Targeted secretion and remodelling of pectin in response to pathogen infection

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Affidavit

Herewith, I declare that the present PhD thesis entitled "Targeted secretion and remodelling of pectin in response to pathogen infection"

was written on my own without other sources and aids than quoted.

Göttingen,

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Athanas Guzha

I.   Table of Contents

II. Abbreviations

ADPG2	ARABIDOPSIS POLYYGALACTURONASE2
AGP	Arabinogalactan proteins
AIR	Alcohol insoluble residue
AOC	ALLENE OXIDE CYCLASE
AOS	ALLENE OXIDASE SYNTHASE
Ara	Arabinose
ARAD1	ARABINAN DEFICIENT1
*At*ERF014	*Arabidopsis thaliana* ethylene responsive factor14
BBE	Berberine Bridge Enzyme
BXL1	BETAXYLOSIDASE1
BXL4	BETAXYLOSIDASE4
BXL6	BETAXYLOSIDASE6
CD	Cellodextrin
CESA	Cellulase synthase
COI1	CORONATINE INSENSITIVE1
CWDEs	Cell wall degrading enzymes
DAMP	Danger associated molecular pattern
dpa	Days post anthesis
eds1	*enhanced disease susceptibility1*
EXPs	Expansins
GAE	GLUCORONATE 4-EPIMERASE
Gal	Galactose
GalA	Galacturonic Acid
GAUT1	GALACTURONOSYLTRANSFERASE1
GAUT8	GALACTURONOSYLTRANSFERASE8
GC-MS	Gas Chromatography-Mass Spectrometry
Glu	Glucose
GluC	Glucuronic Acid
GRP	Glycine rich proteins
HAMPs	Herbivore associated molecular patterns

HG	Homogalacturonan
HG-MT	Homogalacturonan-methyltransferase
HPLC	High performance liquid chromatography
HRGP	Hydroxyproline rich glycoproteins
IDA	INFLORESCENT DEFICIENT IN ABSCISSION
JA	Jasmonic acid
JA-Ile	Jasmonoyl isoleucine
JAZ10	JASMONATE ZIM-DOMAIN10
LLP1	LEGUME LECTIN LIKE PROTEIN1
LOX	LIPOXYGENASE
MAMP	Microbe associated molecular pattern
Man	Mannose
MAPK	Mitogen-activated protein kinase
MBS	Major beam splitter
MOX	Methoxyamine hydrochloride
mpk3	*mitogen-activated protein kinase3*
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
MUCI70	MUCILAGE-RELATED70
OGOX1	OG OXIDASE1
OGs	Oligogalacturonides
PAD3	*PHYTOALEXIN DEFICIENT3*
PAL	PHENYLALANINE AMMONIA LYASE
PDF1.2	*PLANT DEFENSIN1.2*
pgm	*phosphoglucomutase*
PLL	pectate lyase like
PME	Pectin methylesterase
PMEI	pectin methylesterase inhibitor
pmr	*powdery mildew resistant*
PRPs	proline rich proteins
PRRs	Pattern recognition receptors
PRX	Peroxidase

Psm	*Pseudomonas syringae* pv. *maculicola*
QUA1	QUASIMODO1
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
Rha	Rhamnose
ROS	Reactive Oxygen species
RRT	RG-I:rhamnosyltransferase
rwa2	*reduced wall acetylation2*
SA	salicylic acid
TBA2	TESTA ABUNDANT2
TFA	Trifluoroacetic acid
WAK1	WALL ASSOCIATED KINASE1
Ws	Wassilevskia
Xcv	*Xanthomonas campestris vesicatoria*
XG	Xylogalacturonan
XGD1	xylosyltransferase1
XTHs	xyloglucan endo-transglycosylases/hydrolases
XyG	Xyloglucan
Xyl	Xylose

III. Abstract

Plant cell walls are a complex mix of three major classes of polysaccharides; cellulose, hemicellulose and pectin together with a minor amount of proteins, and they surround every plant cell. Besides serving as a structural component helping to maintain the shape and integrity of the plant cell. Cell walls are an interaction interface between plant cells as well as the plant and its environment and they undergo various modifications in response to developmental and environmental changes. The attack of plants by plant pathogens results in the remodelling of the pectin component in response to the pathogens. The remodelling of pectin and its implications on plant-pathogen interactions are largely unknown. Here we firstly developed a robust GC-MS based method to analyse the monosaccharide profile of pectin extracted from leaves, mucilage and commercial pectin. The method is quick and easy, and both neutral sugars and galacturonic acid are quantified within the same run. We also investigate the enzymatic function of BXL4 which belongs to a seven-member betaxylosidase gene family in Arabidopsis. By expressing BXL4 in the seed coat of a *bxl1* mutant, we can rescue the mucilage extrusion phenotype of *bxl1.* Monosaccharide analysis of the complemented *bxl1* and over expression lines in Col-0 show that BXL4 acts on both xylose and arabinose in the cell walls. The *bxl4* mutants exhibit higher amounts of arabinose in the leaf cell walls but this does not affect the general development of the plants. Wounding and *Botrytis cinerea* infection of Arabidopsis show that *BXL4* is a stress induced gene and its expression is JA-Ile dependant. Whilst *BXL4* was acting downstream of JA-Ile the *bxl4* mutants show a reduction in the expression of JA-Ile marker genes *JAZ10* and *PDF1.2* upon infection and mechanical wounding, compared to wild type. The expression of *PAD3* is also reduced in the *bxl4* mutant compared to wild type after *B. cinerea* infection. Accumulation of JA-Ile and camalexin is reduced in the *bxl4* mutants after infection with *B. cinerea* as compared to wild type plants. Infection of the plants with *B. cinerea* shows that the *bxl4* mutants have an enhanced susceptibility to the pathogen. The post secretion remodelling of pectin by BXL4 is thus an important component of plant immunity against *B. cinerea*, and this is likely due to its role in JA-Ile signalling and camalexin biosynthesis.

1 General Introduction

The growing human population, projected to reach 10.9 billion by 2100 (Population and development review, 2013), means an ever-increasing demand for agricultural produce from finite land resources. This calls for the development and adoption of strategies that increase the plant biomass accumulation from a unit landholding in a sustainable manner. The development of novel strategies that protect the crop against pathogen threats is fundamental to attain food security. After the cuticle, the plant cell wall acts as a second barrier that restrict the entry of pathogens. Plant cell walls are also an abundant source of renewable energy. The cell walls of multicellular land plants are a complex mix of various polysaccharides that can be used for various applications in different technological fields. Modification of plant cell walls of important crops to enhance recalcitrance against pests and pathogens is a sustainable approach to mitigate yield losses.

1.1 Botrytis cinerea

Plant pathogens are a ubiquitous group of organisms that contribute to crop yield losses ranging from 13% to 16% across the world every year (Vurro et al., 2010). Generally, plant pathogens are divided into three groups according to their infection strategy which are; necrotrophs, biotrophs, and hemibiotrophs (Laluk and Mengiste, 2010). Biotrophic and hemibiotrophic pathogens keep their host alive at least during some stage of their infection cycle whilst the necrotrophs kill their host tissues and proliferate in the wreckage (Shlezinger et al., 2011; Spanu and Panstruga, 2017). *Botrytis cinerea* is a fungal necrotrophic pathogen with a wide host range, that is responsible for pre- and post-harvest damage in many crops (Williamson et al., 2007) causing economic losses exceeding $10 billion across the world (Weiberg et al., 2013). The necrotrophic pathogen is able to colonise its host through the secretion of an array of cell wall degrading enzymes (Di Matteo et al., 2006). The *B. cinerea* infection cycle involves the attachment of conidiospores on a plant surface, followed by germination and penetration of the plant cell wall via an appressorium, aided by the secretion of cell wall degrading enzymes (Brandhoff et al., 2017). To facilitate development and spread of the hyphae*, B. cinerea* kills the host cells allowing proliferation of the hyphae in the dead tissues (Clark, 1976). The use of brute force is not the only strategy utilised by *B. cinerea* to infect its host, but it is also known to manipulate the host defence mechanism and hinder the activation of defence responses (Laluk and Mengiste,

2010). The infection of plants by *B. cinerea* also requires the active participation of the host. Cuticle penetration together with the development of the primary lesion by *B. cinerea* results in oxidative burst at the plasma membrane and extracellular matrix near the fungal hyphae (Schouten et al., 2002). This results in disruption of the redox status close to the infection site and thereby promotes the development of the fungus (Lyon et al., 2007). *B. cinerea* develops sclerotia within the decomposing host debris. This serves as an important survival strategy, as sclerotia are able to withstand microbial attack, UV radiation and desiccation (Backhouse and Willetts, 1984). Because of its large host range, an array of infection strategies and ability to survive harsh conditions, *B. cinerea* is difficult to control. Natural plant defences against this pathogen are constitutive, involving the pre-formed barriers such as cell walls and cuticle, or inducible chemical and physical barriers (Williamson *et al.*, 2007).

1.2 Plant cell walls

Plant cell walls are an important component that performs a diversity of functions such as provision of mechanical support, cell-to-cell communication, water transport, cell growth and development, and defence against biotic and abiotic factors (Cosgrove, 2005; Caffall and Mohnen, 2009). Plant cell walls usually consist of a primary cell wall found around actively growing and expanding cells, or secondary cell walls which form in specialised cells and may become fortified with lignin depositions such as occurs in vascular elements or fibre cells (Keegstra, 2010).
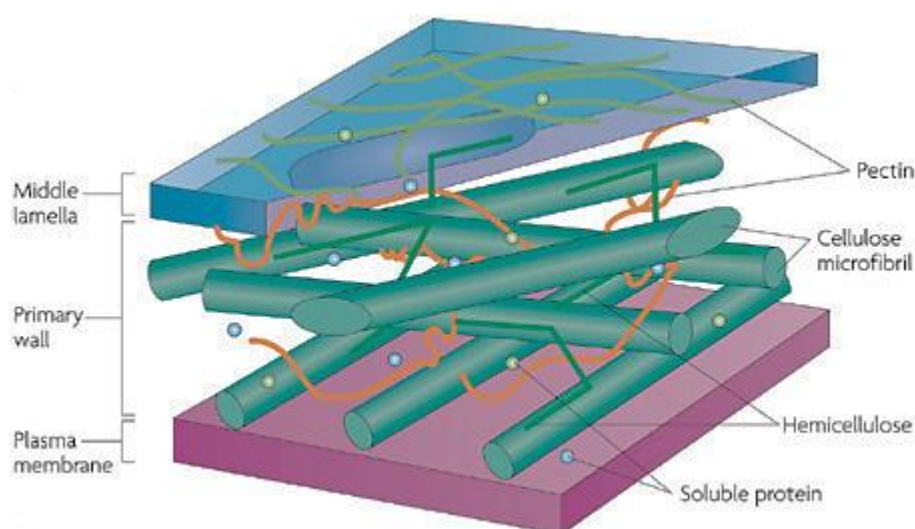

Figure 1*:* **Model showing the major components of the plant cell walls of multicellular land plants**. Cellulose microfibrils form mechanical structures that are

cross-linked by various hemicelluloses, embedded in a pectin matrix. A small component consists of structural proteins and enzymes involved in cell wall modifications. Image adapted from Sticklen, (2008).

1.3 Components of the cell wall

Plant cell walls consist of a heterogeneous mixture of polysaccharides, phenols, proteins and ions (McCann and Carpita, 2008). The polysaccharide composition of cell walls of vascular plants varies from species to species, but on average, cellulose constitutes 30%, hemicellulose 30% and pectin 35% (Ochoa-Villarreal et al., 2012).
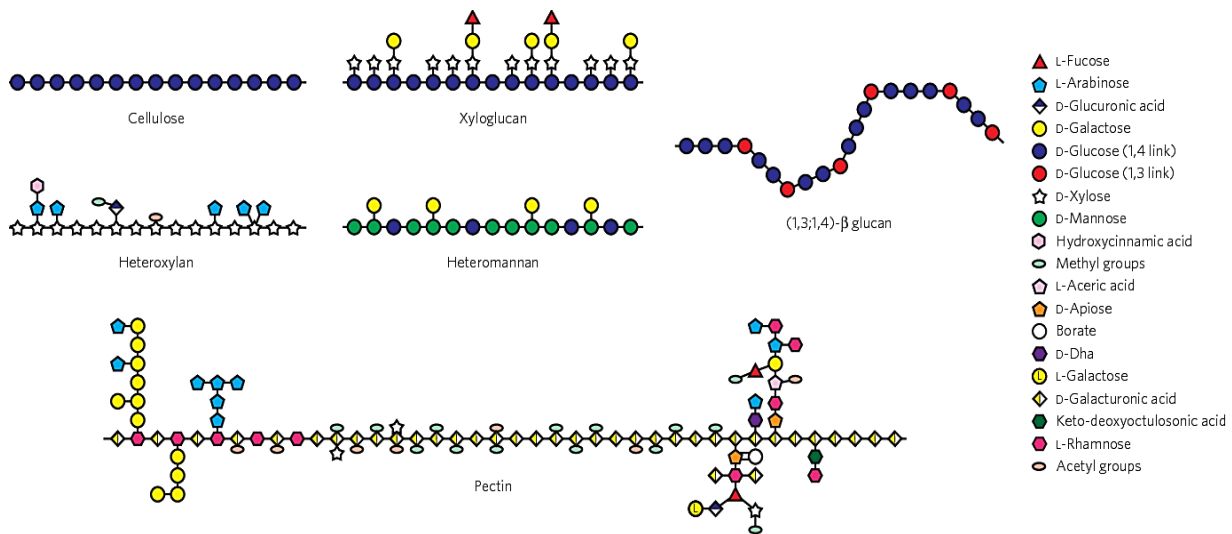

Figure 2: Cell walls are a complex mix of diverse polysaccharides linked by various glycosyl linkages. Image adapted from Burton et al., (2010).

