

Gierlinger, N., and Schwanninger, M. (2006). Chemical imaging of poplar wood cell walls by confocal Raman microscopy. *Plant Physiol.* 140, 1246–1254. doi: 10.1104/pp.105.066993

Gorshkova, T. A., Kozlova, L. V., and Mikshina, P. V. (2013). Spatial structure of plant cell wall polysaccharides and its functional significance. *Biochemistry (Moscow)* 78, 836–853. doi: 10.1134/S0006297913070146

Haas, B., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., et al. (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* 461, 393–398. doi: 10.1038/nature08358

Harris, D. M., Corbin, K., Wang, T., Gutierrez, R., Bertolo, A. L., Petti, C., et al. (2012). Cellulose microfibril crystallinity is reduced by mutating C-terminal transmembrane region residues CESA1A903V and CESA3T942I of cellulose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4098–4103. doi: 10.1073/pnas.1200352109

Hématy, K., Cherk, C., and Somerville, S. (2009). Host–pathogen warfare at the plant cell wall. *Curr. Opin. Plant Biol.* 12, 406–413. doi: 10.1016/j.pbi.2009.06.007

Kaèuráková, M., Capek, P., Sasinková, V., Wellner, N., and Ebringerová, A. (2000). FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. *Carbohydr. Polym.* 43, 195–203. doi: 10.1016/S0144-8617(00)00151-X

Khakimov, B., Amigo, J. M., Bak, S., and Engelsen, S. B. (2012). Plant metabolomics: resolution and quantification of elusive peaks in liquid chromatography-mass spectrometry profiles of complex plant extracts using multi-way decomposition methods. *J. Chromatogr.* 1266, 84–94. doi: 10.1016/j.chroma.2012.10.023

Kirby, A., Gunning, A. P., Waldron, K. W., Morris, V. J., and Ng, A. (1996). Visualization of plant cell walls by atomic force microscopy. *Biophys. J.* 70, 1138–1143. doi: 10.1016/S0006-3495(96)79708-4

Knox, J. P. (2008). Revealing the structural and functional diversity of plant cell walls. *Curr. Opin. Plant Biol.* 11, 308–313. doi: 10.1016/j.pbi.2008.03.001

Kopka, J. (2006). "Gas chromatography mass spectrometry," in *Plant Metabolomics,* eds K. Saito, R. Dixon, and L. Willmitzer (Berlin: Springer-Verlag Press), 3–20.

Krishnan, P., Kruger, N. J., and Ratcliffe, R. G. (2005). Metabolite fingerprinting and profiling in plants using NMR. *J. Exp. Bot.* 56, 255–265. doi: 10.1093/jxb/eri010

Kristensen, J., Thygesen, L., Felby, C., Jorgensen, H., and Elder, T. (2008). Cell-wall structural changes in wheat straw pretreated for bioethanol production. *Biotechnol. Biofuels* 1, 5. doi: 10.1186/1754-6834-1-5

Lacayo, C. I., Malkin, A. J., Holman, H. Y. N., Chen, L., Ding, S. Y., Hwang, M. S., et al. (2010). Imaging cell wall architecture in single *Zinnia elegans* tracheary elements. *Plant Physiol.* 154, 121–133. doi: 10.1104/pp.110.155242

Lee, K. J. D., Marcus, S. E., and Knox, J. P. (2011). Cell wall biology: perspectives from cell wall imaging. *Mol. Plant* 4, 212–219. doi: 10.1093/mp/ssq075

Lichtman, J. W., and Conchello, J. A. (2005). Fluorescence microscopy. *Nat. Methods* 2, 910–919. doi: 10.1038/nmeth817

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., et al. (2007). Overexpression of pectin methylesterase inhibitors in Arabidopsis restricts fungal infection by *Botrytis cinerea*. *Plant Physiol.* 143, 1871–1880. doi: 10.1104/pp.106.090803

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D., and Dangl, J. (2013). Practical innovations for high-throughput amplicon sequencing. *Nat. Methods* 10, 999–1002. doi: 10.1038/nmeth.2634

Ma, J., Ji, Z., Zhou, X., Zhang, Z. H., and Xu, F. (2013). Transmission electron microscopy, fluorescence microscopy, and confocal raman microscopic analysis of ultrastructural and compositional heterogeneity of *Cornus alba* L. Wood cell wall. *Microsc. Microanal.* 19, 243–253. doi: 10.1017/S1431927612013906

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., et al. (2011). Loss-of-function mutation of REDUCED WALL ACETYLATION2 in Arabidopsis leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. *Plant Physiol.* 155, 1068–1078. doi: 10.1104/pp.110.168989

McCann, M. C., Bush, M., Milioni, D., Sado, P., Stacey, N., Catchpole, G., et al. (2001). Approaches to understanding the functional architecture of the plant cell wall. *Phytochemistry* 57, 811–821. doi: 10.1016/S0031-9422(01)00144-3

Mellersh, D. G., and Heath, M. C. (2001). Plasma membrane–cell wall adhesion is required for expression of plant defense responses during fungal penetration. *Plant Cell* 13, 413–424. doi: 10.1105/tpc.13.2.413

Moller, I. E., Pettolino, F. A., Hart, C., Lampugnani, E. R., Willats, W. G., and Bacic, A. (2012). Glycan profiling of plant cell wall polymers using microarrays. *J. Vis. Exp.* 17:e4238. doi: 10.3791/4238

Moran-Mirabal, J. M. (2013). "Advanced-microscopy techniques for the characterization of cellulose structure and cellulose-cellulase interactions," in *Cellulose–Fundamental Aspects,* eds V. T. van de and L. Godbout (Rijeka: InTech Press), 1–44.

Mutwil, M., Debolt, S., and Persson, S. (2008). Cellulose synthesis: a complex complex. *Curr. Opin. Plant Biol.* 11, 252–257. doi: 10.1016/j.pbi.2008.03.007

Nguema-Ona, E., Moore, J., Fagerstrom, A., Fangel, J., Willats, W. G. T., Hugo, A., et al. (2013). Overexpression of the grapevine PGIP1 in tobacco results in compositional changes in the leaf arabinoxyloglucan network in the absence of fungal infection. *BMC Plant Biol.* 13:46. doi: 10.1186/1471-2229-13-46

Nguema-Ona, E., Moore, J. P., Fagerström, A., Fangel, J. U., Willats, W. G. T., Hugo, A., et al. (2012). Profiling the main cell wall polysaccharides of tobacco

leaves using high-throughput and fractionation techniques. *Carbohydr. Polym.* 88, 939–949. doi: 10.1016/j.carbpol.2012.01.044

NREL National Renewable Energy Laboratories. (2000). *Technical Report: Determining the Cost of Producing Ethanol from Corn Starch and Lignocellulosic Feedstocks*. NREL/TP-580–28893. Golden, CO: NREL Press.

Nwaneshiudu, A., Kuschal, C., Sakamoto, F. H., Anderson, R. R., Schwarzenberger, K., and Young, R. C. (2012). Introduction to confocal microscopy. *J. Invest. Dermatol.* 132, e3. doi: 10.1038/jid.2012.429

Obel, N., Erben, V., Schwarz, T., Kühnel, S., Fodor, A., and Pauly, M. (2009). Microanalysis of plant cell wall polysaccharides. *Mol. Plant* 2, 922–932. doi: 10.1093/mp/ssp046

Paredez, A. R., Somerville, C., and Ehrhardt, D. W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. *Science* 312, 1491–1495. doi: 10.1126/science.1126551

Park, Y. B., Lee, C. M., Kafle, K., Park, S., Cosgrove, D. J., and Kim, S. H. (2014). Effects of plant cell wall matrix polysaccharides on bacterial cellulose structure studied with vibrational sum frequency generation spectroscopy and x-ray diffraction. *Biomacromolecules* 15, 2718–2724. doi: 10.1021/bm500567v

Pattathil, S., Avci, U., Baldwin, D., Swennes, A. G., McGill, J. A., Popper, Z., et al. (2010). A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiol.* 153, 514–552. doi: 10.1104/pp.109.151985

Pattathil, S., Avci, U., Miller, J., and Hahn, M. G. (2012). "Immunological approaches to plant cell wall and biomass characterization: glycome profiling," in *Biomass Conversion*, Vol. 908, ed M. E. Himmel (New York, NY: Humana Press), 61–72.

Pedersen, H. L., Fangel, J. U., McCleary, B., Ruzanski, C., Rydahl, M. G., Ralet, M. C., et al. (2012). Versatile high resolution oligosaccharide microarrays for plant glycobiology and cell wall research. *J. Biol. Chem.* 287, 39429–39438. doi: 10.1074/jbc.M112.396598

Pogorelko, G., Lionetti, V., Bellincampi, D., and Zabotina, O. (2013). Cell wall integrity: targeted post-synthetic modifications to reveal its role in plant growth and defense against pathogens. *Plant Signal. Behav.* 8:e25435. doi: 10.4161/psb.25435

Richter, S., Müssig, J., and Gierlinger, N. (2011). Functional plant cell wall design revealed by the Raman imaging approach. *Planta* 233, 763–772. doi: 10.1007/s00425-010-1338-z

Roessner, U., and Bowne, J. (2009). What is metabolomics all about? *Biotechniques* 46, 363–365. doi: 10.2144/000113133

Sant'Anna, C., Costa, L. T., Abud, Y., Biancatto, L., Miguens, F. C., and de Souza, W. (2013). Sugarcane cell wall structure and lignin distribution investigated by confocal and electron microscopy. *Microsc. Res. Tech.* 76, 829–834. doi: 10.1002/jemt.22235

Sappl, P. G., and Heisler, M. G. (2013). Live-imaging of plant development: latest approaches. *Curr. Opin. Plant Biol.* 16, 33–40. doi: 10.1016/j.pbi.2012.10.006

Sarkar, P., Bosneaga, E., and Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *J. Exp. Bot.* 60, 3615–3635. doi: 10.1093/jxb/erp245

Schwessinger, B., Ronald, P. C., Schwessinger, B., and Ronald, P. C. (2012). Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* 63, 451–482. doi: 10.1146/annurev-arplant-042811-105518

Shaw, S. L. (2006). Imaging the live plant cell. *Plant J.* 45, 573–598. doi: 10.1111/j.1365-313X.2006.02653.x

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., et al. (2004). Toward a systems approach to understanding plant cell walls. *Science* 306, 2206–2211. doi: 10.1126/science.1102765

Stephens, D. J., and Allan, V. J. (2003). Light microscopy techniques for live cell imaging. *Science* 300, 82–86. doi: 10.1126/science.1082160

Sumner, L. W. (2006). "Current status and forward looking thoughts on LC/MS metabolomics," in *Plant Metabolomics*, Vol. 57, eds K. Saito, R. Dixon, and L. Willmitzer (Berlin: Springer Press), 21–32.

Sumner, L. W., Yang, D. S., Bench, B. J., Watson, B. S., Li, C., and Jones, A. D. (2011). "Spatially—resolved metabolomics—challenges for the future," in *The Biology of Plant Metabolomics,* Chapter 11, ed R. D. Hall (London: Blackwell-Wiley Press), 343–366.

Thomma, B. P. H. J., Cammue, B. P. A., and Thevissen, K. (2002). Plant defensins. *Planta* 216, 193–202. doi: 10.1007/s00425-002-0902-6

Timischl, B., Dettmer, K., Kaspar, H., Thieme, M., and Oefner, P. J. (2008). Development of a quantitative, validated Capillary electrophoresis-time of flight–mass spectrometry method with integrated high-confidence analyte identification for metabolomics. Electrophoresis 29, 2203–2214. doi: 10.1002/elps.200700517

Tong, C. B. S., and Gross, K. C. (1988). Glycosyl-linkage composition of tomato fruit cell wall hemicellulosic fractions during ripening. *Physiol. Plant.* 74, 365–370. doi: 10.1111/j.1399-3054.1988.tb00644.x

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Front. Plant Sci.* 3:85. doi: 10.3389/fpls.2012.00085

Updegraff, D. M. (1969). Semimicro determination of cellulose inbiological materials. *Anal. Biochem.* 32, 420–424. doi: 10.1038/nature11336

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. (2002). PMR6, a pectate lyase–like gene required for powdery mildew susceptibility in Arabidopsis. *Plant Cell* 14, 2095–2106. doi: 10.1105/tpc.003509

Vorwerk, S., Somerville, S., and Somerville, C. (2004). The role of plant cell wall polysaccharide composition in disease resistance. *Trends Plant Sci.* 9, 203–209. doi: 10.1016/j.tplants.2004.02.005

Wallace, I. S., and Anderson, C. T. (2012). Small molecule probes for plant cell wall polysaccharide imaging. *Front. Plant Sci.* 3:89. doi: 10.3389/fpls.2012.00089

Wang, T., Park, Y. B., Caporini, M. A., Rosay, M., Zhong, L., Cosgrove, D. J., et al. (2013). Sensitivity-enhanced solid-state NMR detection of expansin's target in plant cell walls. *Proc. Nat. Acad. Sci. U.S.A.* 110, 16444–16449. doi: 10.1073/pnas.1316290110

Wilson, R. H., Smith, A. C., Kačuráková, M., Saunders, P. K., Wellner, N., and Waldron, K. W. (2000). The mechanical properties and molecular dynamics of plant cell wall polysaccharides studied by Fourier-transform infrared spectroscopy. *Plant Physiol.* 124, 397–406. doi: 10.1104/pp.124.1.397

Wilt, B. A., Burns, L. D., Wei Ho, E. T., Ghosh, K. K., Mukamel, E. A., and Schnitzer, M. J. (2009). Advances in light microscopy for neuroscience. *Annu. Rev. Neurosci.* 32, 435–506. doi: 10.1146/annurev.neuro.051508.135540

Xia, Y., Lei, L., Brabham, C., Stork, J., Strickland, J., Ladak, A., et al. (2013). Characterization of culturable bacterial endophytes of switchgrass (*Panicum virgatum L.*) and their capacity to influence plant growth. *GCB Bioenergy* 5, 674–682. doi: 10.1111/j.1757-1707.2012.01208.x

Xia, Y., Lei, L., Brabham, C., Stork, J., Strickland, J., Ladak, A., et al. (2014). Acetobixan, an inhibitor of cellulose synthesis identified by microbial bioprospecting. *PLoS ONE* 9:e95245. doi: 10.1371/journal.pone.0095245

Zhang, M., Chen, G., Kumar, R., and Xu, B. Q. (2013). Mapping out the structural changes of natural and pretreated plant cell wall surfaces by atomic force microscopy single molecular recognition imaging. *Biotechnol. Biofuels* 6, 147–152. doi: 10.1186/1754-6834-6-147

Zhang, M., Wu, S. C., Zhou, W., and Xu, B. Q. (2012). Imaging and measuring single-molecule interaction between a carbohydrate-binding module and natural plant cell wall cellulose. *J. Phys. Chem.* 116, 9949–9956. doi: 10.1021/jp304686q

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2014; accepted: 23 September 2014; published online: 14 October 2014.