1.3.1 Cellulose

Cellulose represents the main structural component of the cell walls. It consists of an unbranched polymer of β-(1,4)-D-Glucose moieties that interact together through hydrogen bonding and Van der Waals forces forming insoluble paracrystalline structures (Nishiyama et al., 2002; Nishiyama et al., 2003; Somerville, 2006). Cellulose microfibrils are built by the aggregation of cellulose chains forming insoluble structures, which are resistant to enzyme attack and are chemically stable. Cellulose is composed of two types of microfibrils, namely cellulose Iα and Iβ (Brown, 1996). Cellulose biosynthesis is performed by plasma membrane bound cellulase synthase (CESA) complexes that catalyse the polymerisation of glucan moieties. Ten *CESA* genes have so far been identified in Arabidopsis (Holland et al., 2000; Richmond, 2000). CESA

complexes comprise of a number of subunits, which form a rosette of six CESA subunits that synthesise cellulose chains.

1.3.1.1 Biological activity of cellulose derived oligosaccharins

Hydrolysis of cell wall polysaccharides results in the generation of short chain sugars joined by glycosidic linkages that are termed oligosaccharides (Côté and Hahn, 1994). Some of these oligosaccharides can elicit a biological response in plants and they are called oligosaccharins (Darvill et al., 1992). Until recently, it was thought that only fragments generated from degradation of non-cellulosic components had biological activity. It has however been observed that cellulose derived oligasaccharins known as cellodextrins (CDs) trigger a number of plant defence mechanisms in grape plants (Aziz et al., 2007). Cellodextrins are oligomers with a linear β-(1,4)-glucose backbone and result from the hydrolysis of cellulose. *PHENYLALANINE AMMONIA LYASE* (*PAL*) genes are activated upon exposure of plant cells to CD leading to the enhanced production of phytoalexins, lignin, and salicylic acid (Chaliha et al., 2018). These then activate β-1,3-glucanases and chitinases, which hydrolyse fungal cell wall β-1,3-glucanases and chitin (Farmer et al., 1991; De Lorenzo and Ferrari, 2002). Cellodextrin applied exogenously as an elicitor has been shown to trigger ROS production, activate biosynthesis of chitinases and β-1,3-glucanases, and to trigger defence related genes and the accumulation of cytosolic Ca^{2+}, providing resistance against *B. cinerea* (Aziz et al., 2007). Arabidopsis treatment with CD activates the innate immunity by triggering MAPKs and accumulating cytosolic calcium after infection with *Pseudomonas syringae* (Souza et al., 2017).

1.3.2 Hemicellulose

Hemicelluloses are low molecular weight polysaccharides consisting of a β-(1→4)-linked backbone made up of glucose, xylose, or mannose with short side chains that are diverse (Scheller and Ulvskov, 2010). Hemicelluloses are important for cell wall strengthening through their interaction with cellulose microfibrils and lignin in secondary cell walls (Kurata et al., 2018).

1.3.2.1 Xyloglucan

Xyloglucans (XyG) are the most abundant hemicelluloses and have been identified in all plant species, and some mosses (Moller et al., 2007; Popper, 2008). In dicotyledonous species, the most common structure consists of a repeating heptamer

of three glucose molecules with an α-D-xylose substitution, followed by an unsubstituted glucose (Wilder and Albersheim, 1973).

However, there are many variations found of this polysaccharide. A β-Gal or α-l-Araf side chain sometimes substitutes the xylose residues. The branched XyG is more soluble than unbranched, which may correlate with their function (Scheller and Ulvskov, 2010). Cellulose and xyloglucans exist in equal proportions in dicotyledonous plants, with XyG side chains attached to cellulose microfibrils contributing to the mechanical strength of cells walls. Arabidopsis mutants with deficiencies in the biosynthesis of XyG (*xx1 xx2*) are slightly smaller than the wild type with weakened hypocotyls of the seedlings, suggesting a possible role in cell wall strengthening (Cavalier et al., 2008).

1.3.2.2 Xylans

Xylans form a widely varied group of hemicelluloses, whose backbone is characterised by β-(1,4)-linked xylose moieties possessing side branches of α-(1,2) linked glucuronic acid together with 4-*O*-methyl glucuronic acid. Xylans that contain many of these side chains are referred to as glucuronoxylans, and they are the most common non-cellulosic component of the secondary cell walls of dicotyledonous species. If the xylan backbone is decorated with arabinan residues, it is called arabinoxylan or glucuronoarabinoxylan. This type of xylan is more common in grasses than in dicotyledonous plants (Darvill et al., 1980; Bochicchio and Reicher, 2003).

1.3.2.3 Mannans and Glucomannans

These are β-(1→4)-linked hemicelluloses, consisting of a mannose (mannans) or glucose and mannose in a non-repeating manner (glucomannans and galactoglucomannans). Acetylation usually occurs on the mannans and glucomannans, which act as seed storage polysaccharides (Ebringerová et al., 2005; Popper, 2008).

1.3.2.4 Biological activity of hemicellulose derived oligosaccharins

The major hemicellulose in the apoplast is xyloglucan, and the biological activity of xyloglucan-derived oligosaccharins relates to their physiological function including cell elongation and expansion. In *Pisum sativum*, xyloglucan oligomers could trigger accelerated cell elongation of peeled stem segments (Takeda et al., 2002), whilst in *Nicotiana tabacum* suspension cell cultures, they led to cell expansion and cell division

(Kaida et al., 2010). Xyloglucans were shown to elicit the activation of MAPK and defence gene expression in *Vitis vinifera* and Arabidopsis triggering resistance to various pathogens including *B. cinerea* and *Hyaloperonospora arabidopsidis* (Claverie et al., 2018). Wheat seedlings coleoptile growth was promoted by addition of an octasaccharide derived from xyloglucan (Vargas-Rechia et al., 1998). A three-component oligosaccharide (FucGalXyl) of xyloglucan increased the callus tissue weight, stimulated embryogenesis, and inhibited ethylene biosynthesis in *Gossypium hirsutum* (Rakitin et al., 2001). Galactoglucomannan, whose backbone structure consists of glucose and mannose and side chains of galactose, also produces oligosaccharides with proven biological activity. In mung bean (*Vigna radiata* L.) and buckthorn (*Karwinskia humboldtiana*), it was proven that the root morphology was modulated by galactoglucomannan oligosaccharins (Kollárová et al., 2005, 2007).

1.3.3 Pectin

Pectin is a complex and abundant polysaccharide component of the primary cell walls of plants performing diverse functional roles (Voragen et al., 2009). All pectin polysaccharides contain galacturonic acid making them acidic (Caffall and Mohnen, 2009). Pectin consists of more than 17 different monosaccharides bound together by various linkages (Ridley et al., 2001; O'Neill et al., 2004). The composition of pectin varies with the plant tissues together with developmental stage of cells, tissues and organs, environment and species (Zablackis et al., 1995). There are 4 major types of pectin, namely homogalacturonan (HG), rhamnogalacturonans I (RG-I) and II (RG-II) and xylogalacturonan (XG). There is no consensus on the way the different pectins are linked together. However, it is generally thought that the different types of pectin are covalently cross-linked because their isolation requires treatment with strong chemicals (Caffall and Mohnen, 2009). Therefore models support the notion that HG, XG, RG-I and RG-II link through their backbones (Coenen et al., 2007; Ishii and Matsunaga, 2001).
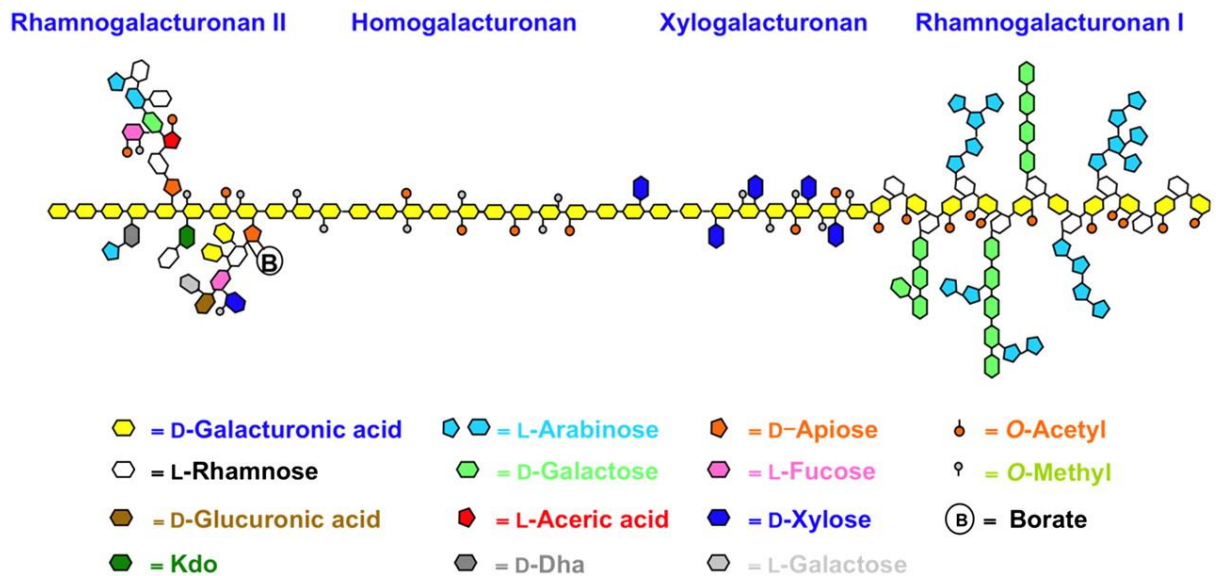
Figure 3: Pectin consists of 4 pectin polysaccharides. Pectin is characterised by the presence of a (1→4) α-D-galacturonic acid backbone, with the exception of rhamnogalaturonan I, which has a backbone consisting of interchanging galacturonic acid-rhamnose residues. Depending on the type of pectin, the backbone can also be methylated or acetylated and can carry side chains. Image adapted from Harholt et al., (2010).

Homogalacturonan

HG is the most abundant pectic polysaccharide in the leaves of Arabidopsis. HG constitutes up to 60% of all pectin polysaccharides in cell walls (O'Neill et al., 1990). HG is the least complex of all pectic polysaccharides consisting of an unbranched backbone of (1→4) α-D-galacturonic acid residues (McNeil et al., 1984; Mouille et al., 2007). Depending on the plant species, the carboxyl groups of HG are normally methyl-esterified at the *C*-6 position, whilst the *O*-2 and *O*-3 positions are acetyl-esterified (Ochoa-Villarreal et al., 2012). Pectin methylesterases (PMEs) are involved in the demethylation of HG (Körner et al., 2009), which is carried out in a random manner, or in a continuous block-wise manner, exposing negatively charged carboxyl groups. If at least ten galacturonic acid residues are demethylesterified, Ca^{2+} crosslinks this pectin forming an egg-box structure and inducing gelling of the pectin as shown in figure 4 (Raj and Ranganathan, 2012).
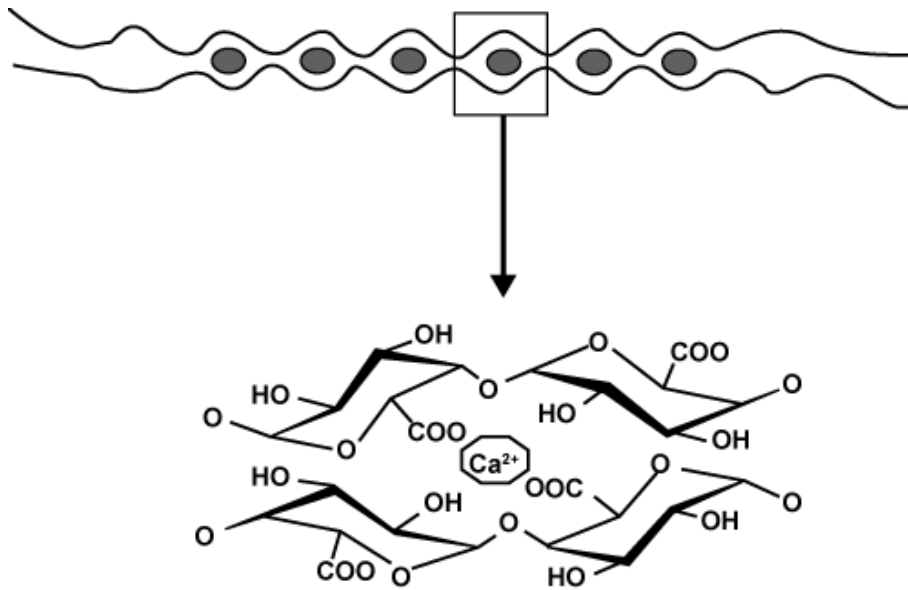
Figure 4: Demethylesterified homogalacturonan blocks are cross-linked by calcium resulting in formation of a gel. Image adopted from Raj et al., (2012).

Rhamnogalacturonan I

RG-I constitutes up to 20-35% of pectin in the plant cell wall of Arabidopsis (Mohnen, 2008). RG-I is a unique type of pectin with a backbone consisting of a repeating galacturonic acid-rhamnose disaccharide (α-1,4-d-GalA-α-1,2-l-Rha) (Mohnen, 2008; Harholt et al., 2010). The rhamnose residue is usually substituted with side chains that are usually β-(1,4) D-galactans and/or α-(1,5) L-Arabinans (ØBro et al., 2004). The galacturonic residue may be acetylated on the C2 and/or C3 (Kravtchenko et al., 1992). The side chains of RG-I are heterogeneous and vary depending with plant species or tissues suggesting diverse functional roles (Mohnen, 2008).

Rhamnogalacturonan II

RG-II is the most complex pectin, and it is very conserved in the plant kingdom. RG-II is characterised by a galacturonic acid backbone with four distinct side chains namely A, B, C and D, which contain peculiar sugars such as apiose, 3-deoxy-manno-2-octulosonic acid and 3 deoxy-lyxo-2-heptulosaric acid (Ishii and Matsunaga, 2001; O'Neill et al., 2001; Ridley et al., 2001). RG-II complexity is shown by the fact that it is made of at least 12 different sugar residues with up to 20 different glycosidic linkages involved (O'Neill et al., 2004). Despite its low abundance (0.5-8% in dicotyledons), RG-

II is important for proper cell wall development. RG-II is cross-linked by borate, which is important for cell wall strength (Ishii and Matsunaga, 2001; Ishii et al., 1999).
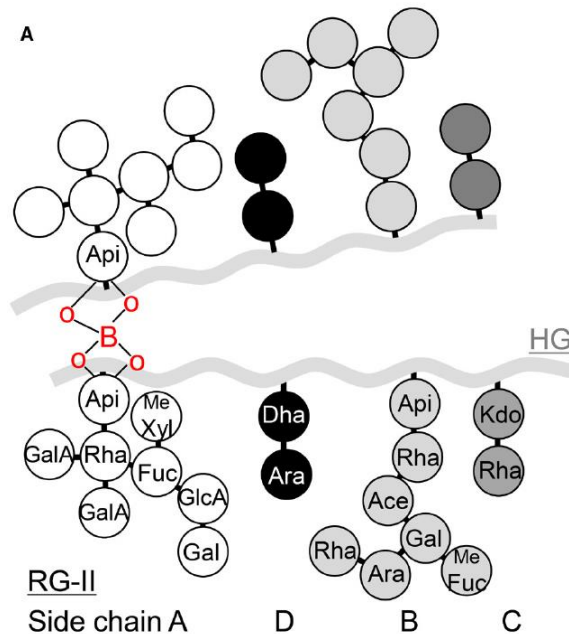

Figure 5 Model showing RG-II structure cross-linked with borate. There is the formation of a borate diester bond that covalently crosslinks the apiose residues in side chain A. Image adapted from Funakawa and Miwa, (2015).

Xylogalacturonan

 The pectin xylogalacturonan consists of a backbone similar to homogalacturonan, which is a polymer of (1→4)-linked α-D-galacturonic acid residues. However, the backbone is substituted with β-(1-3)-xylose monomers (Zandleven et al., 2006; Jensen et al., 2008a). Xylogalacturonan is present in most plant tissues, and accounts for 7%, 6% and 2,5% of pectin extracted from young leaves, mature leaves and stems of Arabidopsis, respectively (Zandleven et al., 2007).

1.3.3.1 Pectin Biosynthesis

It is generally agreed that pectin biosynthesis occurs in the Golgi as most pectic biosynthetic activities co-fractionate with Golgi markers (Mohnen, 2008). However, the involvement of the endoplasmic reticulum during the early stages cannot be ruled out (Sterling et al., 2001; Nunan, 2003; Geshi et al., 2004). To date, few enzymes, out of the estimated 67 glycosyltransferases, acetyltransferases and methyltransferases predicted to be players in pectin biosynthesis, have been identified, and these all localize in the Golgi (Mohnen *et al.* 2008). *In vitro* enzymatic reactions have shown that glycosyltransferases involved in pectin biosynthesis utilise nucleotide sugars as

substrate. The biosynthesis of nucleotide sugars needed to produce pectin occurs in the cytosolic side of the Golgi (Ridley et al., 2001). Transferase reactions needed to synthesis the pectin backbone together with associated side chains then takes place in the Golgi. The mechanism involved in the transport of nucleotide sugars across the Golgi membrane involves nucleotide-sugar:nucleoside monophosphate antiporters. This was confirmed in work carried out in animal systems (Capasso and Hirschberg, 1984) as well as plants (Wang et al., 1997; Neckelmann and Orellana, 1998).
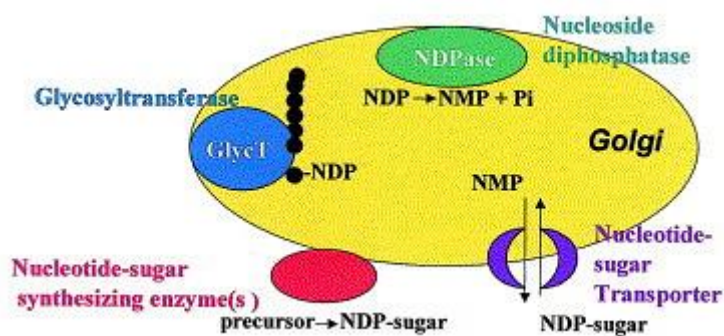

Figure 6: Model showing the biosynthetic pathway of pectin. Image taken from Ridley, et al., (2001). Nucleotide sugars are synthesised in the cytosolic side of the Golgi before nucleotide-sugar:nucleoside monophosphate antiporters transport them into the Golgi lumen. Glycosyltransferases act on the nucleotide sugars substrate by adding the glycosyl units to the growing polysaccharide chains. A nucleoside-5′-diphosphatase located in the Golgi then hydrolyses the released nucleoside diphosphate to an inorganic phosphate and nucleoside monophosphate which is then made available for the nucleotide-sugar:nucleoside monophosphate antiporters (Orellana et al., 1997)

1.3.3.2 Homogalacturonan biosynthesis

There are two major enzymes catalysing the biosynthesis of homogalacturonan, namely homogalacturonan-α-1,4 galacturonosyltransferase designated as GALACTURONOSYLTRANSFERASE (GAUT) (Sterling et al., 2006) and homogalacturonan-methyltransferase. Work by Scheller et al (1999) showed that an *α*-1,4-GalA transferase added galacturonic acid monomers to the non-reducing growing end of homogalacturonan chain *in vitro* (Scheller et al., 1999). GAUT1 has a predicted N-terminal transmembrane domain with the globular domain located in the Golgi lumen. In Arabidopsis, the GAUT family contains 15 members and belongs to group 8 of CAZy GTs. Earlier work had also implicated one member of this family (QUASIMODO1 (QUA1; GAUT8)) as being a player in the production of

homogalacturonan (Bouton et al., 2002). It is known that the methylation and polymerisation of homogalacturonan are two interdependent events during homogalacturonan biosynthesis (Mouille et al., 2007). HG-methyltransferase (HG-MT) has been identified in microsomal preparations and has been shown to methylate the pectin homogalacturonan exclusively (Mouille et al., 2007). This enzyme has been identified in mung bean (Crombie, H.J. and Reid, 1998), tobacco (Goubet and Mohnen, 1999), flax (Vannier et al., 1992), Arabidopsis (Mouille et al., 2007) and soy bean (Ishikawa et al., 2000). In the membranes of mung bean and tobacco, the methylesterification of homogalacturonan is accelerated by simultaneous addition of UDP-GalA, which is required for the synthesis of homogalacturonan (Goubet et al., 1998). The catalytic site of tobacco membrane bound HG-MT faces the Golgi lumen (Goubet and Mohnen, 1999), which is consistent with earlier observation that HG-MTs from mung bean are compartmentalised in membranes (Kauss and Swanson, 1969) and also that pea epicotyls methyltransferases localise to the Golgi (Baydoun et al., 1999). HG-MT and GalAT calatytic sites co-localise in the Golgi lumen supporting the assertion that they act one after the other in a unit during homogalacturonan biosynthesis.

The activity of a pectin *O*-acetyltransferase, whose acetate donor is acetyl-CoA, has been observed in microsomal preparations obtained from suspension cell cultures of potatoes (Pauly and Scheller, 2000). In Arabidopsis, two mutants with a decrease in leaf pectin acetylation have been found and they are termed *reduced wall acetylation2 (rwa2).* These proteins were identified based on homology to an acetyltransferase from the fungus *Cryptococcus neoformans,* Cas1p, which is responsible for acetylation of a coat polysaccharide (Janbon et al., 2001).

1.3.3.3 Rhamnogalacturonan I biosynthesis

RG-I is a more complex pectin compared to homogalacturonan, as it possesses arabinan and galactan derived side chains (Mohnen, 2008). The increased complexity of RG-I means more enzymes are involved in its biosynthesis. Analysis of cell free homogenates of mung bean has confirmed galactosyltransferase activity, and the presence of synthesised β-1,4-galactan could confirm the presence of rhamnogalacturonan I galactosyltransferases (Panayotatos and Villemez, 1973). Membrane preparations made from hypocotyls of mung bean led to the identification of β-1,4-galactosyltransferase activity (Brickell and Grant Reid, 1996), and also in flax

(Peugnet et al., 2001) and potatoes (Geshi et al., 2002) activity was detected. The transfer of Gal from UDP-Gal to acceptors of RG-I has been shown and the products could only be digested by a galactosidase and not an endo-β-1,4-galactanase (Geshi et al., 2002).

Plants possess mutases that are able to convert UDP-arabinopyranose to UDP-arabinofuranose, and work by Konishi et al., (2007) could prove the activity of the purified protein. Konishi et al., (2006) showed the efficient transfer of arabinofuranose onto growing oligosaccharide chains in vitro. Nevertheless, knowledge on glycosyl transferases involved in the biosynthesis of RG-I is still limited, with only a few glycosyl transferases published. Two proteins (ARABINAN DEFICIENT1 and XYLOGALACTORONAN DEFICIENT1) are membrane bound type II proteins that are localised in the Golgi (Harholt et al., 2005; Jensen et al., 2008) and involved in RG-I biosynthesis. ARABINAN DEFICIENT1 (ARAD1) is a putative arabinosyltransferase as the *arad1* mutants have reduced cell wall arabinose (Harholt et al., 2005). Recently another transferase, RG-I:rhamnosyltransferase (RRT) was identified from microsomal extracts of azuki beans (Uehara et al., 2017). In vitro assays indicated that the protein could synthesis the RG-I backbone using RG-I backbone derived oligoasacharides. The transferase localised in the Golgi and endoplasmic reticulum (Uehara et al., 2017). Another glycosyl transferase *MUCILAGE-RELATED70* (*MUCI70*) has been implicated to be involved in the biosynthesis of mucilage RG-I (Voiniciuc et al., 2018a).

1.3.3.4 Rhamnogalacturonan II biosynthesis

Not much is known about the biosynthesis of rhamnogalacturonan II, but a group of proteins (RGXT1, RGXT2, and RGXT3) belonging to *Arabidopsis* CAZy GT77 family was identified as having RG-II biosynthetic function (Egelund et al., 2006, 2008). The proteins are able to synthesise glycosidic linkages that only exists in RG-II and are made by an *α*-1,3-xylosyltransferase that transfers xylose to fucose from UDP-xyl (Harholt et al., 2010). However, the activity of these proteins has only been tested *in vitro* using proteins isolated from insect cells that are transfected with *Baculovirus* (Egelund et al., 2008), and *Pichia pastoris* (Petersen et al., 2009).

Work by Bourlard et al., (1997) demonstrateded that a pectin methyltransferase extracted from flax cell suspension cultures can use RG-II as an acceptor *in vitro*. However, the exact position where this methylation occurs is yet to be identified. Given

the complexity of RG-I, many glycosyl transferases must be required for its biosynthesis

1.3.3.5 Xylogalacturonan biosynthesis

A xylogalacturonan xylosyltransferase (XGD1) has been characterised and is found in CAZy GT47 family. Monosaccharide and linkage analysis of mutants deficient in the enzyme have a reduction in xylogalacturonan content (Jensen et al., 2008). Microsomal preparations used in *in vitro* assays and transient expression assays in *Nicotiana benthamiana* indicated that xylose was transferred from UDP-Xyl to GalUA oligosaccharides confirming the xylosyltransferase activity of XGD1 (Jensen et al., 2008).