*Citation: Xia Y, Petti C, Williams MA and DeBolt S (2014) Experimental approaches to study plant cell walls during plant-microbe interactions. Front. Plant Sci. **5**:540. doi: 10.3389/fpls.2014.00540*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 03 June 2014
doi: 10.3389/fpls.2014.00249

Uncovering plant-pathogen crosstalk through apoplastic proteomic studies

Bertrand Delaunois, Philippe Jeandet, Christophe Clément, Fabienne Baillieul, Stéphan Dorey and Sylvain Cordelier*

Laboratoire Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne-EA 4707, Université de Reims Champagne-Ardenne, Reims, France

Edited by:
Jean-Pierre Metraux, Université de Fribourg, Switzerland

Reviewed by:
Murray Grant, University of Exeter, UK
Richard Bostock, University of California, Davis, USA

Correspondence:
Sylvain Cordelier, Laboratoire Stress, Défenses et Reproduction des Plantes, Unité de Recherche Vignes et Vins de Champagne-EA 4707, Université de Reims Champagne-Ardenne, Moulin de la Housse – BP 1039, 51687 Reims cedex 2, France
e-mail: sylvain.cordelier@univ-reims.fr

Plant pathogens have evolved by developing different strategies to infect their host, which in turn have elaborated immune responses to counter the pathogen invasion. The apoplast, including the cell wall and extracellular space outside the plasma membrane, is one of the first compartments where pathogen-host interaction occurs. The plant cell wall is composed of a complex network of polysaccharides polymers and glycoproteins and serves as a natural physical barrier against pathogen invasion. The apoplastic fluid, circulating through the cell wall and intercellular spaces, provides a means for delivering molecules and facilitating intercellular communications. Some plant-pathogen interactions lead to plant cell wall degradation allowing pathogens to penetrate into the cells. In turn, the plant immune system recognizes microbial- or damage-associated molecular patterns (MAMPs or DAMPs) and initiates a set of basal immune responses, including the strengthening of the plant cell wall. The establishment of defense requires the regulation of a wide variety of proteins that are involved at different levels, from receptor perception of the pathogen via signaling mechanisms to the strengthening of the cell wall or degradation of the pathogen itself. A fine regulation of apoplastic proteins is therefore essential for rapid and effective pathogen perception and for maintaining cell wall integrity. This review aims to provide insight into analyses using proteomic approaches of the apoplast to highlight the modulation of the apoplastic protein patterns during pathogen infection and to unravel the key players involved in plant-pathogen interaction.

Keywords: apoplast, cell wall, proteomics, secretome, pathogen, defense, MAMP

INTRODUCTION

Higher plants interact continuously with microbes such as viruses, bacteria, oomycetes or fungi, some of which are phytopathogens, leading to plant diseases. The lifestyle of the pathogen determines the nature of this interaction (Doehlemann and Hemetsberger, 2013). Biotrophic pathogens have developed specific strategies to interact with the cell wall and keep plant cells alive during their life cycles. In contrast, necrotrophic pathogens feed on dead plant cells. Plants naturally display preformed defenses, which include the cell wall and cuticle acting as a physical barrier. However, these preformed defenses are sometimes not strong enough to stop the invading pathogen. Successful resistance then comes from a rapid activation of the plant's innate immune system (Boller and Felix, 2009). Plant perception of conserved molecules characteristic of many microbes is the first step in this innate immune response. These molecules, more commonly called general elicitors, are also referred to microbe-associated molecular patterns (MAMPs) (Jones and Dangl, 2006; Pel and Pieterse, 2013). MAMPs are recognized by pattern recognition receptors (PRRs), which are generally localized at the level of the plasma membrane. MAMP perception leads to the establishment of the so-called MAMP-triggered immunity (MTI) (Boller and Felix, 2009). The small peptide flg22 derived from bacterial flagellin and the elongation-factor Tu peptide elf18 are the most extensively studied MAMPs (Felix and Boller, 2003; Zipfel, 2009; Trdá et al., 2013). Many other MAMPs have been identified (reviewed in Newman et al., 2013) such as eicosapolyenoic acids (Bostock et al., 1981; Savchenko et al., 2010), β-glucans (Umemoto et al., 1997; Klarzynski et al., 2000), peptidoglycans (Willmann et al., 2011), lipopolysaccharides (Newman et al., 2002; Erbs and Newman, 2012), rhamnolipids (Varnier et al., 2009; Sanchez et al., 2012), or chitin oligomers (Kaku et al., 2006; Miya et al., 2007). Pathogens can suppress MTI by secreting effector proteins that act either by inhibiting the MAMP-PRR interaction or downstream signaling. The direct or indirect recognition of effectors (previously called specific elicitors) by plant resistance gene products results in the so-called effector-triggered immunity (ETI) (Pel and Pieterse, 2013). ETI is usually quantitatively stronger than MTI and associated with more sustained and robust immune responses including localized cell death (HR, hypersensitive response) (Tsuda and Katagiri, 2010). Some plant-derived molecules called damage-associated molecular patterns (DAMPs) are also recognized by the plant itself and can trigger an immune response (Boller and Felix, 2009). The well-known systemin or oligogalacturonides released upon cell wall damage were shown to act as DAMPs (Pearce et al., 1991; Schweizer et al., 1996; Denoux et al., 2008; Brutus et al., 2010; Ferrari et al., 2013). Recently, the plant endogenous peptides AtPeps

have also been characterized as powerful DAMPs (Yamaguchi and Huffaker, 2011).

Induction of defenses by MAMPs, DAMPS or effectors starts within minutes after signal perception with ion fluxes, MAPK kinase activation, the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide (NO) (Garcia-Brugger et al., 2006; Scheler et al., 2013). ROS and NO can act in signaling and have direct antimicrobial effects. ROS are also involved in plant cell wall strengthening by oxidative cross-linking of polymers. During the plant immune response, the phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play a key role in signal transduction (Robert-Seilaniantz et al., 2011). The importance of JA and SA as primary signals in the regulation of plant immune responses has been well established (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). The JA pathway is primarily induced by and effective in mediating resistance against necrotrophic pathogens, whereas the SA pathway is primarily induced by and effective in mediating resistance against biotrophic pathogens (Glazebrook, 2005). Nonetheless, this is an over-simplistic view of disease resistance mechanisms as there are complex repertoires of plant hormones that play a role in defense signaling pathways. Indeed, other hormones such as auxin, abscisic acid, cytokinins, and brassinosteroids function as modulators of the plant immune signaling network (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). Not surprisingly, pathogenic microbes have developed strategies to manipulate plant hormonal pathways in order to divert the immune signaling mechanism for their own benefit. Recent studies suggest that these pathogen-induced modulations of signaling pathways via hormones contribute to virulence (Pieterse et al., 2012). Therefore, the complex crosstalk and induced hormonal changes modulate disease and resistance with the outcomes dependent on pathogen lifestyles and the genetic constitution of the host (Robert-Seilaniantz et al., 2011; Gimenez-Ibanez and Solano, 2013). Thus, pathogens have adapted different types of complex interactions. Biotrophs depend on the living plant metabolism as their nutritional source, and therefore interact intimately with the host cells to modify metabolic processes (Glazebrook, 2005; Horbach et al., 2011). Necrotrophs, on the other hand, invade and kill the plant tissue, feeding on the dead tissue debris. To this end, they usually macerate plant tissues by secreting toxins and abundant hydrolytic enzymes that degrade cell wall polymers (Laluk and Mengiste, 2010). As for the plant, defense responses occur upon pathogen recognition through reinforcement of the plant cell wall to counter pathogen invasion, while production of antimicrobial compounds such as phytoalexins (Jeandet et al., 2013) and synthesis of pathogenesis-related (PR) proteins including hydrolytic enzymes like β-1,3-glucanases and chitinases (Van Loon et al., 2006) contribute to the alteration of pathogen integrity.

Studies of plant-pathogen interactions are numerous in the literature and include a wide range of physiological, molecular and biochemical approaches. Proteomics has become an important tool for large-scale analysis of the proteins involved in the complex plant-pathogen interactions in the post-genomic area (for review see Quirino et al., 2010; Jayaraman et al., 2012). Characterization of a set of proteins under specific plant-pathogen interactions provides a more direct view of cellular processes than DNA or RNA analysis. Proteomics provides insight into protein localization, protein–protein interactions, enzymatic complexes or post-translational modifications that are essential to a better understanding of plant-pathogen interactions. Proteomic approaches have been used in recent years to further characterize plant interactions with viruses (Casado-Vela et al., 2006; Giribaldi et al., 2011; Li et al., 2011; Di Carli et al., 2012), bacteria (Jones et al., 2006; Afroz et al., 2009, 2013; Li et al., 2012), or fungi (Kim et al., 2004a; Geddes et al., 2008; Bhadauria et al., 2010; Mukherjee et al., 2010; Shah et al., 2012). The general picture of changes occurring in the plant-host proteome highlights common features among the broad range of interaction analyzed. A common response, observed in almost all studies, is related to plant photosynthetic activity, which is negatively regulated by pathogen infection, most probably reflecting allocation of energy resources to a general plant defense regulatory mechanism. In parallel, plants counteract to pathogen infection by modulating the accumulation of defense- or stress-associated proteins and proteins involved in ROS metabolism. However, most of the key proteins involved in the plant-pathogen interaction are probably produced at low levels and the majority of studies only detected the most abundant pathogen protein, such as coat protein for virus. These global approaches also present significant technical challenges, as they generally need to differentiate between plant and pathogen proteins (Mathesius, 2009). Simplified models have been developed to circumvent these technical challenges. In some studies, the plant was treated with a MAMP from a pathogen (Chivasa et al., 2006; Liao et al., 2009) or with a signal molecule (Rajjou et al., 2006; Macarisin et al., 2009) to characterize the proteomic changes within the plant. Other studies focused on the pathogen secretome alone (Brown et al., 2012; Girard et al., 2013) or in the presence of plant extracts (Phalip et al., 2005; Fernandez Acero et al., 2009). However, the absence of one of the actors in these simplified models underestimates the complexity of the events occurring during the plant-pathogen crosstalk. The large dynamic range of protein abundance present in plant-pathogen samples, such as pathogen-infected leaves represents an additional difficulty (Bindschedler and Cramer, 2011). In fact, many important proteins are present at low level and are thus difficult to isolate from complex mixtures containing more highly abundant proteins. As the resolution of analytical separation methods is too limited to dissect the total proteome of a cell or a tissue, less abundant proteins are often masked by those produced at higher levels. Sub-cellular proteomics has the advantage not only of relating proteins to a functional compartment of eukaryotic cells, but also of reducing the complexity of the whole cell or tissue protein extracts (Brunet et al., 2003). However, the isolation of subcellular proteins typically requires a series of labor-intensive steps. Thus, efficient protocols for sub-cellular fractionation, purification, and enrichment are necessary for each cellular compartment (Lee et al., 2013).

Important processes such as development, intercellular communications or defense mechanisms take place in the apoplast (Sakurai, 1998). The apoplastic proteins are involved in different physiological and biological processes related to growth regulation, biotic and abiotic stresses and cell wall maintenance

(Ellis et al., 2007; Tseng et al., 2009). The apoplast or apoplastic space is one of the first physiological compartments of pathogen-host exchanges and the key processes that occur there during microbial infections therefore determine the fate of the interaction (Doehlemann and Hemetsberger, 2013). The apoplast is defined as the extracellular matrix or plant cell wall and the intercellular spaces where the apoplastic fluid circulates (Agrawal et al., 2010). The apoplastic fluid plays a key role in intercellular and intracellular communications and is composed of many substances, notably nutrients, polysaccharides, secondary metabolites and secreted proteins. Lohaus et al. (2001) showed low metabolite concentrations in the apoplastic solution from *Vicia faba*, *Spinacia oleracea*, *Hordeum vulgare,* and *Zea mays*. The sucrose concentration was about 1–2 mM in all plant species, whereas the concentration of hexoses differed strongly between the species. Similarly, the highest concentration of amino acids in the apoplastic solution was found in *Vicia faba* (about 10 mM), the lowest in *Zea mays* (about 2 mM). It is also well known that the redox and pH control in the apoplast serves as a mechanism to respond to environmental signals. The main cation in the apoplastic solution of the analyzed plant species was potassium, representing about 70–80% of the total cation concentration when the main anions were nitrate and chloride. In *Spinacia oleracea*, oxalate was also an important anion, while apoplast from *Hordeum vulgare* contained high amounts of malate. The charge balance was equal and total contents of cations and anions were between 10 and 20 mM, respectively. The activity of plasma membrane-bound H^+-ATPase and membrane transport of solutes determine the pH condition of apoplast but pectic substances in the cell walls also affect the ion concentrations and pH in apoplast. The measured pH range of apoplast by pH electrode in apoplast of different plants varies from 4.5 to 7 (Sakurai, 1998).

The plant cell wall is mainly composed of polysaccharides such as celluloses, hemicelluloses and pectins, which interlock to form a dense and complex network. Additional compounds such as lignins, waxes or cutins are synthesized to form the secondary wall in specific differentiated cells (Carpita and Gibeaut, 1993). The cell wall acts as a passive barrier limiting the access of pathogens to plant cells and in turn, pathogens, especially necrotrophs, actively synthesize cell wall-degrading enzymes to penetrate or kill the plant cell (Laluk and Mengiste, 2010). The plant cell wall is actively remodeled and reinforced specifically at discrete sites of interaction with pathogens (Hamann, 2012; Underwood, 2012). This active reinforcement through the deposition of cell wall appositions, referred to as papillae, is one of the earliest responses to pathogen attacks (Micali et al., 2011). However, the cell wall proteome under a biotic stress is still poorly characterized. Identifying the proteins present in the apoplast during a pathogen infection is therefore essential to understanding the perception and regulation processes occurring between the two protagonists. A proteomic analysis that provides an overview of the protein pool at a given time is thereby an appropriate tool to address this issue and identify the actors involved in the interaction. Through this review, we will begin by pointing out the technical constraints to recovering apoplastic samples for proteomic analyses. We will then highlight the major findings obtained from the apoplastic proteome patterns during plant-pathogen interactions. In perspective, we will suggest future approaches to characterize early protein interconnections taking place between the pathogen and its host in the apoplast.

SECRETOME AND APOPLASTIC PROTEOME ISOLATION

Despite the importance of apoplastic proteome during a given plant-pathogen interaction, it remains poorly characterized compared to the intracellular proteome. This is especially due to the difficulty in obtaining sufficient apoplastic material without damaging the plant cell and in avoiding potential contamination of the sample with cytoplasmic proteins. The proteins secreted in the apoplast are either soluble in the apoplastic fluid or ionically bonded to the plant cell wall (Soares et al., 2007). The literature uses different terms for the extracellular proteome present in the apoplast, the most common being "secretome" and plant "cell wall proteome." For clarity and convenience, in this review, we will use the term "secretome" to designate secreted proteome obtained from *in vitro* cell suspension cultures, "apoplastic proteome" for soluble proteins present in the apoplastic fluid (generally extracted by the VIC method discussed below) and "cell wall proteome" for the secreted proteins that are loosely ionically bonded to the cell wall. The cell wall proteome is generally obtained from purified cell walls produced by disruptive methods. The cell wall proteome has been well investigated and excellent reviews have been published recently (Jamet et al., 2008; Rose and Lee, 2010; Albenne et al., 2013; Komatsu and Yanagawa, 2013).

Several studies have used suspension cell cultures such as *in vitro*-simplified models to develop practical, simple and non-destructive methods to isolate secreted proteins. The suspension cell cultures are easy to maintain, handle and scale up/down and the secreted proteins in the culture medium can be easily separated from suspended cells by filtration without cell disruption. Therefore, this system facilitates the extraction of freely soluble secreted proteins by the plant cells in suspension cultures and greatly limits potential contamination by cytoplasmic proteins. This simplified approach has been used to characterize basal secretomes of different species like *Arabidopsis* (Oh et al., 2005), alfalfa (Kusumawati et al., 2008), tobacco (Okushima et al., 2000), or rice (Cho et al., 2009). These *in vitro* systems have been used to assess cell responses more easily following signal molecules or fungal-derived elicitor treatments (**Table 1**). The effect of the well-known signal molecules SA and JA was characterized on *Arabidopsis* and grapevine cell suspension secretomes, respectively (Oh et al., 2005; Cheng et al., 2009; Martinez-Esteso et al., 2009). Comparative secretome studies have been performed with chitosan and *Fusarium*-based elicitors on *Arabidopsis* and maize (Ndimba et al., 2003; Chivasa et al., 2005). These studies revealed proteome changes in response to individual MAMPs but without taking into account the complexity of the responses triggered during a typical plant-microorganism interaction. To our knowledge, only two studies have directly used the pathogen itself, demonstrating that the study of plant-pathogen interactions has proven to be very difficult in these *in vitro* systems (Kaffarnik et al., 2009; Kim et al., 2009). Even then, the *in vitro* secretome analysis only provides partial identification of the secreted proteins

Table 1 | Main secretome studies on *in vitro* plant cell suspension under an elicitor or pathogen treatment.