1.3.3.6 Functions of pectin

Pectin performs diverse functional roles during plant development. The different structural domains present in the pectin heteropolysaccharide give various physical properties attributed to pectin. Pectin can influence the way cellulose microfibrils are deposited, which in turn influences cell mechanical properties (Chanliaud and Gidley, 1999). Pectin is known to influence the ion homeostasis and pH of the cell wall matrix through its ability to bind calcium and boron. The binding of calcium to demethylesterified homogalacturonan forma a gel affecting the porosity and mechanical properties of cell walls (Willats et al., 2001). Pectin present in the extracellular matrix is important for cell-to-cell adhesion, and also the controlled degradation of bonds present in this pectin is a requisite for the formation of intercellular spaces present in parenchymatous tissues (Knox, 1992).

The pectin network is an important player in plant developmental processes such as fruit ripening, pod dehiscence and fruit and leaf and fruit abscission (Wen et al*.,* 1999; Roberts, 2000). Pectin acts as a storage polymer and is important for the germination of seeds of some plant species. The arabinan and galactan side chains of RG I, because of their easy degradation, are the first polymers mobilised during germination (Tang et al., 1999). In Arabidopsis, the removal of Arabinans prior to germination negatively influences the germination process (Gomez et al., 2009). The active opening and shutting of the stomata in Arabidopsis is a function of the arabinan content present in pectin. It was shown that lack of arabinans in guard cells affected their ability to open or close (Jones et al., 2003, 2005; Merced and Renzaglia, 2019). The regulation of pollen tube tip growth is dependent on pectin and chemical nature of the

pectin. It is known that the growing tip of pollen tubes is exclusively made out of pectin, which is strong enough to prevent bursting and at the same time flexible enough to promote elongation for successful fertilisation to take place. Altering the nature or deposition of this pectin affects pollen tube growth with negative effects on pollination (Bosch, 2005; Ischebeck *et al.*, 2008). Homogalacturonan degradation generates oligalactoronides with biological activity influencing the expansion of cells and the triggering of plant defences (Dumville and Fry, 2000; Kohorn and Kohorn, 2012)

1.3.3.7 Role of pectin in plant pathogen defence

The importance of pectin in plant immunity is shown by the wide array of pectin hydrolysing proteins secreted by pathogens during their infection cycle. Silencing of some these enzymes renders the pathogen unable to infect the plant (Bellincampi et al., 2014; Lionetti et al., 2012). Bethke et al., (2016) indicated that reduction in pectin biosynthesis increase the susceptibility of Arabidopsis to various pathogens. Mutations in glucoronate 4-epimerase result in Arabidopsis plants with reduced cell wall pectin, because the protein is required for the biosynthesis of pectin building block UDP-D-galacturonic acid from its precursor UDP-D-glucuronic acid. Knockout mutants have a compromised basal resistance to specific *B. cinerea* isolates and to *P. syringae.* An Arabidopsis nuclear-localised transcription factor *AtERF014* was found to play a role in pectin biosynthesis, as it activates the genes involved in this process (Nakano et al., 2012). Arabidopsis *AtERF014*-RNAi knockdown lines have a reduction in cell wall pectin and were more susceptible to *P. syringae*, but were more resistant to *B. cinerea (Zhang* et al., 2016)*.*

An Arabidopsis starch deficient mutant*, phosphoglucomutase* (*pgm*), that has reduced levels of cell wall pectin was shown to be susceptible to the biotrophic pathogen *Colletotrichum higginsianum* (Engelsdorf et al., 2017). The Arabidopsis mutant *mur8-1* also exhibited an increased susceptibility to *C. higginsianum* (Engelsdorf et al., 2017)*.* MUR8 codes for a protein involved in rhamnose biosynthesis (Mertz et al., 2012), and the knockout mutant *mur8* has reduced rhamnose and RG-I content in the cell walls. However, the effects of pectin on plant defences are not always straight forward, as complex resistance phenotypes can result from changes in pectin content. For example the *powdery mildew resistant 5* (*pmr5*), *pmr6* double mutant, while being less susceptible to powdery mildew, exhibits a decreased resistance to *P.syringae* and *Hyaperonospora arabidopsidis* compared to wild type plants (Vogel, 2002; Vogel et al., 2004). *PMR6* is a putative pectate lyase, thus explaining the increased pectin

abundance in the mutant plant cell walls, whilst *PMR5* codes for a protein of a still unknown function (Maeda et al., 2009).

Changes to pectin that occur in response to interactions with pathogens are not limited only to the amount of pectin in the cell walls but also to the post-synthesis modifications. It is known that pectin upon deposition in the apoplast undergoes various modifications such as methylation, acetylation (Atmodjo et al., 2013), or trimming of galactan or arabinan side chains in RG-I (Arsovski et al., 2009; Dean et al., 2007). Multiple lines of evidence indicate that such changes to pectin composition influence plant-pathogen interactions. Homogalacturonan methylesterification is controlled by the action of pectin methylesterases, which are in turn regulated by pectin methylesterase inhibitors (PMEIs) (Lionetti et al., 2012). *At*PMEI10, *At*PMEI11 and *At*PMEI12 are pectin methylesterase inhibitors that regulate the methylesterification of pectin during infection with *B. cinerea* (Lionetti et al., 2017). Some AtPMEI overexpression lines exhibited an increased susceptibility to viral infections highlighting the importance of pectin methylesterification to plant pathogen interaction (Lionetti et al., 2014). It is thought that *At*PMEI10 and *At*PMEI11 are part of a plant defence system activated by the phytohormones jasmonate or ethylene (Overmyer et al., 2003) and help in resistance against *B. cinerea* in Arabidopsis (Lionetti et al., 2017). Wieczorek et al., (2014) presented evidence that pectate lyase like (*PLL*) genes together regulate the complexity of pectin including the degree of pectin methylesterification. In Arabidopsis, the infection with some cyst nematodes is reduced if the activity of PLL is abrogated, as the development and maintenance of the syncytia is impaired (Wieczorek et al., 2014).

Arabidopsis mutant plants with reduced wall acetylation have been identified (*rwa2*). The reduction in acetylation occurs on both pectin and non-pectin polysaccharides (Manabe et al., 2011). The *rwa2* Arabidopsis plants show a reduced susceptibility to *B. cinerea* (Manabe et al., 2011; Pogorelko et al., 2013). Plants produce endogenous polygalacturonases which depolymerise pectic polysaccharides during different developmental processes such as cell expansion, leaf and flower abscission and pollen tube development (D'Ovidio et al., 2004; Hadfield and Bennett, 1998). Wang et al., (2017) could show that *P. syringae* is able to trigger the induction of *IDA-like* 6 (IDL6) in Arabidopsis, which then induces the expression of an Arabidopsis polygalacturonase (ADPG2). The ADPG2 overexpression lines and the *adpg2* knockdown mutants had an increased resistance and susceptibility to *P. syringae*

respectively. The activity of polygalacturonases is inhibited by polygalacturonase inhibitor proteins (PGIPs), and as a defence response to phytopathogen polygalacturonase, plant produce PGIPs. Different plant species that overexpress PGIPs have been shown to have enhanced resistance to bacteria and necrotrophic fungi (Agüero et al., 2005; Ferrari et al., 2012).

1.3.3.8 Biological activity of pectin derived oligosaccharins

Pectin depolymerisation results in the generation of fragments containing various sugar residues including galacturonic acid, rhamnose, arabinose, xylose, mannose and galactose (Ridley et al., 2001). Oligogalacturonides (OGs) are derived from the degradation of homogalacturonan and thereby form linear galacturonic acid oligomers, which may or may not be methylesterified or acetylated. OGs produced from homogalacturonan degradation by pathogens act as danger associated molecular patterns (DAMPs) that activate various plant defence responses (Boller and Felix, 2009; Tör et al., 2009; Ranf et al., 2011). Activated defence responses include callose deposition, induction of ROS species (Galletti et al., 2008), production of chitinases and glucanases (Davis and Hahlbrock, 1987; Broekaert and Peumans, 1988), and the accumulation of phytoalexins (Davis et al., 1986; Chassot et al., 2008; Selim et al., 2017).

The plant defence responses elicited by OGs have led to the development of the theory that plants developed a pectin integrity monitoring system (PIMS), and that OGs are key players in this system that monitors pectin perturbation either from pathogens or during normal developmental processes (De Lorenzo et al., 2011). However, the degree of polymerisation is important for OGs to elicit biological responses. Only OGs with a degree of polymerisation of 10-15 elicit biological responses (Côté and Hahn, 1994). PGIPs which retard the activity of polygalacturonases are thus an important player in plant defences, as they not only impede pectin hydrolysis but also promote the production of OGs that elicit biological responses (De Lorenzo et al., 994, 2001). However, there have been OGs with a degree of polymerisation of 2-6 that have been shown to elicit biological responses, though they suppress plant defence responses in wheat (Moerschbacher et al., 1999). OGs are known to trigger oxidative bursts in Arabidopsis resulting in the deposition of callose in the apoplast (Zhang et al., 2007;

Galletti et al., 2008). Arabidopsis treated with OGs exhibits reduced susceptibility to phytopathogens including *B. cinerea* (Ferrari et al., 2007; Rasul et al., 2012).

OGs come with various degrees of methylesterification and acetylation, and it has been proven that the formation of haustoria of *Blumeria graminis* is impaired by acetylated OGs (Pelloux et al., 2007). Work by Randoux et al., (2010) showed that acetylated and unacetylated OGs triggered hydrogen peroxide accumulation at fungal penetration site. Osorio et al., (2008) also proved that transgenic strawberries (*Fragaria vesca* L.) that produced demethylesterified OGs were more resistant to *B. cinerea*, thus demonstrating the effect of methylesterification on the biological activity of OGs. Biological responses elicited by pectin oligosaccharides are not only confined to plant defences, but can trigger various developmental and physiological processes. OGs are antagonistic to auxins and suppress the expression of some auxin-induced genes upon perception of auxins (Mauro et al., 2002). Consequently, coleoptile growth in maize (*Zea mays* L.) seedlings was reduced, and lateral rooting induced by OGs resulted in modified root structure (Hernández-Mata et al., 2010).

1.3.4 Plant cell wall proteins

Plant cell wall proteins constitute a minor component of cell wall, approximately 5-10% (Fry, 2004), but they are a critical player in cell wall development and responses to environment (Fry, 2004; Passardi et al., 2004). Cell wall proteins can be classified into two broad groups: structural proteins and cell wall modifying proteins.

Structural proteins vary in their abundance according to growth conditions, plant tissues and plant species. Classification of structural proteins is based on their predominant amino acids with the most common being glycine rich proteins (GRPs), hydroxyproline rich glycoproteins (HRGPs), arabinogalactan proteins (AGPs) and proline rich proteins (PRPs) (Albenne et al., 2013). Arabinogalactan proteins have been implicated to also play a role in cell signalling (Seifert and Blaukopf, 2010).

Other cell wall proteins do not have a structural role, but are capable of modifying cell wall polysaccharides. The most widely studied cell wall modifying proteins are the expansins (EXPs), which, however, have no enzymatic function that has clearly been characterised. EXPs are thought to modify interactions existing between cellulose and xyloglucans in a manner that is pH-dependent (Marowa et al., 2016). The pH-dependent regulation of EXPs forms the basis of the acid-growth hypothesis, which states that cell walls expand in acidic conditions (Cosgrove, 2005). EXPs promote cell

wall relaxation, which in turn allows for cell expansion and gives other cell wall modifying proteins access to cell wall polymers. EXPs also enhance the activity of many other cell wall-modifying proteins such as xyloglucan endo-transglycosylases/hydrolases (XTHs) that are able to cleave and re-ligate xyloglucans (Eklof and Brumer, 2010).

Other cell wall proteins do not actively act on the cell polysaccharides, but their activity indirectly modifies the cell walls. Arabidopsis class III peroxidases localised in the apoplast have been identified including PRX33 and PRX34, which act as sources of apoplastic oxidative bursts in plants infected with avirulent *P. syringae* strains (Bindschedler et al., 2006). O'Brien et al., (2012) could show that Arabidopsis cell suspensions, when treated with MAMPs, produced H_2O_2, of which 50% was attributed to PRX33 and PRX34. It was also shown that PRX33 and PRX34 were important for resistance against various pathogens including *B. cinerea*, *Golovinomyces cichoracearum* and *P. syringae* (Bindschedler et al., 2006). Another group of cell wall-localised proteins contributing to ROS bursts are amine oxidases and polyamine oxidases, which catabolise polyamines such as spermidine resulting in H_2O_2 production, leading to programmed cell death or triggering of stress tolerance signals (Petrov et al., 2015). Infiltration of Arabidopsis leaves with polyamines results in oxidative bursts that trigger programmed cell death (Yoda et al., 2009). Table 1 gives a summary of some major cell wall proteins identified in Arabidopsis. Many proteins modifying pectin have also been characterised for instance HG is cleaved by pectate lyases, which is important for re-arrangement of the pectin matrix (Domingo et al., 2002; Marin-Rodriguez, 2002). HG also undergoes modifications by pectin methyesterases, acetylases and polygalacturonases which have already been highlighted (Micheli, 2001; Babu and Bayer, 2014; Philippe et al., 2017). In Arabidopsis, an enzyme that oxidises OGs named OG OXIDASE1 (OGOX1) was identified (Benedetti et al., 2018). The cell wall-localised protein belongs to a large gene family of putative FAD-binding proteins named Berberine Bridge Enzyme like (BBE-like). The oxidation of OGs was found to be important for the deactivation of biologically active OGs produced after pathogen infection (Benedetti et al., 2018).

The pectin RG-I undergoes various modifications including degradation of the backbone by a rhamnogalacturonan lyase during fruit ripening (Guillermo Berumen-Varela and Martín-Ernesto Tiznado-Hernández, 2018). Galactosidases and

arabinosidases are also thought to act on pectin RG-I. Their activity is important for production of mucilage with correct hydration properties in Arabidopsis seed coat epidermal cells (Dean et al., 2007; Arsovski et al., 2009).

Table 1 Classification of Arabidopsis cell wall proteins according to predicted functional domains. Proteins are classified according to their functional domains determined using Pfam, InterPro and PROSITE bioinformatics program (Albenne, et al., 2013).

Functional class	% of identified proteins
Proteins acting on polysaccharides	**25.7**
Glycoside hydrolase Esterases/lyases Expansins	
Oxido-reductases	**14.6**
Peroxidases Berberine Bridge enzymes	
Structural proteins	**1.6**
Proteins involved in signalling	**6.6**
Proteases	**11.2**
Proteins with interacting domains	**11**
Lectin domain LRR domain Enzyme inhibitors	
Proteins related to lipid metabolism	**5.8**
Miscellaneous	**11**
Unknown function	**12.5**

1.3.4.1 Arabidopsis BXL gene family

The analysis of Arabidopsis crude protein extracts from stem tissues led to the identification of two proteins exhibiting betaxylosidase activity namely; BXL1 and BXL4 (Minic et al., 2004). Previous work had also identified BXL1 in a screen for proteins with a possible function in secondary cell wall thickening (Goujon et al., 2003). Additional work confirmed that BXL1 belonged to a small gene family of xylosidases or beta-xylosidase due to the presence of a glycosyl hydrolase domain and their similarity to bacterial and fungal beta-xylosidases (Goujon et al., 2003). The other members of this gene family are BXL2, 3, 4, 5, 6 and 7 (figure 7). *BXL1* is the only fully characterised member of this gene family and according to Arsovski et al., (2009), it is a bifunctional β-d-xylosidase/α-l-arabinofuranosidase. Mutations in *BXL1* result in plants with seed mucilage that cannot extrude due to an increase in Ara side chains of RG-I (Arsovski et al., 2009). *BXL1* expression is confined to tissues undergoing thickening of the

secondary cell walls and the knockout mutants have a reduced beta-xylosidase activity (Goujon et al., 2003). BXL2, 4 and 5 all have a predicted extracellular localisation, and are therefore possibly involved in cell wall remodelling as well (Goujon et al., 2003).
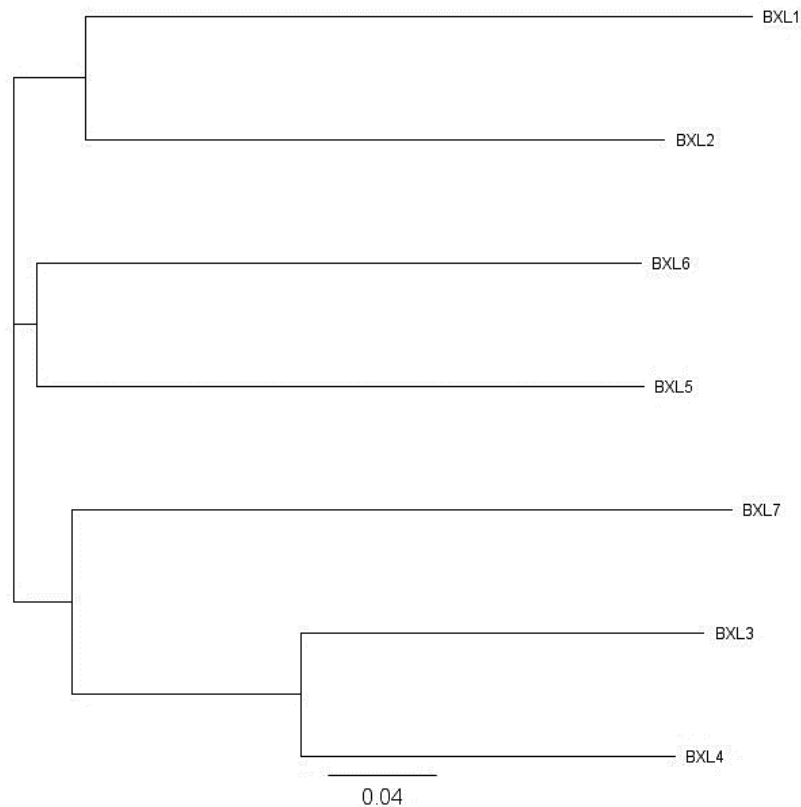

Figure 7: Phylogenetic tree of betaxylosidases (BXLs) from _Arabidopsis thaliana_. Image generated with Geneious version 8.1 created by Biomatters (_http://www.geneious.com_) using Jukes-Cantor genetic distance model and Neighbor-joining tree build method

1.4 Aims of the thesis

As already highlighted Arabidopsis BXL1 is a known bifunctional β-d-Xylosidase/α-l-Arabinofuranosidase belonging to a seven member gene family in Arabidopsis and acts on xylan and arabinan chains present in cell walls (Goujon et al., 2003; Minic et al., 2004; Arsovski et al., 2009). The enzyme activity of the other members of this BXL family are yet to be ascertained, presenting the first objective of this thesis. This work aims to determine the functional activity of BXL4 with regards to cell wall modifications through expression in Arabidopsis seed coat epidermal cells of the _bxl1_ knockout mutant and analysis of cell walls of _bxl4_ knockout mutants.

The importance of plant cell modifications in plant pathogen defence responses in Arabidopsis has already been indicated in this paper (Bacete et al., 2018; Novaković et al., 2018). Transcriptome databases such as eFP browser and Genvestigator show that *BXL4* is upregulated upon infection of Arabidopsis by various phytopathogens (Breitenbach et al., 2014) thus, might be involved in cell wall remodelling to mitigate pathogen development in Arabidopsis. The second aim was to determine the cell wall modifications induced by BXL4 and its effect on plant immunity. *bxl4* mutants were assayed for their susceptibility/resistance to *B. cinerea*.

The third aim of the thesis is to develop a gas chromatography–mass spectrometry (GC-MS) based protocol to analyse monosaccharide compositions of pectin extracted from various tissues including mucilage and leaves. The protocol developed should reliably determine monosaccharide compositions of both neutral sugars and galacturonic acid of extracted pectin and is needed to pursue the first aim.

2 Manuscript A: A GC-MS-based method of water extracted pectin monosaccharide analysis.

This manuscript is due for submission to Plant Methods journal.

Individual contribution:

Athanas Guzha performed all the experiments together with Patricia Scholz. He also did the data analysis and wrote the manuscript with the assistance of Patricia Scholz and Till Ischebeck.

A GC-MS-based method of water extracted pectin monosaccharide analysis.

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Abstract

The plant cell wall consists of a complex mix of polysaccharides that undergo various modifications during a plant cell's lifetime. Cell wall modifications are induced in response to environmental changes and physiological development needs. Identification of changes occurring in these cell walls is critical to get a better understanding of the link between cell wall composition and function and requires suitable methods to determine cell wall composition. Here, we describe a GC-MS-based method to measure the monosaccharide composition of pectin derived sugars. We used this method for fast and straightforward monosaccharide composition analysis of pectin derived from different organs including leaves, seeds and fruit peel as proof of concept. We provide an improved GC-MS method as a useful tool to study pectin composition from various sources.

All plants have cell walls that are important for mechanical support, cell-to-cell-communication, plant morphogenesis and plant defence responses. To fulfil these diverse functions, plant cell walls are composed of an intricate assembly of carbohydrates, proteins and phenolic compounds, varying between different cell types and developmental stages (Carpita and Gibeaut, 1993; Freshour et al., 2003; Knox et al., 1990). Primary cell walls consist up to 90 % of polysaccharides like cellulose, hemicelluloses and pectin (Caffall and Mohnen, 2009; Höfte and Voxeur, 2017; Pettolino et al., 2012). Cellulose and hemicelluloses form a strong network that is embedded into the amorphous matrix formed by pectin (Carpita and Gibeaut, 1993). Pectin consists of several different polysaccharide domains, each characterised by a high content of galacturonic acid linked in O1- and O4-positions. The most abundant and least structurally complex pectic polysaccharide, homogalacturonan (HG), has a backbone of $\alpha(1\rightarrow4)$-linked D-galacturonic acid (GalA) monomers. GalA monomers in a HG chain may be O-acetylated in O2 or O3 position; furthermore, GalA may be substituted with D-xylose in O3 position or D-apiofuranose in O2-/O3-position to form xylogalacturonan and apiogalacturonan, respectively (Anderson, 2016; Atmodjo et al., 2013; Mohnen, 2008; Caffall and Mohnen, 2009). Similar to HG, rhamnogalacturonan II (RG II) possesses a backbone formed by $\alpha(1\rightarrow4)$-linked D-GalA monomers, however, it is substituted with four different structurally complex side chains (Mohnen, 2008; Anderson, 2016; Voiniciuc et al., 2018). Unlike the other two pectic polysaccharides, rhamnogalacturonan I (RG I) has a backbone formed by the disaccharide repeat of [-α-D-GalA - 1,2-α-L-Rha-1,4 -]. Depending on cell type and developmental states, the backbone carries different arabinan, galactan or arabinogalactan side chains (Mohnen, 2008; Anderson, 2016; Bidhendi and Geitmann, 2016).

Matrix polysaccharides of pectin are synthesised in the Golgi, transported in secretory vesicles and further modified *in mura* (Harholt et al., 2010; Mohnen, D., Bar-Peled, M. and Somerville, 2008; Ridley et al., 2001). Modifications in the cell wall occur due to enzymes localised in the apoplast and are often linked to responses to pathogen attack (Körner et al., 2009; Wang et al., 2017). To monitor changes and modifications of pectin it is therefore necessary to develop methods to identify alterations of its biochemical composition. Furthermore, description of the various enzymes involved in pectin biosynthesis and their respective Arabidopsis mutants requires reliable analysis

of pectin composition. However, the insoluble yet amorphous properties of pectic compounds makes a detailed characterisation of their structure *in mura* a challenging task. The use of different fluorescent probes, monoclonal antibodies and sugar analogues derived from click chemistry for monitoring of cell wall pectin has been described, however, it gives limited information about the relative amounts of the different monosaccharides (Bethke and Glazebrook, 2019; Hoogenboom et al., 2016; Voiniciuc et al., 2018). Complete biochemical analysis of cell wall components combines analysis of the components, their respective abundance and the linkages they undergo in the cell wall. Full linkage analysis is a time-consuming process, as it requires methylation of cell wall samples for the differentiation of free hydroxyl groups ahead of cell wall hydrolysis and component analysis (Biswal et al., 2017; York et al., 1986; Pettolino et al., 2012). Additionally, for several purposes such as monitoring of pathogen induced cell wall remodelling, a composition analysis alone already provides the required information.

Pectin for analysis can derive from different plant sources. Cell wall samples, e.g. of leaves, are often prepared in form of alcohol insoluble residue (AIR) (Gille et al., 2009; Pettolino et al., 2012; Biswal et al., 2017). An established alternative for the analysis of RG I is seed coat mucilage, an extracellular matrix that is rapidly extruded from mucilage secretory cells of Arabidopsis seeds upon hydration (Arsovski et al., 2010; Voiniciuc et al., 2015). Independent of the source, pectin polysaccharides are subsequently hydrolysed into their respective monosaccharides. These can then be quantified by a variety of methods including colorimetric assays, GC-MS- and HPLC-based approaches (Willför et al., 2009; Pettolino et al., 2012; Biswal et al., 2017; Bethke and Glazebrook, 2019). For the use of GC-MS detection, hydrolysis products are further derivatised to be volatile enough for GC separation. Two main methods for volatilisation are described in the literature: reduction and acetylation to form alditol acetates or methanolysis of cell wall samples followed by trimethylsilylation (York et al., 1986; Merkle and Poppe, 1994; Biswal et al., 2017). Derivatisation to alditol acetates will impair the detection of uronic acids, as the carboxyl-group is reduced in the reduction step. For the analysis of pectin composition, the analysis of alditol acetates is therefore unsuitable. However, methanolysis of cell wall samples is a time-consuming processing requiring incubation times of 15 hours or more. Additionally, methanolysis results in several different analytes formed from one monosaccharide,

leading to several signals in the GC chromatogram and the methylation of monosaccharides complicates absolute quantification of hydrolysed sugars (York et al., 1986; Biswal et al., 2017).