Plant	Proteome type	Biotic treatment	Sample preparation	Analysis method	Key findings	References
Arabidopsis thaliana	S; CWP	Chitosan and Fusarium-based elicitor	Filtration and acetone precipitation	2D-PAGE and MALDI-MS or ESI-MS/MS	2 glucanases, 1 peroxidase, 1 chitinase, 1 polyglacturonase, 1 carboxypeptidase, and 1 receptor-like kinase differentially expressed in S or CWP. Potential extracellular phosphorylation of glucanase, chitinase and receptor-like kinase	Ndimba et al., 2003
Arabidopsis thaliana	S	SA 0.5 mM	Membrane filtration under vacuum lyophilisation and dialysis	2D-PAGE and MALDI-TOF/MS	13 differentially expressed proteins identified. Identification and characterization of GDSL motif lipase (GLIP1) involved in *A. brassisicola* resistance trough ethylene pathway	Oh et al., 2005
Arabidopsis thaliana	S	SA 0.5 mM and SA 1 mM	Membrane filtration, lyophilisation, dialysis, acetone precipitation	2D-PAGE and LC-MS and Q-TOF-MS	63 differentially expressed proteins within 2 h after SA treatment, mainly involved in metabolism (34%), defense (13%) or binding function (12%)	Cheng et al., 2009
Arabidopsis thaliana	S	*Pseudomonas syringae* p.v. Tomato strains: DC3000 (virulent), AvrRpm1 (avirulent) and HrpA (non-pathogenic)	Filtration and phenol extraction acetone precipitation	iTRAQ combined with LC-MS/MS and Q-TOF-MS	45 differentially expressed proteins identified, mainly involved in metabolism (18%), redox regulation (18%), defense (11%), or cell wall maintenance (26%). DC3000 and AvrRpm1 strains induce proteins without secretion peptide signal	Kaffarnik et al., 2009
Zea mays	S; CWP	Chitosan, H_2O_2 and Fusarium-based elicitor	S: filtration and acetone precipitation CWP: Cell-wall fragment isolation, $CaCl_2$/urea extraction and acetone precipitation	2D-PAGE and MALDI-TOF/MS and nanoHPLC-MS/MS	Glucosaminidase, glyceraldehyde-3P-dehydrogenase, peroxidase, and xylanase inhibitor are treatment-dependent regulated through probable dephosphorylation	Chivasa et al., 2005
Vitis vinifera (cv Gamay)	S	Methylated cyclodextrins 50 mM, and/or methyl jasmonate 0.1 mM	Centrifugation, ethylacetate extraction and TCA precipitation	2D-PAGE and MALDI-TOF/MS or LC-MS/MS	25 differentially expressed protein spots lead to identification of 10 proteins: peroxidases, chitinase, glucanase, thaumatin-like, lipase-like, PR27, endotransglycosylase, subtilisin-like protease	Martinez-Esteso et al., 2009
Oryza sativa	S	*Magnaporthe grisea* and its derived elicitor	Vacuum filtration, phenol-methanol-ammonium extraction and acetone wash	2D-PAGE and MALDI-TOF/MS or μLC-ESI-MS/MS	21 differentially expressed proteins identified with mainly defense-related functions (chitinases, oxalate oxidase) and domain unknown function 26 proteins (DUF26). Stronger and earlier induction of transcripts *in planta* with virulent strains	Kim et al., 2009

S, secretome; CWP, cell-wall proteome.

in comparison to the *in planta* apoplastic proteome analysis as demonstrated by the comparative analysis of *in vitro* secretome and leaf apoplastic proteomes in rice (Jung et al., 2008). Analyses of whole secreted proteins identified 222 protein spots with only 6 protein spots common to both *in planta* and *in vitro* samples. The proteins involved in cell wall metabolism in relation with plant defense mechanisms represent 18% of the total proteins identified *in planta* compared to 64% *in vitro*.

Since the *in vitro* approach does not provide a natural environment for the cells and because physiologically relevant treatments are difficult to apply, recent studies were instead carried out *in planta*. In this case, when a plant organ such as a leaf is required for study, apoplastic fluid is most commonly isolated using the vacuum infiltration centrifugation (VIC) method, well described by Lohaus et al. (2001) and Agrawal et al. (2010). In short, the plant leaves are thoroughly rinsed with a buffer to reduce the leaf surface tension and facilitate the vacuum infiltration. After infiltration with the adapted extraction buffer, the leaf surfaces are quickly dried to avoid sample dilution and the carefully rolled leaves are transferred to 50 ml polypropylene tubes with a washer at the bottom to avoid immersion of leaves into the collected apoplastic fluid. The gentle centrifugation allows the recovery of the apoplastic fluid from which apoplastic proteins are extracted by precipitation. The VIC method has been used to characterize the apoplastic proteome of different plant species such as *Arabidopsis* (Casasoli et al., 2008), rice (Cho et al., 2009), tobacco (Delannoy et al., 2008), maize (Witzel et al., 2011), alfalfa (Soares et al., 2007), pea (Wen et al., 2007), tomato (Konozy et al., 2012), or grapevine (Delaunois et al., 2013). This VIC method has also been used to assess the apoplastic proteome changes occurring after an elicitor, a pathogenic or non-pathogenic treatment (**Table 2**). Casasoli et al. (2008) have used oligogalacturonides on *Arabidopsis* to identify apoplastic candidate proteins involved in the response to this elicitor and the proteins associated with H_2O_2 response was investigated in the rice root apoplast (Zhou et al., 2011). Apoplastic proteome changes were studied during plant interactions with pathogenic bacteria (*Agrobacterium tumefaciens* and *Pseudomonas syringae*) and fungal pathogens, mainly *Verticillium longisporum* and *Magnaporthae oryzae* (**Table 2**). However, methodological adaptations might be necessary for efficient sample preparation. For instance, a water-displacement method was developed to obtain apoplast fluids from stem tissues in the poplar/*Melampsora medusa* interaction (Pechanova et al., 2010). The VIC technique allows the apoplastic proteome extraction without much cell damage. However, at times, the fragility of the samples leads to the rupture of the cytoplasmic membrane, triggering varying contamination by cytoplasmic proteins. The apoplastic fluid sample requires more stringent assessment of intracellular contamination to ensure apoplastic fraction purity. To estimate cytoplasmic contamination, enzyme activity, immunoblotting or electrolyte leakage can be used. Malate dehydrogenase activity is the most commonly used measure to estimate the level of membrane damage caused by the VIC method. Antibodies directed against malate dehydrogenase, RuBisCo or ATPase are also frequently used to determine the contamination level (Delaunois et al., 2013). Electrolyte leakage and concentration of malondialdehyde, which is a breakdown product of membrane lipid peroxidation, can also be used (Zhou et al., 2011).

PROTEIN SEPARATION,IDENTIFICATION, AND QUANTIFICATION TECHNIQUES

The main problem with the VIC technique is the extremely low yield implying that either very large volume of samples have to be produced and concentrated or very sensitive methods have to be used for proteomic analysis. A significant amount of information has been gained from proteomic studies using classical gel-based separation, as resolved proteins can often be identified and further characterized by mass spectrometry (MS). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) involves resolving proteins by isoelectric point (p*I*) and molecular weight (Görg et al., 2004; Kav et al., 2007). The 2D-PAGE has mainly been used for identifying plant protein abundance alterations in secretome or apoplastic proteomes in response to biotic stresses and still remains a viable technique (Shenton et al., 2012). Fluorescence difference gel electrophoresis (DIGE) was developed as a more quantitative form of 2D-PAGE. Here, samples are differentially covalently labeled with fluorophores, allowing for distinction between proteins resolved on the same gel (Casasoli et al., 2008). Casasoli et al. (2008) used this technique in *Arabidopsis* to identify the differential expression of 62 proteins in the same gel between control and oligogalaturonide-treated apoplastic proteomes. Both 2D-PAGE and DIGE approaches control for gel-to-gel variation, but do not overcome the issues of spot overlap (Campostrini et al., 2005). The gel-free methods utilizing liquid chromatography (LC) techniques for separating peptides after sequence-specific digestion can overcome this issue and significantly increase proteome coverage (Roe and Griffin, 2006). For example, multidimensional protein identification technology (MudPIT), which combines strong cation exchange with reversed phase chromatography, has been used on rice leaves infected with the fungus *Magnaporthe oryzae* (Kim et al., 2013). During this interaction, over 730 secretory proteins were identified in the apoplast by combining 2D-PAGE and MudPIT techniques, 40% and 60% of these corresponding to rice and *Magnaporthe oryzae*, respectively. Increasingly, gel-based, and gel-free separation methods are used together as complementary techniques to increase the number of identified proteins (De-La-Pena et al., 2008; Cheng et al., 2009; Pechanova et al., 2010; Kim et al., 2013).

Protein identification is performed by mass spectrometry (MS) analysis. The three main steps are protein or peptide ionization, ion separation and detection. Matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the two main ionization techniques that are applied in apoplastic proteome studies while ion separation and detection uses mainly time-of-flight (TOF) or quadrupole mass analyzer. Tandem MS (MS/MS) is now commonly used to improve the sensitivity and accuracy of peptides/proteins identification and the different techniques are very often combined (like MALDI-TOF/MS or ESI-MS/MS, **Tables 1**, **2**). The identification of proteins present in the apoplast in a given plant-pathogen interaction implies an access to a proteome and/or a genome database of the two organisms, which is not always the case. Alexandersson et al. (2013) suggest the use of a combined plant-pathogen protein database

Table 2 | Main apoplastic proteome studies after an elicitor or pathogen treatment.

Plant	Proteome type	Biotic treatment	Sample preparation	Analysis method	Key findings	References
Arabidopsis thaliana	AP	Oligogalacturonides (100 mg/mL)	Vacuum infiltration centrifugation and TCA precipitation	2D-DIGE and MALDI-TOF/MS or LC-MS/MS	16 differentially expressed proteins identified like polygalacturonase inhibitor, α-glucosidase, LRR protein, DUF26 receptor like Identified proteins putatively involved in pathogen perception and protein PTM regulated by OGs	Casasoli et al., 2008
Arabidopsis thaliana	AP	*Verticillium longisporum*	Vacuum infiltration centrifugation and TCA precipitation	2D-PAGE and ESI-LC-MS/MS	Specific increase in 6 proteins (3 peroxidases, 1 serine carboxypeptidase, 1 α-galactosidase, and 1 germin-like protein GLP3). Main function in carbohydrates modifications (25%), oxidoreductions (21%) proteases (18%),defense, and cell wall modification	Floerl et al., 2012
Arabidopsis thaliana and *Medicago sativa*	REP	*Pseudomonas syringae* p.v. Tomato DC3000 and *Sinorhyzobium meliloti* Rm1021	Filtration, concentration through ultracentrifugal filter and TCA precipitation	2D-PAGE and nanoHPLC-MS/MS	More than 100 identified proteins are differentially accumulated during plant microbe interaction.Seven plant proteins and four bacterial proteins increased during *S. meliloti/alfalfa* interaction and nine plant defense-related proteins increased during *P.syringae* DC3000/Arabidopsis compatible interaction	De-La-Pena et al., 2008
Brassica napus	AP	*Verticillium longisporum* spores on roots	Vacuum infiltration centrifugation and TCA precipitation	2D-PAGE and ESI-LC-MS/MS	Four differentially expressed proteins identified: basic glucanase, β-1,3-glucanase, basic endochitinase and peroxidase	Floerl et al., 2008
Populus deltoides	AP	*Melampsora medusae* and *Melampsora larici-populina*	Fluid exudation under pressure and phenol-methanol-ammonium extraction and acetone wash	2D-PAGE MALDITOF-MS/MS and 2D-LC ESI-MS/MS	Leaf and stem apoplast proteomes were analyzed with mainly stress/defence related proteins, cell wall metabolism, and antioxidative function (chitinases, glucanases, peroxidase, and antioxydant enzymes are the more represented)	Pechanova et al., 2010
Nicotiana benthamiana	AP	*Agrobacterium tumefaciens*	Vacuum infiltration centrifugation and chloroforme-methanol precipitation	2D-PAGE and LC-MS/MS	PR proteins increased greatly upon infection representing 45% of the spot volume and cell wall-modifying enzymes represents 15% of the total protein content	Goulet et al., 2010
Oryza sativa	AP	0.3 and 0.6 mM of hydrogen peroxide (H_2O_2)	Vacuum infiltration centrifugation, filtration, centrifugal concentration and TCA precipitation	2D-PAGE and MALDI-TOF/TOF and MS/MS	35 differentially expressed proteins identified, with around half related to redox state regulation and other involved in cell wall modification, signal transduction, cell defence, and carbohydrate metabolism	Zhou et al., 2011

(Continued)

Table 2 | Continued

Plant	Proteome type	Biotic treatment	Sample preparation	Analysis method	Key findings	References
Oryza sativa ssp. Japonica cv Kakehashi	AP	*Magnaporthe oryzae* Ken54-20 (incompatible) or Ina186-137 (compatible)	Vacuum infiltration centrifugation and TCA precipitation	2D-PAGE and ESI-LC-MS/MS	Three differentially expressed domain unknown function 26 proteins (DUF26) identified at 12 hpi and 5 defense-related proteins at 72 hpi	Shenton et al., 2012
Oryza sativa ssp. Japonica cv Jinheung	AP	*Magnaporthe oryzae* KJ401 (incompatible) or KJ301 (compatible)	Vacuum infiltration centrifugation and phenol-methanol-ammonium extraction and acetone wash	2D-PAGE/MudPIT and MALDI-TOF/MS or nESI-LC-MS/MS	More than 730 identified proteins with 40% and 60% derived from rice and *M oryzae*, respectively, mainly stress and ROS-related function for rice and metabolism and cell wall hydrolysis for *M oryzae*. Differential expression under compatible/incompatible interaction was confirmed by RT-PCR	Kim et al., 2013

AP, apoplastic proteome; REP, root exudates proteome.

extended with a random sequence database to avoid false positive hits from host peptides when matching pathogen peptides.

Plant-pathogen interaction mechanisms involve the fine modulation of protein amount. Precise and sensitive quantification methods of proteins become essential. Staining on polyacrylamide gels with Coomassie blue or silver nitrate is generally performed for spot quantification. However, quantifications on stained spots are difficult to interpret for several reasons: overlapping spots can occur, different proteins can be present in the same spot or some proteins can be present in different spots due to post-translational modifications (PTM), and degradation or maturation of proteins. The labeling of proteins with fluorescent dyes prior to electrophoresis (DIGE) can partially overcome some of these issues (Casasoli et al., 2008). More recently, protein quantification was significantly improved in proteomics using *in vitro* chemical (ICAT or iTRAQ) or *in vivo* metabolic (SILAC or ^{15}N-labeling) isotope-assisted quantification methods. For *in vivo* metabolic stable isotope labeling, cell suspension cultures or plants are grown on media supplemented with heavy isotope-containing amino acids or ^{15}N-labelled nutrients, allowing for the labeling of proteins as they are synthesized (Jayaraman et al., 2012). However, this approach is not always easy to implement *in planta* and requires long and powerful bioinformatics analysis. Kaffarnik et al. (2009) analyzed the secretome of *Arabidopsis* in response to infection by *Pseudomonas syringae* using a recently developed technique known as isobaric tag for relative and absolute quantification (iTRAQ). In this method, labeling is chemically performed *in vitro* on amines of digested peptide samples with commercially available iTRAQ (isobaric tags) reagents. The major advantage is that this strategy can be applied to directly compare up to eight separate samples in one experiment. With this technique Kaffarnik et al. (2009) compared apoplastic proteomes of *Arabidopsis* infected with three different strains of *Pseudomonas syringae* p.v. tomato, strain DC3000 (virulent), strain DC3000 carrying AvrRpm1 (avirulent) and strain DC3000 knocked-out for HrpA (non-pathogenic), suggesting a pathogen-mediated manipulation of apoplastic proteins. The development and the more systematic application of these isotope-assisted quantification and gel-free methods should allow the identification of low-abundance apoplastic proteins or small variations in their level of expression in the near future.

LEADERLESS SECRETION PROTEINS

Plant proteins are secreted to the apoplast mainly via the classical ER-Golgi route. SignalP or TargetP software is widely used to predict signal peptides from the sequences (Emanuelsson et al., 2007; Petersen et al., 2011) and to identify proteins that are secreted through the classical ER-Golgi. However, there is increasing evidence that a subset of apoplastic proteins is likely to be secreted by non-classical pathways. Non-classical or leaderless secretion is common to all eukaryotes, including plants. Computational analysis using different algorithms have been developed to assist in the identification of unexpected secreted proteins. SecretomeP allows secretion prediction based on sequence features conserved or frequently observed in secreted bacterial and mammalian proteins (Bendtsen et al., 2004). Cheng et al. (2009) found that 60% of the Leaderless Secreted Proteins (LSPs) identified in

the *Arabidopsis* secretome were predicted to be secreted with SecretomeP. Among these predicted LSPs actually found in the apoplast, we can mention calmodulin, jacalin, or superoxide dismutase. Although no identified plant superoxide dismutase has a signal peptide, extracellular superoxide dismutase activity in stressed or pathogen-infected plants has been previously reported (Hernández et al., 2001; Karpinska et al., 2001). In the same way, calmodulin is known to be an intracellular calcium sensor, but it has recently been suggested that calmodulin could serve as a dual messenger with roles either inside or outside the cell depending on stress factors (Cui et al., 2005). However, it should be noted that SecretomeP software may not be well-adapted to plant proteins since it has been designed for mammalian proteins. In addition, only a small proportion of the LSPs identified in apoplastic proteome studies gave a score above threshold (Cheng et al., 2009; Fernandez et al., 2012). The difficulties in preserving membrane integrity and extracting non-contaminated apoplastic fluids combined with the limitations of the bioinformatics programs in predicting sub-cellular localization have to be taken into account to understand the contrasting variations of LSPs content between experiments. New computational tools such as software or databases are emerging and should help to predict more precisely and with greater certainty the proteins produced through alternative secretion pathways. LocTree3 is a new software program that predicts protein subcellular localization through a consistent new framework with a high prediction success especially for secreted proteins (https://rostlab.org/services/loctree3) (Goldberg et al., 2012). The comparative platform OrysPSSP is composed of a core "small secreted protein" (SSP) database and a dynamic web interface that integrates a variety of user tools and resources and allows the screening of SSP on the genome scale and across the phylogeny of plant species (http://www.genoportal.org/PSSP/index.do) (Pan et al., 2013).

The existence of these alternative secretory routes could be explained by the need of rapid and effective regulation of secretion to provide a selective advantage to the plant cell. There is growing evidence of complex and highly coordinated spatiotemporal protein secretion in plants. Kaffarnik et al. (2009) showed that most of the proteins induced in *Arabidopsis* by the virulent *Pseudomonas syringae* DC3000 or the avirulent AvrRpm1 strains had no secretion signals as compared to non-pathogenic HrpA strains. Cheng et al. (2009) showed in *Arabidopsis* cell-suspensions treated with SA that 65%, 50%, and 35% of the secreted proteins lack a peptide signal after 1, 2, and 6 h, respectively. These results suggest that external stresses rapidly induce enhanced protein secretion. Other explanations could be the accumulation of inactive pre-proteins prior elicitation or post-translational modifications made by the ER environment, which may not be required for specific activity of apoplastic proteins (Rose and Lee, 2010).

POST-TRANSLATIONAL MODIFICATIONS

Proteomic studies lead very often to the identification of the same protein in different spots suggesting different post-translational modifications (PTMs) of the same protein (Chivasa et al., 2005). Indeed, PTMs are known to control many physiological processes by affecting protein structure, activity, and stability. Proteins can undergo different PTMs such as glycosylation, phosphorylation, carbonylation, or nitrosylation (Jayaraman et al., 2012; Albenne et al., 2013). The secretome analysis of *Arabidopsis* cell suspensions in response to oligogalacturonides highlighted several protein isoforms, such as an alpha xylosidase and a receptor-like kinase, showing differential PTMs (Casasoli et al., 2008). This observation may suggest a role for PTMs in the plant response to pathogens. Glycosylation is one of the most common and complex PTM. There are two main types of glycosylation, namely *N*- and *O*-glycosylation, but plant glycoproteins still remain poorly characterized. Glycoproteomics are currently applied to plants (Albenne et al., 2013). ConA lectin chromatography approaches were used to specifically isolate *N*-glycoproteins from *Arabidopsis* (Minic et al., 2007) and tomato (Català et al., 2011). The use of a multi-dimensional lectin chromatography system increased the coverage of the *Arabidopsis* cell wall glycoproteome leading mainly to the identification of *N*-glycosylated proteins (Zhang et al., 2011). The regulation of enzymes putatively involved in glycosylation, such as the disappearance of a $\beta - N-$ acetylglucosaminidase in the elicitor-treated maize secretome (Chivasa et al., 2005), suggests that glycosylation might also occur in the apoplast. The modulation of post-translational glycosylation would quickly regulate the activity and/or structure of targeted proteins, potentially strengthening the cell wall through stronger cross-linking.

Phosphorylation plays a key role in signal transduction and is based on the reversible regulation of the transfer of a phosphoryl group bonded to an aminoacid by protein kinases or removed by phosphatases. Although different phosphospecific staining techniques were developed for phosphoproteomic studies, LC-MS/MS analysis following gel-free separation and phosphopeptide enrichment is often the method of choice (Grimsrud et al., 2010). Upon perception of microbial signals, kinases and phosphatases target specific proteins, often modifying complex signaling cascades that allow for rapid defense responses. Ndimba et al. (2003) have shown that chitosan treatment of *Arabidopsis* cell-suspensions induce phosphorylation of a receptor-like kinase, endochitinases and glucanases. Similarly, Chivasa et al. (2005) have positively identified in the maize secretome phosphotyrosine protein spots that are rapidly dephosphorylated in response to *Fusarium*-elicitor treatment. The presence of elicitor-induced changes in the phosphorylation status of extracellular proteins suggests the existence of pathogen-induced, phosphorylation/dephosphorylation-regulated intercellular signaling via the extracellular matrix. Moreover the presence of phosphatases in the extracellular proteome of *Arabidopsis* infected by *Pseudomonas syringae* suggests that potential phosphorylation/dephosphorylation reversible regulation could occur in the apoplast (Kaffarnik et al., 2009). A recent comparison of *Lotus japonicus* roots elicited with symbiotic-(Nod factors) and the MAMP flg22 revealed differential phosphorylated protein patterns between symbiotic and defense responses (Serna-Sanz et al., 2011).

Carbonylation is considered as a marker of protein oxidation, which results from the direct oxidation of various aminoacids. This PTM is involved in the control of the protein function and can lead to their degradation (Lounifi et al., 2013). The early

oxidative burst in response to pathogen attacks is leading to an increase of protein carbonylation (Zhang et al., 2007). Although there is a strong link between ROS and pathogen attack on one side and the ROS and protein carbonylation on the other side, so far no large-scale study has been conducted on the regulation of protein carbonylation during a given plant-pathogen interaction. As mentioned before, analysis of the apoplastic proteome under biotic stress has revealed an important part of the proteome changes involved in the ROS metabolism. This correlation suggests that large changes in ROS metabolism-related proteome in the apoplast would influence the redox balance and consequently the protein carbonylation level. The resulting rapid PTM activates other defense-related proteins.

Protein nitrosylation is considered as one of the key mechanism regulating protein function (Lounifi et al., 2013). Since nitrosylation refers to the covalent bonding of a NO molecule to the cysteine amninoacid it becomes apparent that NO species produced upon plant pathogen interactions can exert their signaling action through nitrosylation of specific proteins (Corpas et al., 2008; Spoel and Loake, 2011). A large-scale proteomic study conducted on *Arabidopsis* leaves treated with gaseous NO led to the identification of 25 nitrosylated proteins which are involved in stress response, redox status, signaling, and cytoskeleton functional categories. *Pseudomonas syringae* infection of *Arabidopsis* seedlings leads to a hypersensitive response accompanied by an NO burst and triggers an increase of nitrosylated proteins. Most of the identified nitrosylated proteins were enzymes involved in intermediary metabolism, signaling, and antioxidant defenses (Romero-Puertas et al., 2008). Therefore the extent of protein nitrosylation could be expected to change in response to NO-originated stimuli governed by pathogen infection. Moreover, the occurrence of a biological connection between protein oxidation and nitrosylation in plants appears to be increasingly documented (Lin et al., 2012). Since the ROS-based protein carbonylation and the NO-based protein nitrosylation, as well as their interactions, seem to act as major regulatory systems in stress responses, the characterization of protein oxidation and nitrosylation in plant-pathogen interactions becomes crucial to the understanding of the various physiological processes occurring in the apoplast. Plant PTM proteomics is still in its early stages and is undoubtedly a promising approach to gain new insights into the structure and function of apoplastic proteins during pathogen infection.

MAIN FINDINGS FROM APOPLASTIC PROTEOMICS CASE STUDIES

PERCEPTION AND SIGNAL TRANSDUCTION

As mentioned before, early perception of the pathogen occurs in the apoplastic compartment and several proteomic studies have highlighted the regulation of apoplastic proteins potentially involved in pathogen perception and signal transduction. These apoplastic proteins generally feature LRR-type motifs suggesting a potential receptor role for these proteins and they sometimes undergo PTM like phosphorylation suggesting an involvement in signal transduction cascades. In the secretome of *Arabidopsis* suspension cultures elicited with chitosan, the phosphorylation of a cell wall bound putative receptor-like protein suggests that elicitor-treatment involves signal transduction cascades initiated in the apoplast through accumulation of phosphorylated extracellular receptor-like proteins (Ndimba et al., 2003). In the apoplast of *Arabidopsis* leaves elicited by oligogalacturonides, Casasoli et al. (2008) observed the accumulation of a disease resistance related LRR protein, characterized by a LRR domain composed of 13 repeats of the extracytoplasmic type (eLRRs), and previously localized in the plant cell wall (Borderies et al., 2003; Bayer et al., 2006) (**Figure 1**). The correlation of this LRR protein accumulation with the induction of its corresponding transcript as well as the reported induction of its gene during the incompatible interaction with *Alternaria brassicicola* suggest a role in pathogen perception (Schenk et al., 2003).

Receptor-like kinases with DUF26 domains are another class of interesting proteins related to biotic stress, regulated at both the protein and the transcript level, but with still unknown functions. DUF26 genes were previously found to be up-regulated upon pathogen infection, JA treatment (Kim et al., 2003, 2004b) and wounding (Shen et al., 2003). Recent studies have identified DUF26 proteins regulated upon pathogen infection or elicitor treatment (Kim et al., 2009, 2013; Zhou et al., 2011; Shenton et al., 2012). Several lines of evidence support the fact that these proteins could be involved in the perception of the pathogen or the transduction of the signal after perception. The proteins containing DUF26 domains, which are usually found in serine/threonine kinases, are annotated as small secretory proteins and are associated with plant receptor protein kinase domains in databases. These proteins accumulate earlier in incompatible rice/*Magnaporthe grisae* interactions than in compatible ones (Kim et al., 2009). Their extremely rapid accumulation was also demonstrated in the rice apoplast 12 h after infection with*Magnaporthe oryzea* (Shenton et al., 2012; Kim et al., 2013). Time-course profiling at the transcript level confirmed that their inductions were stronger and earlier in incompatible interactions.

Fine study of the apoplastic proteome during the early phase of the pathogen infection may also lead to the identification of proteins specifically produced by the pathogen and involved in the perception. For instance, a flagellin B homolog from *Agrobacterium tumefaciens* was shown to accumulate in the apoplast of tobacco agro-infiltrated leaves (Goulet et al., 2010) and the putative virulence factor cyclophilin CYP1 from *Magnaporthe* was found in the rice apoplast (Shenton et al., 2012). Similarly, De-La-Pena et al. (2008) showed evidence that bacteria are also able to change the proteins they secrete, depending on the identity of the plant partner. The elongation factor Tu was only secreted in the interaction between *Pseudomonas syringae* DC3000 and alfalfa but not with *Arabidopsis*, suggesting that stimulation of innate immunity by this bacterial protein could be plant-microbe dependent (De-La-Pena et al., 2008).

Kim et al. (2013) recently developed an interesting screening approach to identify putative apoplastic effectors secreted by *Magnaporthe oryzae* in rice leaves showing that some proteins activate the PBZ1 cell death related promoter only when they are expressed in the apoplast, acting putatively as apoplastic effectors. Therefore, apoplastic proteomic approaches may prove to be an interesting tool to discover or unravel key players involved in pathogen perception and signal transduction.

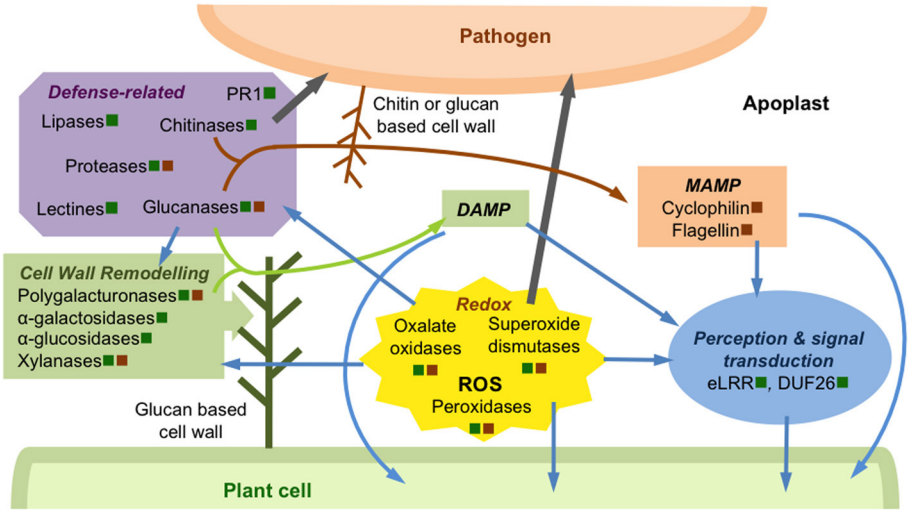


FIGURE 1 | Schematic overview of some events occurring in apoplast during plant pathogen interactions. This illustration, based on studies described in this paper, presents some examples of apoplastic proteins regulated during biotic stresses. These proteins are secreted by the plant cell (green square) and/or pathogen (brown square). Some proteins, modulated in the apoplast after DAMP or MAMP perception, are involved in pathogen perception and signal transduction, leading to the activation of intracellular plant defense signaling pathways (blue arrows). The regulation of a large amount of proteins involved in redox homeostasis modulates the ROS signaling pathway leading to activation of extra- and intracellular plant defense responses (blue arrows). These proteins, such as peroxidases or oxalate oxidases participate also to the plant cell defense through plant cell wall reinforcement or direct pathogen attack (gray arrow). Plant cell wall is actively remodeled and/or reinforced through the regulation of numerous enzymes, such as polyglacturonases or glucanases, which are secreted by the pathogen or its host. Some of the cell wall degradation products can act as DAMP (green arrow) to stimulate plant defense signaling pathways. Most of the defense-related proteins, such as chitinases or glucanases, act directly against the pathogen (gray arrow), releasing degradation products that can be perceived as MAMP by the plant cell (brown arrow).