Here we describe a fast method for GC-MS-based compositional analysis of cell wall pectin extracted from cell wall samples by water extraction using a modified hydrolysis and derivatisation protocol. TFA is used as hydrolysing agent, which greatly reduces hydrolysis time and does not alter the chemical nature of the monosaccharide building blocks. These are then easily derivatised with methoxyamine (MOX) and N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), enabling compositional analysis of pectic samples in 2-3 days.
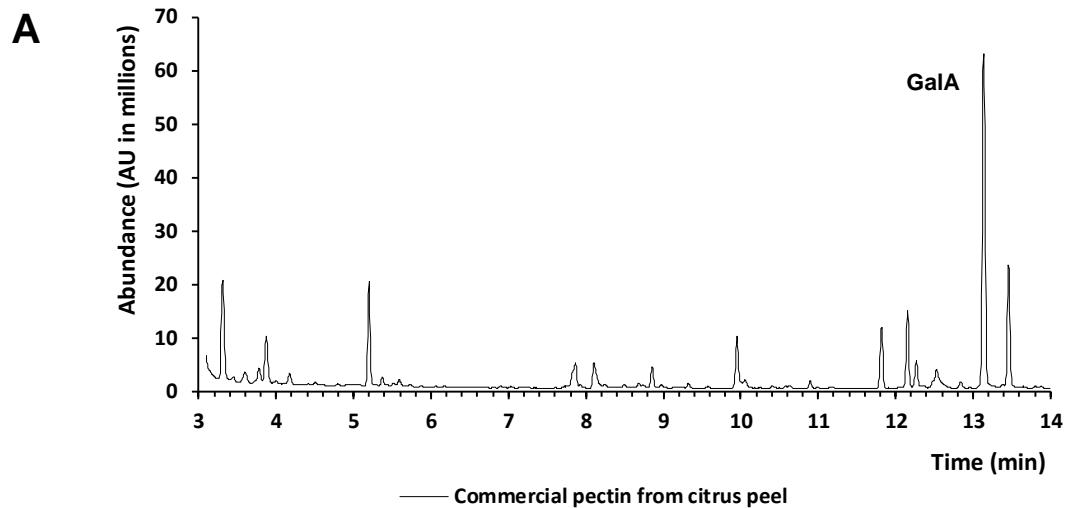
Results and Discussion

Monosaccharide composition of Sigma-Aldrich pectin from citrus.

To test the efficiency and accuracy of our method, commercially available pectin of citrus peels was hydrolysed, derivatised with MOX and MSTFA, and analyzed on the GC-MS. Pectin consists of various sugars including galacturonic acid, galactose, rhamnose, arabinose, fucose, xylose and in minor amounts glucuronic acid (Vincken et al., 2003; Voragen et al., 2009). Other sugars from cell wall polysaccharides such as glucose and mannose can also be detected. All the expected sugars except glucuronic acid could be separated with GC and the expected analytes could be detected by MS (Figure 1A). Some of these monosaccharides are pairs of diastereomers including arabinose/xylose, rhamnose/fucose, galactose/glucose/mannose, and galacturonic acid/glucuronic acid. As of this, they generate similar mass spectra, making MS-based identification a challenge. To counteract this challenge, the individual sugars were obtained as pure substances and the retention times of their derivatives recorded. Derivatisation resulted in the formation of two isomeric analytes in a stable stoichiometric ratio. Consequently, for most monosaccharides two peaks with characteristic retention times and a fixed ratio to each other could be detected. Based on differences in retention time, the individual sugars could be clearly separated.

The most abundant monosaccharide in the pectin of citrus peels was galacturonic acid that contributed 79.5% to the total amount of monosaccharides (Figure 1B). This is

consistent with the product description of at least 74% galacturonic acid content. The most abundant neutral sugars that we observed were galactose, arabinose and rhamnose. Our results are consistent with established pectin structure of a galacturonic acid derived backbone and galactan and arabinan side chains (Mohnen, 2008; Zdunek et al., 2014).
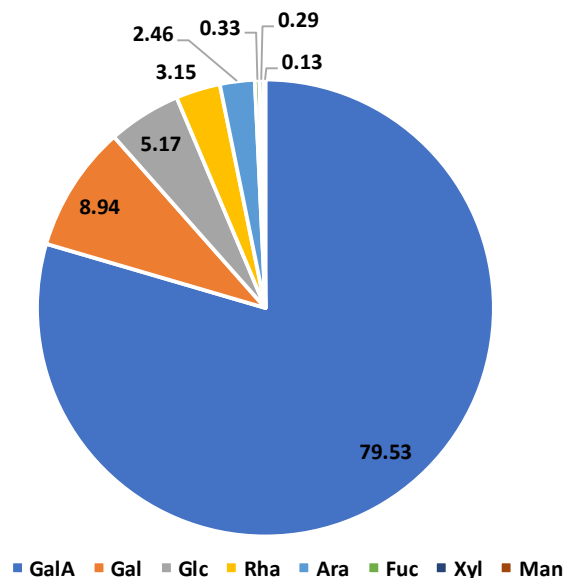

Figure 1: Monosaccharide composition (%) of commercial pectin from citrus peel (Sigma-Aldrich).

(A) Chromatogram of GC-MS run showing the expected analytes eluting within the first 14 minutes. (B) Galacturonic acid (GalA), galactose (Gal), glucose (Glc),

rhamnose (Rha), arabinose (Ara), fucose (Fuc), xylose (Xyl), mannose (Man) could all be detected and quantified. Data represents averages of 4 replicates.

Mucilage monosaccharide composition varies between Arabidopsis ecotypes and mutants

The relative composition of water-extracted mucilage from previously described Arabidopsis lines was investigated to make a comparison with already published data. Mucilage was extracted from Col-0, Ws and a mutant, *bxl1*, in the Ws background which was shown to have higher arabinose levels in its mucilage (Arsovski et al., 2009). Mucilage, being a type of cell wall with all the basic components such as pectin, cellulose, hemicellulose and cell wall proteins, provides an ideal model system to analyse cell wall variances in different ecotypes and modifications due to activity of
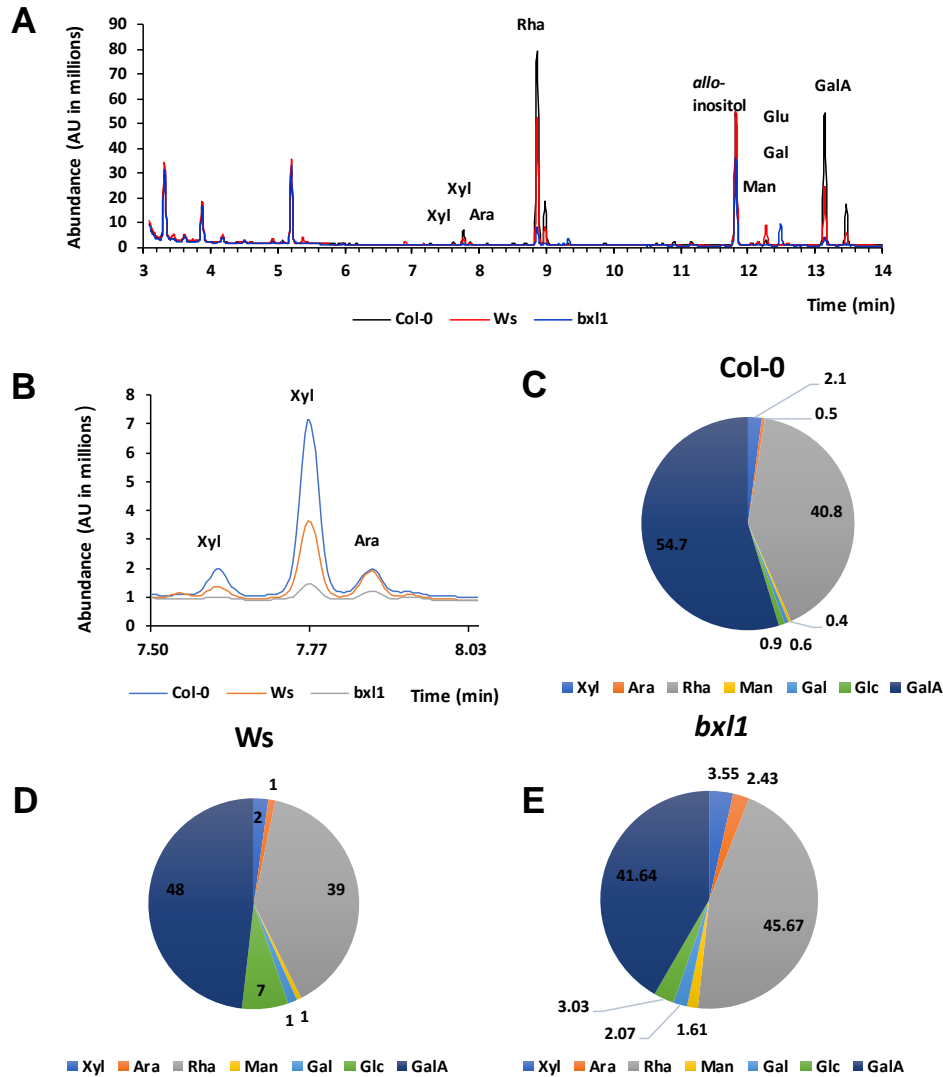
Figure 2: Chromatograms and monosaccharide composition (%) of different Arabidopsis ecotypes and mutants.

(A) Chromatograms indicating mucilage pectin sugars eluting within the first 14 minutes. (B) Enlarged section of the chromatogram showing the elution of xylose and arabinose in Col-0, Ws and *bxl1* mutant. (C-E) Mucilage monosaccharide composition (%) of Col-0 (C) Ws (D) and *bxl1* (E) data represents the average of 4 replicates.

various proteins (Western et al., 2000; Arsovski et al., 2010). All the expected sugars could be detected with the exception of the minor sugar fucose that was probably below the detection level. Rhamnose and galacturonic acid were the dominant sugars (Figure 2A & B) with relative amounts of 39.3% (rhamnose) and 48.2% (galacturonic acid) in Ws and 45.7% (rhamnose) and 41.6% (galacturonic acid) in *bxl1*. This is consistent with the fact that mucilage consists mostly of the pectin RG I, which has an

alternating rhamnose-galacturonic acid backbone (Haughn and Western, 2012). BXL1 is a bifunctional β-D-xylosidase/α-L-arabinofuranosidase, so the chromatograms generated with regard to xylose and arabinose separation were of interest (Figure 2C). After calculating monosaccharide composition, we could observe a 2-fold increase in arabinose content in the mucilage of *bxl1* compared to the Ws wild type (Figure 2D). This was consistent with previously published data (Arsovski et al., 2009). The mucilage monosaccharide compositions obtained from Col-0 showed galacturonic acid and rhamnose contributing 54.7% and 40.8% of total monosaccharides, respectively. The other side chain sugars present in RG I, derived from galactose and arabinose, could also be detected and are in proportions similar to other publications that used high-performance anion-exchange chromatography with pulsed amperometric detection to measure the sugars (Voiniciuc and Günl, 2016).

Analysis of water-soluble pectin from leaf alcohol insoluble residue.

Water-soluble pectin was extracted from the alcohol insoluble residue prepared from Arabidopsis Col-0 leaves. It is known that the extraction method used in pectin extraction affects its composition (Rose et al., 1998). Cell wall disintegration due to heating allows for the extraction of water soluble pectin, whilst acid and enzyme addition allows for the extraction of insoluble pectin that tightly interacts with other cell wall polysaccharides (Assoi et al., 2014). Monosaccharide analysis of the water soluble pectin indicated that galacturonic acid was the dominant monosaccharide (Figure 3A & B), constituting 72% of the extracted monosaccharides (Figure 3C). Our data is consistent with a previously described galacturonic acid content of ca. 70% in pectin (Mohnen, 2008). The extracted pectin also confirmed the presence of neutral sugars, with the most abundant being galactose, arabinose and rhamnose. The monosaccharide profile obtained indicates that the extracted component from the AIR is predominantly pectin, given the high abundance of galacturonic acid and rhamnose, and that the galactose and arabinose are mostly from pectin side chains (Zdunek et al., 2014; Vincken et al., 2003). In water-extracted pectin of leaf cell walls, there is normally an abundance of glucose derived from starch, and to minimise the amount of glucose detected, plants can be incubated in the dark for 48 hours. However, for monosaccharide composition analysis of pectin, glucose does

not present a problem because it is not a component of pectin and can be ignored. It is also clearly separated from its diastereomer galactose so it does not interfere with absolute quantification.
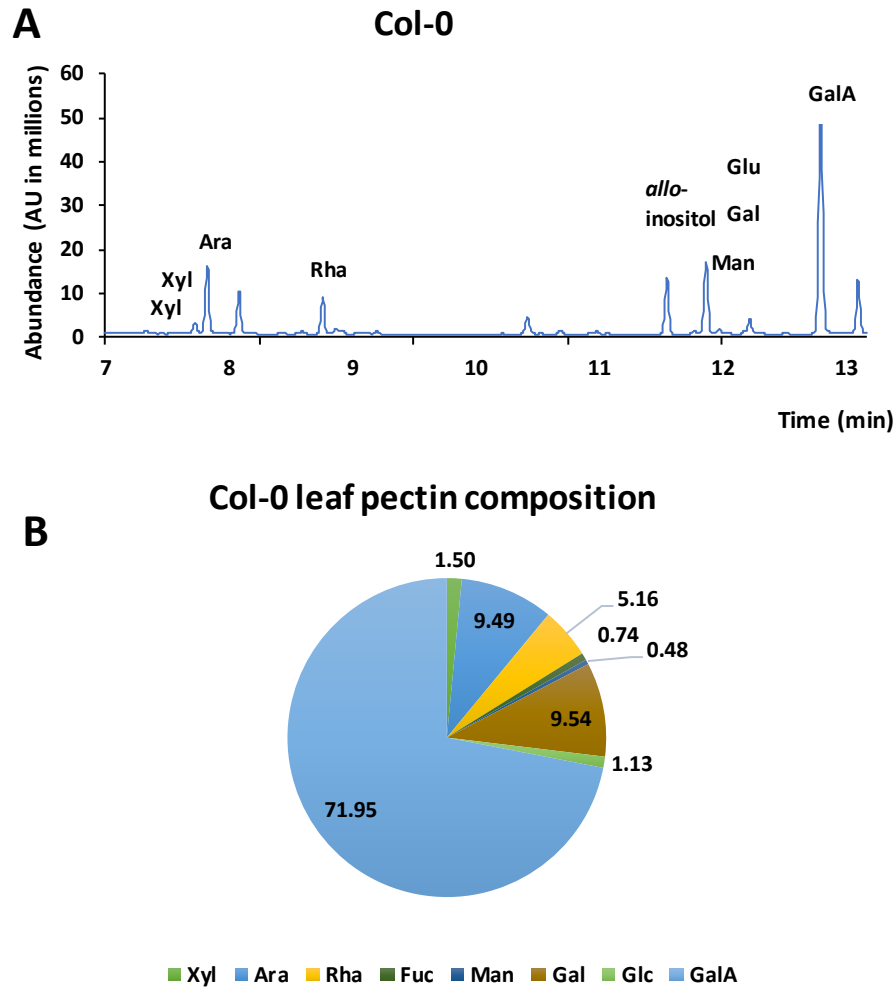

Figure 3: Chromatogram and monosaccharide composition of water extracted pectin from leaf AIR.