ROS AND REDOX REGULATION

Secretome or apoplastic proteome studies under biotic stress conditions generally reveal changes of proteins involved in ROS metabolism, representing 10–30% of the secreted proteome (**Tables 1**, **2**). Members of most of these protein families, such as peroxidases, oxalate oxidases or superoxide dismutases, are generally present in the unstressed leaf apoplast (Delaunois et al., 2013). Unsurprisingly, the H_2O_2 treatment of rice seedlings triggers protein changes in the apoplast, 30% of which are involved in redox homeostasis. These proteins are involved in adjusting redox plant cell status, either triggering defense reactions or overcoming the deleterious effects of oxidative stress (**Figure 1**). It is noteworthy that a number of redox-associated enzymes such as some peroxidases are repressed at protein levels, which might modulate the H_2O_2 concentration to an appropriate level. At the same time, peroxidase accumulation in apoplast has been observed during the *Brassica napus/Verticillium longisporum* interaction (Floerl et al., 2008), in the secretome of grapevine cell suspension treated with MeJA (Martinez-Esteso et al., 2009) or in the secretome of *Arabidopsis* cell suspension treated with SA (Cheng et al., 2009), revealing that either the pathogen or a related molecule signal is able to modulate the level of peroxidases in the apoplast. Moreover, the peroxidase accumulation was correlated with their transcript accumulation in the *Populus/Melampsora* interaction (Pechanova et al., 2010) and the *Arabidopsis/Verticillium* interaction (Floerl et al., 2012). However, the time-course and the degree of transcript accumulation could be different from the corresponding protein levels. Finally, in maize secretome, some peroxidases do not change in quantity but are dephosphorylated after *Fusarium*-elicitor treatment (Chivasa et al., 2005) suggesting a precise regulation of peroxidases in the apoplast itself through PTM. Plants respond to bacterial challenge through quantitative and qualitative changes in peroxidase secretion leading to symbiotic or defense responses. During the rice-*Magnaporthe oryzae* interaction, Kim et al. (2013) have observed the modulation of 20 different peroxidases suggesting an intracellular ROS homeostasis to maintain a delicate equilibrium. Similarly, the root exudate proteome analyzed during the interaction between alfalfa and the bacterial symbiont *Sinorhizobium meliloti* or between *Arabidopsis* plants and an opportunistic bacterial pathogen *Pseudomonas syringae* revealed a complex and fine-tuned regulation of peroxidase amount depending on the plant-bacterium combinations (De-La-Pena et al., 2008). The existence of large multigenic families of peroxidases in plants (with 138 members in rice and 73 members in *Arabidopsis*) with a high number of enzymatic isoforms warrants their complex and fine-tuned regulation. Peroxidases were especially involved in a broad range of plant defense mechanisms such as lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis, and the metabolism of ROS and RNS (Almagro et al., 2009). It should be noted that during most biotic stress responses, the major sources of ROS seem to be due to cell wall localized peroxidases that generate hydrogen peroxide, or plasma membrane-localized NADPH/NADH oxidases that generate superoxide, or both systems operating in tandem (Bolwell, 1999; Daudi et al., 2012). Since the NADPH oxidases are plasma membrane localized

(Lherminier et al., 2009), unsurprisingly, they are not found in the apoplast of pathogen infected plants, whereas the amount of numerous peroxidases is finely modulated. The absence of these proteins in the apoplastic fluid could even be used as a marker of the plasma membrane integrity as well as H^+-ATPases.

Among all the proteins identified in the apoplastic proteome and involved in the regulation of ROS, we can also mention two oxalate oxidases (or germins) that highly accumulate in rice suspension-cultured cells treated with a rice blast fungus elicitor (Kim et al., 2009). The oxalate oxidases are involved in responses to biotic or abiotic stresses by producing H_2O_2 from oxalic acid. Since certain fungal pathogens produce oxalic acid, the oxalate oxidases present in apoplast could degrade the oxalic acid produced upon fungal infection to generate H_2O_2, which in turn may functions as a signal for plant defenses (**Figure 1**).

The antioxidant enzymes superoxide dismutases are also involved in ROS signaling and significantly accumulate in the secretome of *Arabidopsis* cell suspensions treated with SA (Cheng et al., 2009) or inoculated with *Pseudomonas syringae* (Kaffarnik et al., 2009). They also accumulated in the apoplastic poplar proteome infected by *Melampsora medusae* (Pechanova et al., 2010). Superoxide dismutase produce H_2O_2 from superoxides ($O_2^{\cdot -}$), which is further degraded to H_2O by ascorbate peroxidase. The superoxide dismutase could favor the transient nature of the oxidative burst and prevent accumulation of toxic superoxides, limiting the duration of the oxidative burst to an early event in plant defense (Scheler et al., 2013). Similarly, *Sinorhizobium meliloti* secretes higher amounts of superoxide dismutase in alfalfa than *Arabidopsis* roots, suggesting that the bacteria specifically recognize alfalfa to initiate the symbiosis (De-La-Pena et al., 2008).

Overall, these proteomic approaches reveal a strong implication of the apoplastic proteins involved in ROS homeostasis. They also highlight the fine regulation of these proteins requiring the control of their secretion as well as their activation through post-translational modifications depending on the plant-pathogen interaction.

CELL WALL MODIFICATION

As mentioned before, the cell wall is one of the most important barriers to counter pathogen invasion and it is not surprising to find in the unstressed apoplast numerous enzymes involved in cell wall modification or maintenance (Albenne et al., 2013; Komatsu and Yanagawa, 2013). It has already been described how the cell wall is actively remodeled and reinforced during infection specifically at discrete sites of interaction with pathogens (**Figure 1**) (Hamann, 2012; Underwood, 2012). However, a global view of the regulation of the enzymes specifically involved in the cell wall remodeling following a pathogen invasion is still incomplete. Apoplastic proteomic approaches definitely help to decipher the regulation of this complex metabolism involving numerous enzymes. Indeed, in most of the studies listed in **Tables 1, 2**, biotic stresses modulate the accumulation of secreted proteins involved in cell wall modification or maintenance like polygalacturonases, α-galactosidases, α-glucosidases, xylanases, xyloglucanases, and β-1,3-endoglucanases. Peroxidases (see above) also participate in cell wall reinforcement by modifying carbohydrate and structural protein polymer networks (Albenne et al., 2013) or through lignification or suberisation (Ndimba et al., 2003). Enzymes that breach the plant cell wall have also been shown to be important for fungal pathogens that lack specialized penetration structures and for necrotrophic pathogens. For example, the polygalacturonases are among the most extensively studied enzymes. They cleave the linkages between α-1,4 D-galacturonic acid residues, which are the major component of pectin, to produce non-methylated homogalacturonan. Polygalacturonases cause cell separation, tissue maceration, and release of mono- di- and trees-accharides used as nutrients by the pathogen (De Lorenzo et al., 2001). Some of these released fragments, such as oligogalacturonides, are typical DAMPs that elicit defense responses in many plants (Ridley et al., 2001; Sanabria et al., 2008; Ferrari et al., 2013) (**Figure 1**).

In the rice seedling apoplast, 45% of the proteins affected by H_2O_2 are involved in the carbohydrate and cell wall metabolism (Zhou et al., 2011). In this study, the abundance of most of the glycosylhydrolases such as α-galactosidases and β-1,3-glucanases, is found to be down-regulated. The authors suggested that the suppression of these polysaccharide hydrolases under H_2O_2 stress might reduce the hydrolysis of glucan and other polysaccharides altering the dynamic of remodeling of the polysaccharides to withstand the deleterious effects of oxidative stress. In comparison, the activities of α-L-arabinofuranosidases, UDP-glucose pyrophosphorylases and pectinesterases were up-regulated under H_2O_2 treatment suggesting that these enzymes might strengthen the cell wall by modulating polysaccharide degradation and synthesis, and increasing pectin demethylesterification. SA-treatment of *Arabidopsis* cell suspensions induces the accumulation of a large number of the proteins involved in general metabolism (34%) within the 6 first hours. Among them, α-galactosidase, α-1,4-glucan-protein synthase, pectinesterase, or β-fructofuranosidase are putatively involved in cell wall remodeling (Cheng et al., 2009). The fact that half of the identified secreted proteins are LSPs could partially explain the rapid extracellular accumulation of these proteins, which allows a rapid cell wall remodeling in response to defense signaling.

The agroinfiltration of tobacco leaves leads to the accumulation of a number of cell wall-modifying enzymes including galactosidases, α-L-arabinofuranosidases, β-D-xylosidases, peroxidases and proteases accounting for around 15% of the apoplastic proteins. These proteins are likely to be accumulated for cell wall maintenance and to complement constitutive defenses against bacterial pathogens (Goulet et al., 2010). *Verticillium longisporum* infection of *Arabidopsis* results in a specific increase of six extracellular proteins with overlapping functions in defense, development and cell wall metabolism (three peroxidases, germin, serine carboxypeptidase, α-galactosidase) (Floerl et al., 2012). The authors have correlated these changes in infected plants with a new synthesis of cell wall material with enhanced lignification and with a modification of metabolite contents.

Comparison of the apoplastic proteomes of *Arabidopsis* infected with virulent, avirulent or non-pathogenic strains of *Pseudomonas syringae* pv tomato revealed a strain-specific regulation of cell wall-modifying enzymes. For example, α-xylosidase accumulation is increased by MAMPs from the non-pathogenic

mutant strain HrpA. By contrast, amount of the enzyme is decreased by an effector of the virulent strain DC3000, suggesting that this α-xylosidase could be important for *Pseudomonas syringae* resistance (Kaffarnik et al., 2009). In the same way, quantity of two glycosylhydrolases is specifically increased during the compatible *Arabidopsis*/*Pseudomonas syringae* interaction but not in the incompatible interaction with *Sinorhizobium meliloti*, demonstrating a microorganism-dependent regulation of these cell wall enzymes (De-La-Pena et al., 2008).

Out of the 700 proteins identified in the rice apoplast infected by *Magnaporthe oryzae*, 29 proteins from rice and 54 proteins from *Magnaporthe oryzae* were glycosylhydrolases (Kim et al., 2013). Moreover, 17 rice glycosylhydrolase genes were strongly activated at the transcriptional level after infection. Among these 17 genes, 4 glycosylhydrolases were expressed earlier or at a higher level in the incompatible interactions compared to the compatible ones. From the pathogen side, RT-PCR analysis revealed that the transcripts of 6 glycosylhydrolases families from *Magnaporthe oryzae* were differentially expressed in compatible interactions. Therefore, this study demonstrated that the extracellular modulation of both the pathogen and the host glycosylhydrolases have an important role in either promoting successful infection *via* the degradation of the host cell wall, or restricting the pathogen invasion through the reinforcement of the host defenses *via* the cell wall maintenance during early stages of infection.

PR AND OTHER DEFENSE-RELATED PROTEINS

The defense-related proteins have various functions and are generally involved in different metabolisms other than defense. It is therefore relatively difficult to classify these proteins only in terms of their defense function. Some of these proteins such as peroxidases or oxalate oxidases have already been mentioned above. The defense-related proteins represent a large part of the basal apoplastic proteome including pathogenesis-related (PR) proteins, which are the most abundant (Delaunois et al., 2013). Unsurprisingly, the amount of the PR proteins is modulated in response to biotic stress in nearly all the secretome or apoplastic proteome studies listed in **Tables 1, 2**. However, despite the importance of PR proteins in plant defense, they generally only represent 10–15% of the proteins that are regulated in the apoplast following biotic stresses (Cheng et al., 2009; Kaffarnik et al., 2009; Zhou et al., 2011). The well-characterized chitinases degrade the cell walls of pathogen releasing PAMP-derived cell wall fragments that trigger MTI, thereby reinforcing the host defenses (**Figure 1**). Indeed, Kim et al. (2009) have identified up to nine chitinases induced by the rice blast fungus in the rice secretome. While chitinases are largely represented in the apoplast, not all of them are regulated in response to defense signaling. The analysis of the apoplastic proteomes of *Arabidopsis* infected with virulent or non-virulent strains of *Pseudomonas syringae* revealed that the effector of the virulent strain repressed two chitinases but only one is induced by MAMPs from the non-pathogenic strain (Kaffarnik et al., 2009). Similarly, two chitinases were identified in the *Arabidopsis* secretome in response to SA but only one was accumulated within the 2 first hours after treatment (Cheng et al., 2009). In response to H_2O_2, the rice apoplastic proteome analysis revealed the up-regulation of one chitinase and the down-regulation of two others (Zhou et al., 2011). All together, these results clearly indicate a pathogen- or signal-specific regulation of the chitinase pool in the apoplast.

Glucanases represent another large apoplastic protein family often co-induced with chitinases (**Figure 1**). Glucanases are known to limit fungal growth *via* the degradation of the glucans from fungal cell walls. *Verticillum longisporum* infection of *Brassica napus* induces the accumulation of one endochitinase and two β-1,3-glucanases (Floerl et al., 2008). *Agrobacterium* infiltration of tobacco leaves modulates the quantity of several chitinases and glucanases (Goulet et al., 2010). Moreover, the changes in the phosphorylation status of an endochitinase and an endo-1,4-β-glucanase revealed by the chitosan treatment of *Arabidopsis* cells (Ndimba et al., 2003) and the correlation of the transcript accumulation with the increase of acidic chitinases and β-1,3-glucanases in *Melampsora larici*/*Populus deltoides* interaction suggest a close regulation of these PR protein families (Pechanova et al., 2010). It should also be mentioned that glucanases could be involved in host cell wall remodeling putatively leading to the release of DAMPs molecules and thereby reinforcing the host defenses (Casasoli et al., 2008; Martinez-Esteso et al., 2009). Besides direct modifications that glucanases can produce in the cell wall, Finiti et al. (2013) suggested that they might interfere in the signaling network that operates during the defense response. Their enzymatic products, the β-1,3 glucans, can be considered as DAMPs and are known to be general elicitors of plant defense responses. The β-1,3 glucans were shown to induce variety of defense reactions in tobacco (Klarzynski et al., 2000), *Arabidopsis* (Ménard et al., 2004), or grapevine (Aziz et al., 2003, 2007), conferring resistance to viral, bacterial, and fungal pathogens. Moreover previous studies have demonstrated that the absence of the endoglucanases TomCel1 and TomCel2 in tomato and *Arabidopsis* alters the jasmonic acid signaling network limiting the necrotrophic pathogen *Botrytis cinerea* invasion and increasing the susceptibility to the hemibiotrophic *Pseudomonas syringae* DC3000 (Flors et al., 2007; Finiti et al., 2013). These results provide support for the contribution of endoglucanases in the establishment of the appropriate signaling response to pathogens by modifying the properties of the cell wall and/or interfering with signaling pathways.

Other PR proteins are regulated in the apoplast following biotic stress. The PR1 protein is accumulated in the apoplast of agroinfiltrated tobacco or H_2O_2-treated rice and the thaumatin-like protein accumulates in *Melampsora larici* infected poplar (Goulet et al., 2010; Zhou et al., 2011). Some lipases were also thought to act like PR proteins (Jakab et al., 2003). A lipase with a GDSL-like motif was identified in the grapevine secretome in response to JA (Martinez-Esteso et al., 2009) and in the *Arabidopsis* secretome in response to SA (Oh et al., 2005). The *Arabidopsis* lipase (GLIP1) was further characterized for its function in disease resistance and results suggest that GLIP1 may be a critical component in plant resistance. Indeed, the GLIP1 lipase disrupts the fungal spore integrity and triggers systemic resistance signaling in *Alternaria brassicicola* infected plants through the ET pathway (Oh et al., 2005).

Proteolytic enzymes in plants are directly or indirectly involved in most plant cellular processes including disease resistance (Xia

et al., 2004). The induction of the amount of three subtilisin-like proteases, two aspartyl proteases, and one peptidase in the rice apoplast during *Magnaporthe oryzae* infection supports the view that secreted proteolytic enzymes might act as hydrolytic enzymes or mediators of signal transduction in the apoplast during pathogen attack (Kim et al., 2013). In this study, a total of 25 proteases/peptidase proteins from *Magnaporthe oryzae* were identified. These proteins are believed to play roles as pathogenicity factors required to circumvent the host defense responses. Therefore, the study of protease secretion in the apoplast is a promising resource for understanding some facets of plant-pathogen interactions.

Lectins are characterized by the presence of at least one jacalin-like domain that reversibly binds specific mono- or oligosaccharides. According to their carbohydrate specificities, plant lectins are important for a variety of biological processes including host–pathogen interactions. Specifically, they are believed to play a role in pathogen recognition and in plant defense responses (De Hoff et al., 2009). Several studies have shown their accumulation in the apoplast in response to SA treatment, chitosan or oligogalacturonides in *Arabidopsis* (Ndimba et al., 2003; Casasoli et al., 2008; Cheng et al., 2009) or in the rice-*Magnaporthe* interaction (Kim et al., 2009). Their regular identification in the apoplast under biotic-stress conditions reinforces their putative role in plant defense mechanisms.

In the rice-*Magnaporthe* interaction, the accumulation of several PR proteins is induced 72 h after infection in both the compatible and incompatible interactions but with a higher level in the incompatible interaction (Shenton et al., 2012). In the same plant-pathogen interaction, Kim et al. (2013) correlated the PR protein accumulation with upregulation of gene expression in both types of interactions and showed that three chitinase genes were expressed earlier or at a higher level in the incompatible interactions. Six proteins related to defense, such as peroxidases and basic chitinases were highly secreted in *Arabidopsis* 6 h after initial contact with *Pseudomonas syringae* but not in the incompatible interaction with *Sinorhizobium meliloti*, suggesting that *Arabidopsis* can selectively secrete defense proteins at an early stage of compatible interactions (De-La-Pena et al., 2008). Moreover, in the interaction between alfalfa with *Sinorhizobium meliloti* or *Pseudomonas syringae,* three chitinases, a thaumatin-like protein PR-5b, and a PR10-1 protein were secreted in abundance by alfalfa inoculated with *Sinorhizobium meliloti* at 6 h but were not secreted as much when it was inoculated with *Pseudomonas syringae*. The fact that alfalfa responds faster by secreting proteins in the presence of *Sinorhizobium meliloti*, but not in the presence of *Pseudomonas syringae*, suggests that an efficient signaling process similar to that operating during pathogenic interactions takes place during the early interaction with *Sinorhizobium meliloti*.

CONCLUSIONS AND FUTURE PERSPECTIVES

Interest in the plant defense responses occurring in the apoplast is growing as the importance of this dynamic compartment becomes more apparent. The small number of studies indicates the limited availability of information on the potential role of the apoplastic proteome in plant-pathogen interactions. The first secretome analyses were reported using isolated MAMPs or signal molecule onto cell suspension cultures and, to our knowledge, only two secretome studies have been performed using intact pathogens (**Table 1**). A shift from cell-suspensions to *in planta* systems has taken place, but comparative disease-resistance studies are still scarce and little is known about the changes in the secretome during biotic stresses (**Table 2**). Based on the results of the proteomic studies reviewed here, our current understanding of biological processes occurring in the apoplast during plant–pathogen interactions is still rudimentary. Most of the proteins present in the apoplast are involved in the establishment of a basal defense in unstressed plants. Only a small number of these proteins are specifically modulated following the perception of a biotic stress.

Most of the studies listed in this review highlighted the regulation of the same families of proteins occurring in the apoplast during a biotic stress. The regulated proteins potentially involved in the mechanisms of perception and signal transduction such as DUF26 or LRR-like proteins appear to be less identified and/or characterized. The regulation of peroxidases, glucanases and chitinases is also emphasized since these large families of proteins are involved in the regulation of the cell redox status, the cell wall reorganization and the establishment of specific defenses (**Figure 1**). The general consensus suggests that accurate control of the speed and intensity of the protein secretion determines the establishment of effective resistance against a given pathogen.

The LSPs identified in most of the apoplastic proteomes in response to pathogen attacks may be one of the solutions used to increase the speed of protein secretion. Indeed, this secretory mechanism, independent of the classical ER-Golgi secretory pathway, could allow the rapid and efficient secretion of specific proteins providing a selective advantage in response to pathogen infection (Rose and Lee, 2010). Since most of the non-classically secreted proteins have established intracellular functions, it was suggested that they had dual roles with still unknown extracellular functions. There is growing evidence about the role of LSPs in plant defenses and the precise identification and characterization of these secreted proteins remain an exciting and challenging area of research.

Another way to increase the speed and specificity of the defense response in the apoplast may be the modulation of the PTMs of a pool of pre-proteins already present or the existence of several alternative PTMs affecting the final destination of the protein. Most of the apoplastic proteome studies in response to pathogen attacks suggested modulation of PTMs. Modification of PTMs could rapidly activate or repress the specific proteins involved in pathogen perception (like glycosylation) or signal transduction (like phosphorylation). It was demonstrated that the phosphorylation status of extracellular proteins rapidly changes in response to elicitor treatment, suggesting a possible role for the apoplastic proteins in early signal transmission of pathogen defenses through the activation of pathways regulated by external kinases and phosphatases (Casasoli et al., 2008). The establishment of plant phosphoproteomes has made remarkable progress and is now moving from qualitative to quantitative. However, more work needs to be done to investigate the precise phosphorylation nature and phosphorylation patterns. Moreover, there has

been no global study of the glycosylation of apoplastic proteins upon pathogen infection. Plant glycoproteomics is only in its early stages but is a very promising approach toward an integrated study of both sugars and protein moities to gain new insight into the function of glycoproteins in plant defenses. Similarly, little is known about the oxidation of apoplastic proteins even if ROS and NO are important molecules playing key roles in apoplastic plant defenses. The study of PTMs is still in its initial phases, and although instrumentation and separation techniques can be improved, for many PTMs there are some existing methods available that can be adapted to plant disease proteomics research. Undoubtedly, future work needs to be directed toward a better understanding of the possible extracellular PTM events since the ability to define the dynamic proteome is crucial for unraveling novel mechanisms of plant-pathogen signaling.

It was suggested that secreted proteins might be a critical component in the process of signaling and recognition occurring between compatible and incompatible interactions. Infection of rice or *Arabidopsis* with an incompatible pathogen leads to a much earlier induction of genes and proteins than for a compatible interaction (Kaffarnik et al., 2009; Kim et al., 2013). These results highlight the importance of the early stages in the infection process and demonstrate the need for kinetic studies addressing complex organism interactions. More in-depth analyses of the spatial and temporal distribution of responding proteins will improve the understanding of pathogen invasion strategies and the complex interplay between hosts and pathogens. Future studies should focus on differential approaches based on compatible/incompatible interactions by using virulent/avirulent pathogen strains or sensitive/resistant host species. However, these studies should also include kinetics of apoplastic proteome and cell wall proteome at the early steps of the infection process to obtain an dynamic view of identified soluble and ionically bonded proteins from both the plant and the pathogen. Since a down-regulation of a protein upon pathogen attack might indicate regulation by pathogen effectors, functional analysis of a subset of identified secretory proteins from the pathogen implies that a number of them are likely to act as apoplastic effectors that can be recognized by receptors (Kim et al., 2013). As more and more evidence points to the biological role of the fungal effectors that manipulate plant immunity in favor of fungal virulence, the development of reliable quantitative proteomics will indeed be crucial to identifying putative effector targeting in the apoplastic proteome.

Thus, it is important to build a comprehensive inventory of the experimentally identified plant–pathogen secretome to predict secreted proteins more accurately, and then to address the question of their biological role. Apoplastic proteome analyses of plant–pathogen interactions have provided a better understanding of plant defense responses. However, the lack of published studies using quantitative and *in vivo* proteomic techniques is still striking. The improvement of peptide resolution sensitivity based on gel-free technology and the precise and absolute peptide quantification based on isotopic labeling approaches, such as iTRAQ technology, should greatly increase the number of identified apoplastic proteins upon pathogen challenge. The utility of absolute quantification of individual secreted proteins was clearly demonstrated in application to complex, time- and dose dependent experimental designs. There is also a need for performing more biological conditions rather than just technical replicates in experiments for quantification. Moreover, combining proteomic analyses with genetics and other omic approaches would strengthen the biological significance of many studies. A more systematic integration of these complementary approaches will provide useful information that will allow for better prediction and manipulation of plant responses to pathogens. Nevertheless, one of the main challenges in the near future will be to validate and explore the roles of individual secreted proteins involved in plant-pathogen interactions. While most proteomic studies provide protein identification and functional predictions, most of them do not test their hypotheses using genetics. Further studies will then be needed to assign functional roles to these secreted proteins in plant-pathogen interactions.

ACKNOWLEDGMENTS

The authors are grateful to Grace Stockton for editorial assistance. This work was supported by the "Region Champagne Ardennes" through the project VINEAL2 and by "Comité Champagne" (Epernay, France).

REFERENCES

Afroz, A., Khan, M. R., Ahsan, N., and Komatsu, S. (2009). Comparative proteomic analysis of bacterial wilt susceptible and resistant tomato cultivars. *Peptides* 30, 1600–1607. doi: 10.1016/j.peptides.2009.06.005

Afroz, A., Zahur, M., Zeeshan, N., and Komatsu, S. (2013). Plant-bacterium interactions analyzed by proteomics. *Front. Plant Sci.* 4:21. doi: 10.3389/fpls.2013.00021

Agrawal, G. K., Jwa, N.-S., Lebrun, M.-H., Job, D., and Rakwal, R. (2010). Plant secretome: unlocking secrets of the secreted proteins. *Proteomics* 10, 799–827. doi: 10.1002/pmic.200900514

Albenne, C., Canut, H., and Jamet, E. (2013). Plant cell wall proteomics: the leadership of Arabidopsis thaliana. *Front. Plant Sci.* 4:111. doi: 10.3389/fpls.2013.00111

Alexandersson, E., Ali, A., Resjö, S., and Andreasson, E. (2013). Plant secretome proteomics. *Front. Plant Sci.* 4:9. doi: 10.3389/fpls.2013.00009

Almagro, L., Gómez Ros, L. V., Belchi-Navarro, S., Bru, R., Ros Barceló, A., and Pedrenō, M. A. (2009). Class III peroxidases in plant defence reactions. *J. Exp. Bot.* 60, 377–390. doi: 10.1093/jxb/ern277

Aziz, A., Gauthier, A., Bézier, A., Poinssot, B., Joubert, J.-M., Pugin, A., et al. (2007). Elicitor and resistance-inducing activities of β-1,4 cellodextrins in grapevine, comparison with β-1,3 glucans and α-1,4 oligogalacturonides. *J. Exp. Bot.* 58, 1463–1472. doi: 10.1093/jxb/erm008

Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bezier, A., Lambert, B., et al. (2003). Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. *Mol. Plant Microbe Interact.* 16, 1118–1128. doi: 10.1094/MPMI.2003.16.12.1118

Bayer, E. M., Bottrill, A. R., Walshaw, J., Vigouroux, M., Naldrett, M. J., Thomas, C. L., et al. (2006). *Arabidopsis* cell wall proteome defined using multidimensional protein identification technology. *Proteomics* 6, 301–311. doi: 10.1002/pmic.200500046

Bendtsen, J. D., Jensen, L. J., Blom, N., Von Heijne, G., and Brunak, S. (2004). Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng. Des. Sel.* 17, 349–356. doi: 10.1093/protein/gzh037

Bhadauria, V., Banniza, S., Wang, L.-X., Wei, Y.-D., and Peng, Y.-L. (2010). Proteomic studies of phytopathogenic fungi, oomycetes and their interactions with hosts. *Eur. J. Plant Pathol.* 126, 81–95. doi: 10.1007/s10658-009-9521-4

Bindschedler, L. V., and Cramer, R. (2011). Quantitative plant proteomics. *Proteomics* 11, 756–775. doi: 10.1002/pmic.201000426

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by

pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Bolwell, G. P. (1999). Role of active oxygen species and NO in plant defence responses. *Curr. Opin. Plant Biol.* 2, 287–294. doi: 10.1016/S1369-5266(99)80051-X

Borderies, G., Jamet, E., Lafitte, C., Rossignol, M., Jauneau, A., Boudart, G., et al. (2003). Proteomics of loosely bound cell wall proteins of Arabidopsis thaliana cell suspension cultures: a critical analysis. *Electrophoresis* 24, 3421–3432. doi: 10.1002/elps.200305608

Bostock, R. M., Kuc, J. A., and Laine, R. A. (1981). Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiterpenes in the potato. *Science* 212, 67–69. doi: 10.1126/science.212.4490.67

Brown, N. A., Antoniw, J., and Hammond-Kosack, K. E. (2012). The predicted secretome of the plant pathogenic fungus *Fusarium graminearum*: a refined comparative analysis. *PLoS ONE* 7:e33731. doi: 10.1371/journal.pone.0033731

Brunet, S., Thibault, P., Gagnon, E., Kearney, P., Bergeron, J. J., and Desjardins, M. (2003). Organelle proteomics: looking at less to see more. *Trends Cell Biol.* 13, 629–638.

Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9452–9457. doi: 10.1073/pnas.1000675107

Campostrini, N., Areces, L. B., Rappsilber, J., Pietrogrande, M. C., Dondi, F., Pastorino, F., et al. (2005). Spot overlapping in two-dimensional maps: a serious problem ignored for much too long. *Proteomics* 5, 2385–2395. doi: 10.1002/pmic.200401253

Carpita, N. C., and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* 3, 1–30. doi: 10.1111/j.1365-313X.1993.tb00007.x

Casado-Vela, J., SelleìS, S., and Martínez, R. B. (2006). Proteomic analysis of tobacco mosaic virus-infected tomato (*Lycopersicon esculentum* M.) fruits and detection of viral coat protein. *Proteomics* 6, 196–206. doi: 10.1002/pmic.200500317

Casasoli, M., Spadoni, S., Lilley, K. S., Cervone, F., De Lorenzo, G., and Mattei, B. (2008). Identification by 2-D DIGE of apoplastic proteins regulated by oligogalacturonides in *Arabidopsis thaliana*. *Proteomics* 8, 1042–1054. doi: 10.1002/pmic.200700523

Català, C., Howe, K. J., Hucko, S., Rose, J. K. C., and Thannhauser, T. W. (2011). Towards characterization of the glycoproteome of tomato (*Solanum lycopersicum*) fruit using Concanavalin A lectin affinity chromatography and LC-MALDI-MS/MS analysis. *Proteomics* 11, 1530–1544. doi: 10.1002/pmic.201000424

Cheng, F.-Y., Blackburn, K., Lin, Y.-M., Goshe, M. B., and Williamson, J. D. (2009). Absolute protein quantification by LC/MSE for global analysis of salicylic acid-induced plant protein secretion responses. *J. Proteome Res.* 8, 82–93. doi: 10.1021/pr800649s

Chivasa, S., Hamilton, J. M., Pringle, R. S., Ndimba, B. K., Simon, W. J., Lindsey, K., et al. (2006). Proteomic analysis of differentially expressed proteins in fungal elicitor-treated Arabidopsis cell cultures. *J. Exp. Bot.* 57, 1553–1562. doi: 10.1093/jxb/erj149

Chivasa, S., Simon, W., Yu, X. L., Yalpani, N., and Slabas, A. (2005). Pathogen elicitor-induced changes in the maize extracellular matrix proteome. *Proteomics* 5, 4894–4904. doi: 10.1002/pmic.200500047

Cho, W. K., Chen, X. Y., Chu, H., Rim, Y., Kim, S., Kim, S. T., et al. (2009). Proteomic analysis of the secretome of rice calli. *Physiol. Plant.* 135, 331–341. doi: 10.1111/j.1399-3054.2008.01198.x

Corpas, F. J., Del Río, L. A., and Barroso, J. B. (2008). Post-translational modifications mediated by reactive nitrogen species. *Plant Signal. Behav.* 3, 301–303. doi: 10.4161/psb.3.5.5277

Cui, S., Guo, X., Chang, F., Cui, Y., Ma, L., Sun, Y., et al. (2005). Apoplastic calmodulin receptor-like binding proteins in suspension-cultured cells of *Arabidopsis thaliana*. *J. Biol. Chem.* 280, 31420– 31427. doi: 10.1074/jbc.M501349200

Daudi, A., Cheng, Z., O'Brien, J., Mammarella, N., Khan, S., Ausubel, F., et al. (2012). The apoplastic oxidative burst peroxidase in *Arabidopsis* is a major component of pattern-triggered immunity. *Plant Cell* 24, 275–287. doi: 10.1105/tpc.111.093039

De Hoff, P. L., Brill, L. M., and Hirsch, A. M. (2009). Plant lectins: the ties that bind in root symbiosis and plant defense. *Mol. Genet. Genom.* 282, 1–15. doi: 10.1007/s00438-009-0460-8

De Lorenzo, G., D'Ovidio, R., and Cervone, F. (2001). The role of polygacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu. Rev. Phytopathol.* 39, 313–335. doi: 10.1146/annurev.phyto.39.1.313

De-La-Pena, C., Lei, Z., Watson, B. S., Sumner, L. W., and Vivanco, J. M. (2008). Root-microbe communication through protein secretion. *J. Biol. Chem.* 283, 25247–25255. doi: 10.1074/jbc.M801967200

Delannoy, M., Alves, G., Vertommen, D., Ma, J., Boutry, M., and Navarre, C. (2008). Identification of peptidases in *Nicotiana tabacum* leaf intercellular fluid. *Proteomics* 8, 2285–2298. doi: 10.1002/pmic.200700507

Delaunois, B., Colby, T., Belloy, N., Conreux, A., Harzen, A., Baillieul, F., et al. (2013). Large-scale proteomic analysis of the grapevine leaf apoplastic fluid reveals mainly stress-related proteins and cell wall modifying enzymes. *BMC Plant Biol.* 13:24. doi: 10.1186/1471-2229-13-24

Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., et al. (2008). Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings. *Mol. Plant* 1, 423–445. doi: 10.1093/mp/ssn019

Di Carli, M., Benvenuto, E., and Donini, M. (2012). Recent insights into plant-virus interactions through proteomic analysis. *J. Proteome Res.* 11, 4765–4780. doi: 10.1021/pr300494e

Doehlemann, G., and Hemetsberger, C. (2013). Apoplastic immunity and its suppression by filamentous plant pathogens. *New Phytol.* 198, 1001–1016. doi: 10.1111/nph.12277

Ellis, J. G., Dodds, P. N., and Lawrence, G. J. (2007). The role of secreted proteins in diseases of plants caused by rust, powdery mildew and smut fungi. *Curr. Opin. Microbiol.* 10, 326–331. doi: 10.1016/j.mib.2007.05.015

Emanuelsson, O., Brunak, S., Von Heijne, G., and Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protocol.* 2, 953–971. doi: 10.1038/nprot.2007.131

Erbs, G., and Newman, M. A. (2012). The role of lipopolysaccharide and peptidoglycan, two glycosylated bacterial microbe-associated molecular patterns (MAMPs), in plant innate immunity. *Mol. Plant Pathol.* 13, 95–104. doi: 10.1111/j.1364-3703.2011.00730.x

Felix, G., and Boller, T. (2003). Molecular sensing of bacteria in plants. *J. Biol. Chem.* 278, 6201–6208. doi: 10.1074/jbc.M209880200

Fernandez Acero, F. J., Colby, T., Harzen, A., Cantoral, J. M., and Schmidt, J. (2009). Proteomic analysis of the phytopathogenic fungus *Botrytis cinerea* during cellulose degradation. *Proteomics* 9, 2892–2902. doi: 10.1002/pmic.200800540

Fernandez, M. B., Pagano, M. R., Daleo, G. R., and Guevara, M. G. (2012). Hydrophobic proteins secreted into the apoplast may contribute to resistance against *Phytophthora infestans* in potato. *Plant Physiol. Biochem.* 60, 59–66. doi: 10.1016/j.plaphy.2012.07.017

Ferrari, S., Savatin, D., Sicilia, F., Gramegna, G., Cervone, F., and De Lorenzo, G. (2013). Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Front. Plant Sci.* 4:49. doi: 10.3389/fpls.2013.00049

Finiti, I., Leyva, M. O., López-Cruz, J., Calderan Rodrigues, B., Vicedo, B., Angulo, C., et al. (2013). Functional analysis of endo-1,4-β-glucanases in response to *Botrytis cinerea* and *Pseudomonas syringae* reveals their involvement in plant–pathogen interactions. *Plant Biol.* 15, 819–831. doi: 10.1111/j.1438-8677.2012.00701.x

Floerl, S., Druebert, C., Majcherczyk, A., Karlovsky, P., Kües, U., and Polle, A. (2008). Defence reactions in the apoplastic proteome of oilseed rape (*Brassica napus* var. napus) attenuate *Verticillium longisporum* growth but not disease symptoms. *BMC Plant Biol.* 18:129. doi: 10.1186/1471-2229-8-129

Floerl, S., Majcherczyk, A., Possienke, M., Feussner, K., Tappe, H., Gatz, C., et al. (2012). *Verticillium longisporum* infection affects the leaf apoplastic proteome, metabolome, and cell wall properties in *Arabidopsis thaliana*. *PLoS ONE* 7:e31435. doi: 10.1371/journal.pone.0031435

Flors, V., Leyva, M. L., Vicedo, B., Finiti, I., Real, M. D., Garcia-Agustin, P., et al. (2007). Absence of the endo-beta-1,4-glucanases cel1 and Cel2 reduces susceptibility to *Botrytis cinerea* in tomato. *Plant J.* 52, 1027–1040. doi: 10.1111/j.1365-313X.2007.03299.x

Garcia-Brugger, A., Lamotte, O., Vandelle, E., Bourque, S. P., Lecourieux, D., Poinssot, B., et al. (2006). Early signaling events induced by elicitors of plant defenses. *Mol. Plant Microbe Interact.* 19, 711–724. doi: 10.1094/MPMI-19-0711

Geddes, J., Eudes, F., Laroche, A., and Selinger, L. B. (2008). Differential expression of proteins in response to the interaction between the pathogen *Fusarium graminearum* and its host, *Hordeum vulgare*. *Proteomics* 8, 545–554. doi: 10.1002/pmic.200700115

Gimenez-Ibanez, S., and Solano, R. (2013). Nuclear jasmonate and salicylate signaling and crosstalk in defense against pathogens. *Front. Plant Sci.* 4:72. doi: 10.3389/fpls.2013.00072

Girard, V., Dieryckx, C., Job, C., and Job, D. (2013). Secretomes: the fungal strike force. *Proteomics* 13, 597–608. doi: 10.1002/pmic.201200282

Giribaldi, M., Purrotti, M., Pacifico, D., Santini, D., Mannini, F., Caciagli, P., et al. (2011). A multidisciplinary study on the effects of phloem-limited viruses on the agronomical performance and berry quality of *Vitis vinifera* cv. Nebbiolo. *J. Proteom.* 75, 306–315. doi: 10.1016/j.jprot.2011.08.006

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Goldberg, T., Hamp, T., and Rost, B. (2012). LocTree2 predicts localization for all domains of life. *Bioinformatics* 28, i458–i465. doi: 10.1093/bioinformatics/bts390

Görg, A., Weiss, W., and Dunn, M. (2004). Current two-dimensional electrophoresis technology for proteomics. *Proteomics* 4, 3665–3685. doi: 10.1002/pmic.200401031

Goulet, C., Goulet, C., Goulet, M.-C., and Michaud, D. (2010). 2-DE proteome maps for the leaf apoplast of *Nicotiana benthamiana*. *Proteomics* 10, 2536–2544. doi: 10.1002/pmic.200900382

Grimsrud, P. A., Den Os, D., Wenger, C. D., Swaney, D. L., Schwartz, D., Sussman, M. R., et al. (2010). Large-scale phosphoprotein analysis in *Medicago truncatula* roots provides insight into *in vivo* kinase activity in legumes. *Plant Physiol.* 152, 19–28. doi: 10.1104/pp.109.149625

Hamann, T. (2012). Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms. *Front. Plant Sci.* 3:1–5. doi: 10.3389/fpls.2012.00077

Hernández, J. A., Ferrer, M., Jiménez, A., Barceló, A. R., and Sevilla, F. (2001). Antioxidant systems and $O_2 \cdot^-$/H_2O_2 production in the apoplast of pea leaves. *Plant Physiol.* 127, 817–831. doi: 10.1104/pp.010188

Horbach, R., Navarro-Quesada, A., Knogge, W., and Deising, H. (2011). When and how to kill a plant cell: infection strategies of plant pathogenic fungi. *J. Plant Physiol.* 168, 51–62. doi: 10.1016/j.jplph.2010.06.014

Jakab, G., Manrique, A., Zimmerli, L., Metraux, J.-P., and Mauch-Mani, B. (2003). Molecular characterization of a novel lipase-like pathogen-inducible gene family of Arabidopsis. *Plant Physiol.* 132, 2230–2239. doi: 10.1104/pp.103.025312

Jamet, E., Albenne, C., Boudart, G., Irshad, M., Canut, H., and Pont-Lezica, R. (2008). Recent advances in plant cell wall proteomics. *Proteomics* 8, 893–908. doi: 10.1002/pmic.200700938

Jayaraman, D., Forshey, K. L., Grimsrud, P. A., and Ane, J. M. (2012). Leveraging proteomics to understand plant-microbe interactions. *Front. Plant Sci.* 3:44. doi: 10.3389/fpls.2012.00044

Jeandet, P., Clément, C., Courot, E., and Cordelier, S. (2013). Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. *Int. J. Mol. Sci.* 14, 14136–14170. doi: 10.3390/ijms140714136

Jones, A. M. E., Thomas, V., Bennett, M. H., Mansfield, J., and Grant, M. (2006). Modifications to the *Arabidopsis* defence proteome occur prior to significant transcriptional change in response to inoculation with *Pseudomonas syringae*. *Plant Physiol.* 142, 1603–1620. doi: 10.1104/pp.106.086231

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jung, Y.-H., Jeong, S.-H., Kim, S. H., Singh, R., Lee, J.-E., Cho, Y.-S., et al. (2008). Systematic secretome analyses of rice leaf and seed callus suspension-cultured cells: workflow development and establishment of high-density two-dimensional gel reference maps. *J. Proteome Res.* 7, 5187–5210. doi: 10.1021/pr8005149

Kaffarnik, F. A., Jones, A. M., Rathjen, J. P., and Peck, S. C. (2009). Effector proteins of the bacterial pathogen *Pseudomonas syringae* alter the extracellular proteome of the host plant, *Arabidopsis thaliana*. *Mol. Cell Proteomics* 8, 145–156. doi: 10.1074/mcp.M800043-MCP200

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., et al. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11086–11091. doi: 10.1073/pnas.0508882103

Karpinska, B., Karlsson, M., Schinkel, H., Streller, S., Süss, K.-H., Melzer, M., et al. (2001). A novel superoxide dismutase with a high isoelectric point in higher plants. *Plant Physiol.* 126, 1668–1677. doi: 10.1104/pp.126.4.1668

Kav, N. N. V., Srivastava, S., Yajima, W., and Sharma, N. (2007). Application of proteomics to investigate plant-microbe interactions. *Curr. Proteomics* 4, 28–43. doi: 10.2174/157016407781387357

Kim, S. G., Wang, Y., Lee, K. H., Park, Z.-Y., Park, J., Wu, J., et al. (2013). In-depth insight into in vivo apoplastic secretome of rice-*Magnaporthe oryzae* interaction. *J. Proteomics* 78, 58–71. doi: 10.1016/j.jprot.2012.10.029

Kim, S. T., Cho, K. S., Yu, S., Kim, S. G., Hong, J. C., Han, C.-D., et al. (2003). Proteomic analysis of differentially expressed proteins induced by rice blast fungus and elicitor in suspension-cultured rice cells. *Proteomics* 3, 2368–2378. doi: 10.1002/pmic.200300577

Kim, S. T., Kang, Y. H., Wang, Y., Wu, J., Park, Z. Y., Rakwal, R., et al. (2009). Secretome analysis of differentially induced proteins in rice suspension-cultured cells triggered by rice blast fungus and elicitor. *Proteomics* 9, 1302–1313. doi: 10.1002/pmic.200800589

Kim, S., Kim, S., Hwang, D., Kang, S., Kim, H., Lee, B., et al. (2004a). Proteomic analysis of pathogen-responsive proteins from rice leaves induced by rice blast fungus, *Magnaporthe grisea*. *Proteomics* 4, 3569–3578. doi: 10.1002/pmic.200400999

Kim, S. T., Kim, S. G., Hwang, D. H., Kang, S. Y., Kim, H. J., Lee, B. H., et al. (2004b). Proteomic analysis of pathogen-responsive proteins from rice leaves induced by rice blast fungus, *Magnaporthe grisea*. *Proteomics* 4, 3569–3578. doi: 10.1002/pmic.200400999

Klarzynski, O., Plesse, B., Joubert, J. M., Yvin, J. C., Kopp, M., Kloareg, B., et al. (2000). Linear beta-1,3 glucans are elicitors of defense responses in tobacco. *Plant Physiol.* 124, 1027–1038. doi: 10.1104/pp.124.3.1027

Komatsu, S., and Yanagawa, Y. (2013). Cell wall proteomics of crops. *Front. Plant Sci.* 4:17. doi: 10.3389/fpls.2013.00017

Konozy, E. H. E., Rogniaux, H., Causse, M., and Faurobert, M. (2012). Proteomic analysis of tomato (*Solanum lycopersicum*) secretome. *J. Plant Res.* 126, 251–266. doi: 10.1007/s10265-012-0516-4

Kusumawati, L., Imin, N., and Djordjevic, M. A. (2008). Characterization of the secretome of suspension cultures of Medicago species reveals proteins important for defense and development. *J. Proteome Res.* 7, 4508–4520. doi: 10.1021/pr800291z

Laluk, K., and Mengiste, T. (2010). Necrotroph attacks on plants: wanton destruction or covert extortion? *Arabidopsis Book* 8, e0136. doi: 10.1199/tab.0136

Lee, J., Lei, Z., Watson, B. S., and W., S. L. (2013). Sub-cellular proteomics of *Medicago truncatula*. *Front. Plant Sci.* 4 :112. doi: 10.3389/fpls.2013.00112

Lherminier, J., Elmayan, T., Fromentin, J., Elaraqui, K. T., Vesa, S., Morel, J., et al. (2009). NADPH oxidase-mediated reactive oxygen species production: subcellular localization and reassessment of its role in plant defense. *Mol. Plant Microbe Interact.* 22, 868–881. doi: 10.1094/MPMI-22-7-0868