(A) The chromatograms generated from GC-MS runs of water extracted pectin from Arabidopsis leaf AIR (A & B). (B) The monosaccharide composition (%) of the various sugars from leaf pectin analysed by GC-MS. The values represent means of 4 biological replicates.

Table 1: Comparative summary of mucilage monosaccharide compositions obtained with GC-MS analysis versus published data. All values are given as percentage of total monosaccharide content.

	Col-0 mucilage	Col-0-mucilage (Voiniciuc and Günl, 2016)	Ws mucilage	Ws mucilage (Arsovski et al., 2009) Ammonium oxalate extraction	*bxl1* mucilage	*bxl1* mucilage (Arsovski et al., 2009) Ammonium oxalate extraction
Xylose	2.1	3.9	2.2	3.7	3.6	3.3
Arabinose	0.5	1.8	1.1	0.7	2.4	1.3
Rhamnose	40.8	41.7	39.3	49.5	45.7	49.7
Fucose	0		0	tr	0	tr
Mannose	0.4	0.9	0.7	1	1.6	1.2
Galactose	0.6	1.6	1.4	1.8	2.1	1.9
Glucose	0.9	1.6	7.1	2.3	3.0	2.1
Galacturonic acid	54.7	48	48.2	40.9	41.6	40.4

Conclusion

The GC-MS method we developed allowed us to quantify monosaccharide compositions of pectin extracted from leaves, seed mucilage and commercial pectin with reasonable accuracy comparing to what has been published before using other techniques. All nine monosaccharides are detected, so acidic and neutral sugars can be analysed in parallel. Due to the MS-based detection, mass spectra can be used for partial confirmation of monosaccharide identity. The use of TFA as hydrolysing reagent greatly reduces hydrolysis time compared to acidic methanolysis, thus sample preparation up to the first derivatisation step can be carried out in one day followed by GC-MS analysis the next day. Furthermore, as monosaccharides are not modified with methyl groups, absolute quantification is easily achieved by calculating area-response factors with the help of external standards. In summary, we provide an improved method for pectin analysis for comparative purposes between different lines or treatments.

Reagents

- Distilled water

- Liquid nitrogen

- Nitrogen gas

- Ethanol

- Methanol

- Chloroform

- Acetone

- MOX (Methoxyamine hydrochloride, Sigma-Aldrich 226904)

- MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide, Sigma-Aldrich 69479)

- Acetonitrile (Sigma-Aldrich 271004)

- TFA (Trifluoroacetic acid, Sigma-Aldrich 302031

- Pyridine Anhydrous (Sigma-Aldrich 270970)

- *allo*-inositol (Sigma-Aldrich 468088)

- L-(-)- Fucose (Sigma-Aldrich F2252)

- D-Glucuronic acid (Sigma-Aldrich G8645)

- D-(+)-Galacturonic acid (Sigma-Aldrich 48280)

- L-(+)-Arabinose (Fluka-Biochemika 10845)

- L-(+)-Rhamnose (CARL-ROTH 4655.2)

- D-(+)-Mannose (Merck 5984)

- D-(+)-Xylose (Merck 8692)

- D-(+)-Galactose (CARL-ROTH 4987.2)

- D-(+)-Glucose (CARL-ROTH 6780.2)

- Citrus pectin (Sigma-Aldrich P9135)

Equipment

- Fume hood

- Retsch ball mill

- Mortar and pestle

- Heating block with shaker for 2 ml Eppendorf tubes (HLC HTM 130)

- Heating block for Duran tubes

- Rotary shaker

- Digital scale

- GC-MS system
- Heat resistant test tube rack
- Test tube heating block/oven
- Vortexer
- Duran tubes
- Glass Pasteur pipettes
- Acid resistant pipettes
- 2 ml GC-MS-Vials + inserts
- Glass beads 3mm

Procedures

AIR analysis

Alcohol Insoluble residue preparation

The method used in this part of protocol is adapted from Gille et al., (2009) and Bethke et al., (2014) and involves the following steps:

1. Plants for analysis are incubated for 48 h in the dark. Incubation in the dark is an easy way to reduce the starch content, and minimize glucose overload.

2. Organs required for analysis (Leaves, stems, roots, whole rosettes) are flash frozen in liquid nitrogen, and pulverized using mortar and pestle. Grinding of material is a crucial step to prevent contamination of wall material with cytosolic derived sugars.

3. Pulverized material (up to 100 mg) is placed in 2 mL Eppendorf tube.

4. AIR is extracted by washing ground material in 1,5 mL 70% (v/v) ethanol.

5. The supernatant is removed by centrifugation for 10 min at 14,000 rpm.

6. Repeat Ethanol wash as described above.

7. Carry out three washes with 1 mL chloroform/ methanol 1:1 (v/v), with the supernatant obtained by centrifugation at 14000 rpm for 10 minutes discarded after each wash. At the end of the last washing step plant material derived from chlorophyll containing tissue should have a dull grey to off-white color.

8. Wash AIR in 1 ml acetone, and centrifuge at 14000 rpm for 10 minutes.

9. Discard the supernatant and air dry the AIR at room temperature overnight.

AIR pectin analysis

1. Prepare 4 replicates for each genotype. Weigh 2 mg of AIR for each replicate and place in a 2 mL Eppendorf tube. Record the exact weight of AIR placed in Eppendorf tube. Use plants grown at the same time and under the same conditions.

2. Add 1.4 mL of ddH$_2$O to the AIR.

3. Add 2 glass bead (3 mm) and homogenise using a Retsch ball mill at 30 Hz for 1.5 mins

4. For hot water extraction of pectin, incubate the samples for 2 hours on a shaker at 90°C.

5. Allow debris to settle and remove 1 mL of supernatant to a Duran glass tube.

6. Dry under N$_2$ stream, heating the samples to 40°C.

7. Add 300 µL of 2 M TFA.

8. For TFA hydrolysis, incubate at 121°C for 1 hour. Do this step under a fume-hood.

9. Evaporate the TFA under N$_2$ stream at room temperature under a fume-hood.

10. Extract hydrolysis products with 500 µL ddH$_2$O plus 100 µL of 0.05 mg/mL *allo*-inositol.

11. For GC-MS: Evaporate 20 µL under N$_2$ stream. The remaining hydrolysis products can be stored for long term at -20°C.

12. Derivatise dried sample with 15 µL 30 mg/mL MOX in anhydrous pyridine at room temperature over-night.

13. Add 30 µL MSTFA and analyse on GC-MS after incubation of 1-6 hours.

Mucilage analysis

1. Prepare 4 replicates for each genotype. Weigh 2 mg of seeds for each replicate and place in a 2 mL Eppendorf tube. Record the exact weight.

2. Add 1.4 mL of ddH$_2$O to the Eppendorf tube

3. Place on rotary shaker and shake for 2 hours to extract the mucilage.

4. Allow the seeds to settle and transfer 1 mL of supernatant to a Duran glass tube.

5. Dry under N$_2$ stream, heating the samples to 40°C.

6. Add 300 µL of 2 M TFA

7. For TFA hydrolysis, incubate at 121°C for 1 hour . Do this step under a fume hood.

8. Evaporate the TFA under N$_2$ stream at room temperature under a fume hood.

9. Extract hydrolysis products with 500 µL ddH$_2$O plus 100 µL of 0.05 mg/mL *allo*-inositol.

10. For GC-MS: Evaporate 20 µL under N$_2$ stream. The remaining hydrolysis products can be stored for long term at -20°C.

11. Derivatise dried sample with 15 µL 30 mg/mL MOX in anhydrous pyridine at room temperature over-night.

12. Add 30 µL MSTFA and analyse on GC-MS after incubation of 1-6 hours. Samples should be measured 1-6 hours after addition of MSTFA.

GC-MS analysis

Samples were analysed with the 7890B GC-System coupled to a 5977B MSD quadrupole set-up from Agilent Technologies. GC-separation was achieved on a HP-5 column (Agilent Technologies) using the following temperature gradient: 150°C for 2 min, 5 K/min gradient for 20 min, 15 K/min to a final temperature of 320°C and 3 min hold at 320°C. For each run, 1 µL of the derivatised sample was injected. Compounds were identified by a combination of retention times compared to external standards and MS-spectra of electron impact mass spectrometry.

Prepared mucilage samples were quantified relative to the internal standard *allo*-inositol. In parallel runs, monosaccharide standards of different concentration were used to determine response factors for area-to-molar amount conversion

allowing absolute quantification. Sugars were normalized to the mass of pectin, seeds or AIR used for extraction.

Results were analyzed using the program "Instrument #1 Data Analysis" from Agilent Technologies. Peaks were identified with the help of standards from the cell wall monosaccharides arabinose, fucose, galactose, galacturonic acid, glucose, mannose, rhamnose and xylose.

For quantification, area-to-molar amount response factors had to be established for each cell wall monosaccharide. To that end, defined molar amounts (1-3 nmol) of each monosaccharide were derivatised and measured like pectin samples. Three replicates of each concentration were analysed. Peak areas of target ions from the identified compounds were integrated and normalized to the peak area of the internal standard *allo*-inositol. The normalized peak areas were then plotted against the molar amounts. From the resulting calibration curve the response factors were determined which could then be used to calculate the molar amount of cell wall monosaccharides present in mucilage samples.

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Author contributions

P.S. and A.G. performed the experiments and wrote the manuscript. T.I. designed the project and assisted with the data analysis.

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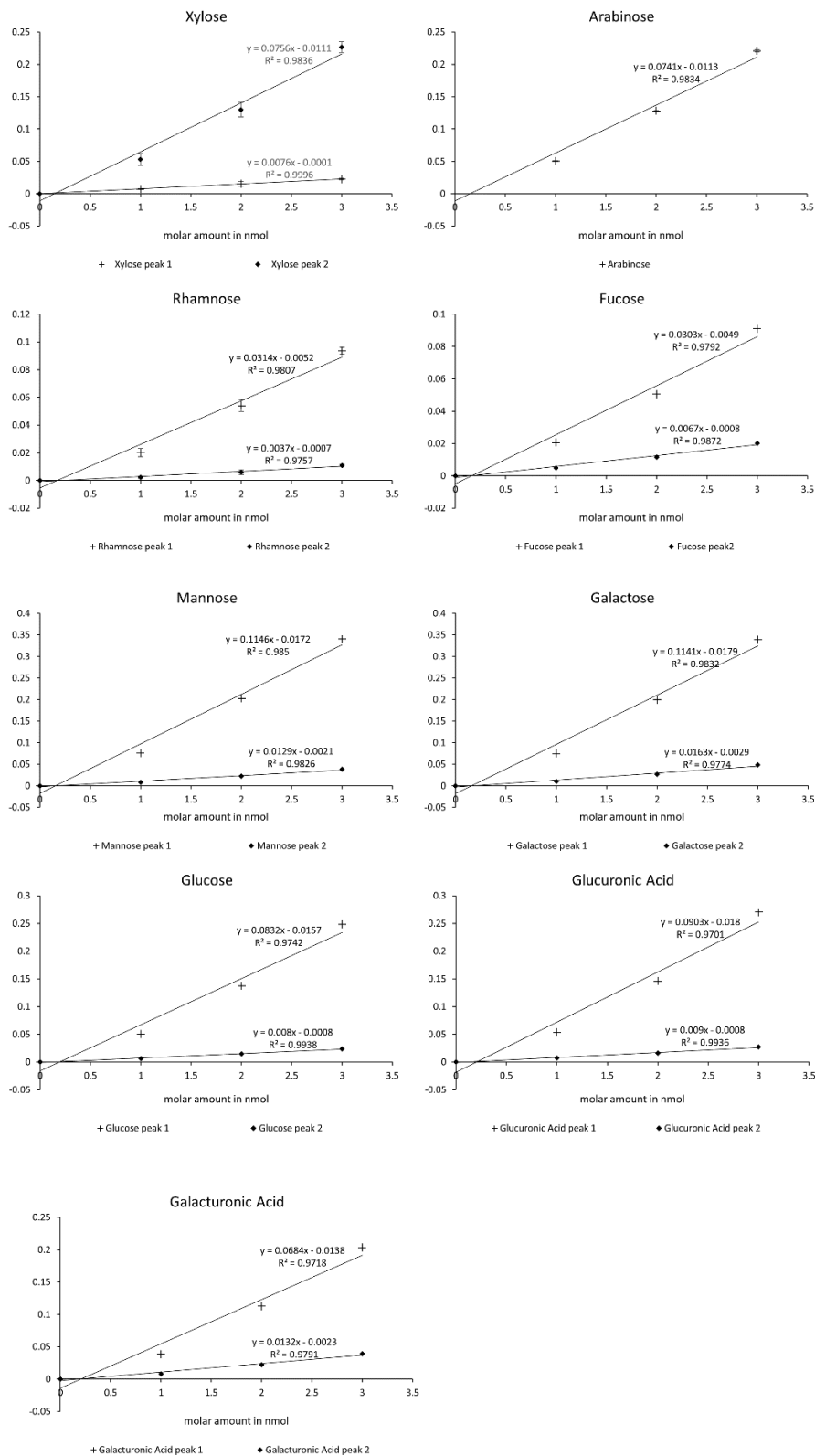
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Supplementary figures