Li, D., Wang, L., Teng, S., Zhang, G., Guo, L., Mao, Q., et al. (2012). Proteomics analysis of rice proteins up-regulated in response to bacterial leaf streak disease. *J. Plant Biol.* 55, 316–324. doi: 10.1007/s12374-011-0346-2

Li, K., Xu, C., and Zhang, J. (2011). Proteome profile of maize (*Zea Mays* L.) leaf tissue at the flowering stage after long-term adjustment to rice black-streaked dwarf virus infection. *Gene* 485, 106–113. doi: 10.1016/j.gene.2011.06.016

Liao, M., Li, Y., and Wang, Z. (2009). Identification of elicitor-responsive proteins in rice leaves by a proteomic approach. *Proteomics* 9, 2809–2819. doi: 10.1002/pmic.200800192

Lin, A., Wang, Y., Tang, J., Xue, P., Li, C., Liu, L., et al. (2012). Nitric oxide and protein S-nitrosylation are integral to hydrogen peroxide-induced leaf cell death in rice. *Plant Physiol.* 158, 451–464. doi: 10.1104/pp.111.184531

Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M., and Hermann Muehling, K. (2001). Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiol. Plant.* 111, 457–465. doi: 10.1034/j.1399-3054.2001.1110405.x

Lounifi, I., Arc, E., Molassiotis, A., Job, D., Rajjou, L., and Tanou, G. (2013). Interplay between protein carbonylation and nitrosylation in plants. *Proteomics* 13, 568–578. doi: 10.1002/pmic.201200304

Macarisin, D., Wisniewski, M. E., Bassett, C., and Thannhauser, T. W. (2009). Proteomic analysis of β-aminobutyric acid priming and abscisic acid – induction of drought resistance in crabapple (*Malus pumila*): effect on general metabolism, the phenylpropanoid pathway and cell wall enzymes. *Plant Cell Environ.* 32, 1612–1631. doi: 10.1111/j.1365-3040.2009.02025.x

Martinez-Esteso, M. J., Sellés-Marchart, S., Vera-Urbina, J. C., Pedreño, M. A., and Bru-Martinez, R. (2009). Changes of defense proteins in the extracellular proteome of grapevine (*Vitis vinifera* cv. Gamay) cell cultures in response to elicitors. *J. Proteomics* 73, 331–341. doi: 10.1016/j.jprot.2009.10.001

Mathesius, U. (2009). Comparative proteomic studies of root–microbe interactions. *J. Proteomics* 72, 353–366. doi: 10.1016/j.jprot.2008.12.006

Ménard, R., Alban, S., De Ruffray, P., Jamois, F., Franz, G., Fritig, B., et al. (2004). β-1,3 glucan sulfate, but not β-1,3 glucan, induces the salicylic acid signaling pathway in Tobacco and *Arabidopsis*. *Plant Cell* 16, 3020–3032. doi: 10.1105/tpc.104.024968

Micali, C. O., Neumann, U., Grunewald, D., Panstruga, R., and O'Connell, R. (2011). Biogenesis of a specialized plant-fungal interface during host cell internalization of *Golovinomyces orontii* haustoria. *Cell Microbiol.* 13, 210–226. doi: 10.1111/j.1462-5822.2010.01530

Minic, Z., Jamet, E., Négroni, L., Der Garabedian, A. P., Zivy, M., and Jouanin, L. (2007). A sub-proteome of *Arabidopsis thaliana* mature stems trapped on Concanavalin a is enriched in cell wall glycoside hydrolases. *J. Exp. Bot.* 58, 2503–2512. doi: 10.1093/jxb/erm082

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., et al. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19613–19618. doi: 10.1073/pnas.0705147104

Mukherjee, A., Carp, M., Zuchman, R., Ziv, T., Horwitz, B., and Gepstein, S. (2010). Proteomics of the response of *Arabidopsis thaliana* to infection with *Alternaria brassicicola*. *J. Proteomics* 73, 709–720. doi: 10.1016/j.jprot.2009.10.005

Ndimba, B. K., Chivasa, S., Hamilton, J. M., Simon, W. J., and Slabas, A. R. (2003). Proteomic analysis of changes in the extracellular matrix of *Arabidopsis* cell suspension cultures induced by fungal elicitors. *Proteomics* 3, 1047–1059. doi: 10.1002/pmic.200300413

Newman, M.-A., Sundelin, T., Nielsen, J. T., and Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* 4:139. doi: 10.3389/fpls.2013.00139

Newman, M.-A., Von Roepenack-Lahaye, E., Parr, A., Daniels, M. J., and Dow, J. M. (2002). Prior exposure to lipopolysaccharide potentiates expression of plant defenses in response to bacteria. *Plant J.* 29, 487–495. doi: 10.1046/j.0960-7412.2001.00233.x

Oh, I. S., Park, A. R., Bae, M. S., Kwon, S. J., Kim, Y. S., Lee, J. E., et al. (2005). Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola*. *Plant Cell* 17, 2832–2847. doi: 10.1105/tpc.105.034819

Okushima, Y., Koizumi, N., Kusano, T., and Sano, H. (2000). Secreted proteins of tobacco cultured BY2 cells: identification of a new member of pathogenesis-related proteins. *Plant Mol. Biol.* 42, 479–488. doi: 10.1023/A:1006393326985

Pan, B., Sheng, J., Sun, W., Zhao, Y., Hao, P., and Li, X. (2013). OrysPSSP: a comparative platform for small secreted proteins from rice and other plants. *Nucleic Acids Res.* 41, D1192–D1198. doi: 10.1093/nar/gks1090

Pearce, G., Strydom, D., Johnson, S., and Ryan, C. A. (1991). A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* 253, 895–897. doi: 10.1126/science.253.5022.895

Pechanova, O., Hsu, C. Y., Adams, J. P., Pechan, T., Vandervelde, L., Drnevich, J., et al. (2010). Apoplast proteome reveals that extracellular matrix contributes to multistress response in poplar. *BMC Genomics* 11:674. doi: 10.1186/1471-2164-11-674

Pel, M. J., and Pieterse, C. M. (2013). Microbial recognition and evasion of host immunity. *J. Exp. Bot.* 64, 1237–1248. doi: 10.1093/jxb/ers262

Petersen, T. N., Brunak, S., Von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. doi: 10.1038/nmeth.1701

Phalip, V., Delalande, F., Carapito, C., Goubet, F., Hatsch, D., Leize-Wagner, E., et al. (2005). Diversity of the exoproteome of *Fusarium graminearum* grown on plant cell wall. *Curr. Genet.* 48, 366–379. doi: 10.1007/s00294-005-0040-3

Pieterse, C. M., Van Der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Quirino, B. F., Candido, E. S., Campos, P. F., Franco, O. L., and Krüger, R. H. (2010). Proteomic approaches to study plant-pathogen interactions. *Phytochemistry* 71, 351–362. doi: 10.1016/j.phytochem.2009.11.005

Rajjou, L., Belghazi, M., Huguet, R., Robin, C., Moreau, A., Job, C., et al. (2006). Proteomic investigation of the effect of salicylic acid on Arabidopsis seed germination and establishment of early defense mechanisms. *Plant Physiol.* 141, 910–923. doi: 10.1104/pp.106.082057

Ridley, B. L., O'Neill, M. A., and Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57, 929–967. doi: 10.1016/S0031-9422(01)00113-3

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Roe, M. R., and Griffin, T. J. (2006). Gel-free mass spectrometry-based high throughput proteomics: tools for studying biological response of proteins and proteomes. *Proteomics* 6, 4678–4687. doi: 10.1002/pmic.200500876

Romero-Puertas, M. C., Campostrini, N., Mattè, A., Righetti, P. G., Perazzolli, M., Zolla, L., et al. (2008). Proteomic analysis of S-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. *Proteomics* 8, 1459–1469. doi: 10.1002/pmic.200700536

Rose, J. K. C., and Lee, S.-J. (2010). Straying off the highway: trafficking of secreted plant proteins and complexity in the plant cell wall proteome. *Plant Physiol.* 153, 433–436. doi: 10.1104/pp.110.154872

Sakurai, N. (1998). Dynamic function and regulation of apoplast in the plant body. *J. Plant Res.* 111, 133–148. doi: 10.1007/BF02507160

Sanabria, N., Goring, D., Nurnberger, T., and Dubery, I. (2008). Self/nonself perception and recognition mechanisms in plants: a comparison of self-incompatibility and innate immunity. *New Phytol.* 178, 503–513. doi: 10.1111/j.1469-8137.2008.02403.x

Sanchez, L., Courteaux, B., Hubert, J., Kauffmann, S., Renault, J. H., Clement, C., et al. (2012). Rhamnolipids elicit defense responses and induce disease resistance against biotrophic, hemibiotrophic, and necrotrophic pathogens that require different signaling pathways in Arabidopsis and highlight a central role for salicylic acid. *Plant Physiol.* 160, 1630–1641. doi: 10.1104/pp.112.201913

Savchenko, T., Walley, J. W., Chehab, E. W., Xiao, Y., Kaspi, R., Pye, M. F., et al. (2010). Arachidonic acid: an evolutionarily conserved signaling molecule modulates plant stress signaling networks. *Plant Cell* 22, 3193–3205. doi: 10.1105/tpc.110.073858

Scheler, C., Durner, J., and Astier, J. (2013). Nitric oxide and reactive oxygen species in plant biotic interactions. *Curr. Opin. Plant Biol.* 16, 534–539. doi: 10.1016/j.pbi.2013.06.020

Schenk, P. M., Kazan, K., Manners, J. M., Anderson, J. P., Simpson, R. S., Wilson, I. W., et al. (2003). Systemic gene expression in Arabidopsis during an incompatible interaction with *Alternaria brassicicola*. *Plant Physiol.* 132, 999–1010. doi: 10.1104/pp.103.021683

Schweizer, P., Felix, G., Buchala, A., Müller, C., and Métraux, J. P. (1996). Perception of free cutin monomers by plant cells. *Plant J.* 10, 331–341. doi: 10.1046/j.1365-313X.1996.10020331.x

Serna-Sanz, A., Parniske, M., and Peck, S. C. (2011). Phosphoproteome analysis of *Lotus japonicus* roots reveals shared and distinct components of symbiosis and defense. *Mol. Plant Microbe Interact.* 24, 932–937. doi: 10.1094/MPMI-09-10-0222

Shah, P., Powell, A. L. T., Orlando, R., Bergmann, C., and Gutierrez-Sanchez, G. (2012). Proteomic analysis of ripening tomato fruit infected by *Botrytis cinerea*. *J. Proteome Res.* 11, 2178–2192. doi: 10.1021/pr200965c

Shen, S., Jing, Y., and Kuang, T. (2003). Proteomics approach to identify wound-response related proteins from rice leaf sheath. *Proteomics* 3, 527–535. doi: 10.1002/pmic.200390066

Shenton, M. R., Berberich, T., Kamo, M., Yamashita, T., Taira, H., and Terauchi, R. (2012). Use of intercellular washing fluid to investigate the secreted proteome of the rice–*Magnaporthe* interaction. *J. Plant Res.* 125, 311–316. doi: 10.1007/s10265-012-0473-y

Soares, N. C., Francisco, R., Ricardo, C. P., and Jackson, P. A. (2007). Proteomics of ionically bound and soluble extracellular proteins in *Medicago truncatula* leaves. *Proteomics* 7, 2070–2082. doi: 10.1002/pmic.200600953

Spoel, S. H., and Loake, G. J. (2011). Redox-based protein modifications: the missing link in plant immune signalling. *Curr. Opin. Plant Biol.* 14, 358–364. doi: 10.1016/j.pbi.2011.03.007

Trdá, L., Fernandez, O., Boutrot, F., Héloir, M.-C., Kelloniemi, J., Daire, X., et al. (2013). The grapevine flagellin receptor VvFLS2 differentially recognizes flagellin-derived epitopes from the endophytic growth-promoting bacterium *Burkholderia phytofirmans* and plant pathogenic bacteria. *New Phytol.* 201, 1371–1384. doi: 10.1111/nph.12592

Tseng, T.-T., Tyler, B. M., and Setubal, J. C. (2009). Protein secretion systems in bacterial-host associations, and their description in the gene ontology. *BMC Microbiol.* 9:S2. doi: 10.1186/1471-2180-9-S1-S2

Tsuda, K., and Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* 13, 459–465. doi: 10.1016/j.pbi.2010.04.006

Umemoto, N., Kakitani, M., Iwamatsu, A., Yoshikawa, M., Yamaoka, N., and Ishida, I. (1997). The structure and function of a soybean β-glucan-elicitor-binding-protein. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1029–1034. doi: 10.1073/pnas.94.3.1029

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Front. Plant Sci.* 3:85. doi: 10.3389/fpls.2012.00085

Van Loon, L. C., Rep, M., and Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162. doi: 10.1146/annurev.phyto.44.070505.143425

Varnier, A. L., Sanchez, L., Vatsa, P., Boudesocque, L., Garcia-Brugger, A., Rabenoelina, F., et al. (2009). Bacterial rhamnolipids are novel MAMPs conferring resistance to *Botrytis cinerea* in grapevine. *Plant Cell Environ.* 32, 178–193. doi: 10.1111/j.1365-3040.2008.01911.x

Wen, F., Vanetten, H. D., Tsaprailis, G., and Hawes, M. C. (2007). Extracellular proteins in pea root tip and border cell exudates. *Plant Physiol.* 143, 773–783. doi: 10.1104/pp.106.091637

Willmann, R., Lajunen, H. M., Erbs, G., Newman, M. A., Kolb, D., Tsuda, K., et al. (2011). Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19824–19829. doi: 10.1073/pnas.1112862108

Witzel, K., Shahzad, M., Matros, A., Mock, H. P., and Muhling, K. H. (2011). Comparative evaluation of extraction methods for apoplastic proteins from maize leaves. *Plant Methods* 7, 48. doi: 10.1186/1746-4811-7-48

Xia, Y., Suzuki, H., Borevitz, J., Blount, J., Guo, Z., Patel, K., Dixon, R. A., and Lamb, C. (2004). An extracellular aspartic protease functions in Arabidopsis disease resistance signaling. *EMBO Journal* 23, 980–988. doi: 10.1038/sj.emboj.7600086

Yamaguchi, Y., and Huffaker, A. (2011). Endogenous peptide elicitors in higher plants. *Curr. Opin. Plant Biol.* 14, 351–357. doi: 10.1016/j.pbi.2011.05.001

Zhang, X., Dai, Y., Xiong, Y., Defraia, C., Li, J., Dong, X., et al. (2007). Overexpression of *Arabidopsis* MAP kinase kinase 7 leads to activation of plant basal and systemic acquired resistance. *Plant J.* 52, 1066–1079. doi: 10.1111/j.1365-313X.2007.03294.x

Zhang, Y., Giboulot, A., Zivy, M., Valot, B., Jamet, E., and Albenne, C. (2011). Combining various strategies to increase the coverage of the plant cell wall glycoproteome. *Phytochemistry* 72, 1109–1123. doi: 10.1016/j.phytochem.2010.10.019

Zhou, L., Bokhari, S. A., Dong, C.-J., and Liu, J.-Y. (2011). Comparative proteomics analysis of the root apoplasts of rice seedlings in response to hydrogen peroxide. *PLoS ONE* 6:e16723. doi: 10.1371/journal.pone.0016723

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Curr. Opin. Plant Biol.* 12, 414–420. doi: 10.1016/j.pbi.2009.06.003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 February 2014; accepted: 15 May 2014; published online: 03 June 2014.
*Citation: Delaunois B, Jeandet P, Clément C, Baillieul F, Dorey S and Cordelier S (2014) Uncovering plant-pathogen crosstalk through apoplastic proteomic studies. Front. Plant Sci. **5**:249. doi: 10.3389/fpls.2014.00249*
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.