Xylose
y = 0.0756x - 0.0111
R² = 0.9836
y = 0.0076x - 0.0001
R² = 0.9996
+ Xylose peak 1   ◆ Xylose peak 2

Arabinose
y = 0.0741x - 0.0113
R² = 0.9834
+ Arabinose

Rhamnose
y = 0.0314x - 0.0052
R² = 0.9807
y = 0.0037x - 0.0007
R² = 0.9757
+ Rhamnose peak 1   ◆ Rhamnose peak 2

Fucose
y = 0.0303x - 0.0049
R² = 0.9792
y = 0.0067x - 0.0008
R² = 0.9872
+ Fucose peak 1   ◆ Fucose peak2

Mannose
y = 0.1146x - 0.0172
R² = 0.985
y = 0.0129x - 0.0021
R² = 0.9826
+ Mannose peak 1   ◆ Mannose peak 2

Galactose
y = 0.1141x - 0.0179
R² = 0.9832
y = 0.0163x - 0.0029
R² = 0.9774
+ Galactose peak 1   ◆ Galactose peak 2

Glucose
y = 0.0832x - 0.0157
R² = 0.9742
y = 0.008x - 0.0008
R² = 0.9938
+ Glucose peak 1   ◆ Glucose peak 2

Glucuronic Acid
y = 0.0903x - 0.018
R² = 0.9701
y = 0.009x - 0.0008
R² = 0.9936
+ Glucuronic Acid peak 1   ◆ Glucuronic Acid peak 2

Galacturonic Acid
y = 0.0684x - 0.0138
R² = 0.9718
y = 0.0132x - 0.0023
R² = 0.9791
+ Galacturonic Acid peak 1   ◆ Galacturonic Acid peak 2

molar amount in nmol

51

Supplementary Figure 1: Calibration curves for the quantification of the different sugars

Supplementary Table 1: Sugar retention times

Sugar	Retention time [min]
Xylose peak 1	7.610
Xylose peak 2	7.760
Arabinose	7.870
Rhamnose peak 1	8.860
Rhamnose peak 2	8.975
Fucose peak 1	9.020
Fucose peak 2	9.230
Mannose peak 1	12.050
Mannose peak 2	12.305
Galactose peak 1	12.150
Galactose peak 2	12.530
Glucose peak 1	12.265
Glucose peak 2	12.590
Glucuronic Acid peak 1	12.985
Glucuronic Acid peak 2	13.245
Galacturonic Acid peak 1	13.140
Galacturonic Acid peak 2	13.460

3 Manuscript B: Modification of pectin rhamnogalacturonan I contributes to plant immunity against *Botrytis cinerea*.

This manuscript is being prepared for submission.

Individual contribution

Athanas Guzha cloned all the constructs used in this work. He was involved in the confocal microscopy work (Figure 1A and B, Supplementary figure 1A and B). He also did the Arabidopsis transformations for mucilage rescue screens and the monosaccharide composition analysis (Figure 2 and 3). He did the gene expression analysis (Figure 4) and the Botrytis infection assays (Figure 6). Athanas Guzha wrote the manuscript with the help of other authors.

Modification of pectin rhamnogalacturonan I contributes to plant immunity against *Botrytis cinerea*.

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ABSTRACT

Plant cell walls constitute a barrier that plant pathogens have to breach to access contents of the plant cells. The primary cell wall of multicellular plants consists of three major classes of polysaccharides: cellulose, hemicellulose and pectin. Cellulose microfibrils are crosslinked by hemicelluloses and are surrounded by a pectic matrix. Cell wall proteins constitute a relatively minor component of cell walls but they play fundamental roles in maintenance of structural integrity as well as modification of the polysaccharide matrix during development and in response to environmental changes. Rhamnogalacturonan I (RG-I) is the second most abundant pectin found in primary cell walls and enzymes that modify it, such as beta-xylosidases/beta-arabinosidases, are found in all plant cell walls. In Arabidopsis, a cell wall localised protein BETAXYLOSIDASE 4 (BXL4) belonging to a seven-member gene family *BETAXYLOSIDASE* (*BXL*), is induced upon infection with various pathogens and wounding in a jasmonoyl isoleucine (JA-Ile) dependent manner. Ectopic expression of *BXL4* in Arabidopsis seed coat epidermal cells suggests

that like another member of the gene family (*BXL1*), the protein it encodes has both xylosidase and arabinosidase activity and acts on RG-I. *Bxl4* knockout mutants exhibit an increased abundance of arabinans, and have a compromised resistance to the necrotrophic pathogen *Botrytis cinerea*. Upon infection, the *bxl4* knockout mutants show reduced levels of JA-Ile and camalexin accumulation. These data suggest that remodelling of the pectin RG-I could be an important component in plant immunity against *B. cinerea*, and might play a role in JA-Ile and camalexin accumulation.

INTRODUCTION

Plants are continuously exposed to a plethora of potential biotic threats such as herbivorous insects and phytopathogens, as well as constantly changing weather and climatic conditions. To help mitigate against these threats, plants have evolved various constitutive and inducible defences against the different biotic and abiotic perturbations.

Together with the cuticle, the plant cell wall forms an important barrier that pathogens have to breach to access cell contents (Vorwerk et al., 2004; Underwood, 2012; Engelsdorf et al., 2017). The importance of plant cell walls in plant immunity is demonstrated by the abundance of cell wall degrading enzymes (CWDEs) that pathogens secrete in order to successfully invade plant tissues (Glass et al., 2013; Quoc & Bao Chau, 2017). Plant cell walls consist of a complex of polysaccharides where cellulose microfibrils are cross-linked by various hemicelluloses and embedded in a pectic matrix. Cellulose consists of (1-4)-β linked D-glucose residues and is synthesised by cellulose synthases located in the plasma membrane (Somerville, 2006; Carpita, 2011). Hemicelluloses are a widely varied group of polysaccharides, with the most abundant in Arabidopsis being xyloglucan which is characterised by a (1,4)-β-linked glucan regularly substituted with α-(1-6)-xylosyl residues (Liepman et al., 2010; Scheller and Ulvskov, 2010). Cellulose and hemicelluloses are embedded in a pectin matrix.

Pectin is the most complex of all cell wall polysaccharides, and its biosynthesis involves at least 67 glycosyltransferases localised in the Golgi (Harholt et al., 2010) before it is exported to the apoplast. Pectin consists of four types of polysaccharides: Homogalacturonan, xylogalacturonan and rhamnogalacturonan II (RG-II), all

characterised by the presence of a (1-4)-α-D-galacturonic acid backbone, and rhamnogalacturonan I (RG-I), which has a backbone of alternating (1-2)-α-L-rhamnose and (1-4)-α-D-galacturonic acid (O'Neill et al., 1990; Ridley et al., 2001; Mohnen, 2008; Mohnen, et al., 2008). RG-I is also characterised by arabinan, galactan and arabinogalactan side chains, and recently xylan side chains were also proposed to be present (Ralet et al., 2016). In Arabidopsis, pectin is the most abundant class of polysaccharide in the primary cell wall, and is important for the regulation of cell wall mechanical properties during growth and development. It also influences water imbibition of seeds, pollen tube growth, leaf and flower abscission, fruit ripening, and signalling (Mohnen, 2008; Arsovski et al., 2009; Harholt et al., 2010; Kohorn and Kohorn, 2012).

The biosynthesis as well as post secretory modifications of pectin have been shown to be important in plant defence against pathogens. Arabidopsis mutants with defects in the biosynthesis of pectin such as a *gae1 gae6* (*glucoronate 4-epimerases*) exhibit a compromised resistance to *B. cinerea* and *Pseudomonas syringae* infection (Bethke et al., 2016). The lines defective in the transcription factor *At*EF014 with reduced pectin content in the cell walls have an increased susceptibility to *P. syringae* pv *DC3000* and enhanced resistance to *B. cinerea* (Zhang et al., 2016). Interestingly, some pectin enriched Arabidopsis mutants (*pmr5* and *pmr6*) with enhanced resistance to powdery mildews (*Erysiphe cichoracearum*) have an enhanced susceptibility to *P. syringae* (Vogel, 2002; Vogel et al., 2004).

Proteins comprise only a small proportion of plant cell wall components, but they are an integral part as they contribute to structural integrity or modify cell wall composition during development and in response to environmental cues (Sommer-Knudsen et al., 1998; Fry, 2004; Passardi et al., 2004). The modifications of pectin such as acetylation and methylesterification (Liu et al., 2018) are known to play a role in cell wall integrity and resistance to plant pathogens. Arabidopsis *rwa2* (*reduced wall acetylation 2*) mutants have a reduction in the acetylation of pectin, and are more resistant to *B. cinerea* (Manabe et al., 2011). Pectin methylestarase inhibitors (PMEI) are known to inhibit the activity of endogenous Arabidopsis pectin methylesterases (PMEs) which remove methylesters present on HG in either a random manner or blockwise fashion, exposing the galacturonic acid units (Willats et al., 2001). Arabidopsis plants overexpressing PMEI-1 and PMEI-2 showed greater resistance to the phytopathogens

B. cinerea and *Pectobacterium carotovorum* (Lionetti et al., 2007). In Arabidopsis, four members of a berberine bridge enzyme like family were found to be responsible for oxidation of oligogalacturonides (OGs) derived from homogalacturonan hydrolysis. The oxidised OGs triggered weaker immune responses, but were more recalcitrant to hydrolysis by *B. cinerea* enzymes. Arabidopsis plants overexpressing these oxidases were shown to be more resistant to this pathogen (Benedetti et al., 2018). However, given the various modifications that occur in pectin, it remains to be fully elucidated how they impact plant pathogen interactions.

In this study, the potential role of cell wall-localised Arabidopsis protein *BXL4* (BETA-XYLOSIDASE 4) in the modification of RG-I and its impact on plant immunity is shown. Arabidopsis has a seven-member betaxylosidase (BXL) family, whose members possess glycosyl hydrolase domains whilst some have signal peptides for extracellular localisation (Goujon et al., 2003). The functional role of most of the members of this group are still to be determined but *BXL1* was shown to be a bifunctional β-D-xylosidase/α-L-arabinofuranosidase (Goujon et al., 2003; Minic, 2004), whose activity is important for extrusion of mucilage after hydration of Arabidopsis seeds (Arsovski et al., 2009). It has been hypothesised that *BXL1* trims arabinan side chains present in RG-I of both the primary cell wall and mucilage of seed coat epidermal cells. According to transcriptomics databases, *BXL4* is upregulated upon infection by various pathogens and could therefore influence plant-pathogen interaction. Here, we provide evidence that *BXL4* acts on both xylose and arabinose in the cell wall. We can confirm that *BXL4* is induced by *B. cinerea* infection and mechanical wounding in a JA-Ile dependent manner. We show that the *bxl4* mutants have higher arabinose content but grow normally. The *bxl4* mutants are more susceptible when challenged with *B. cinerea* and display reduced levels of JA-Ile and camalexin after infection.

MATERIALS AND METHODS

Plant and Botrytis cinerea growth conditions

Arabidopsis plants used for infection assays were grown on semi-sterile soil under short day conditions (8 h light and 16 h darkness) at a temperature of 22°C and a relative humidity of 65% in a Percival (Percival Scientific, Perry, USA). Arabidopsis plants for seed propagation were grown under long day conditions (16 h light and 8 h

darkness), light intensity of 120-150 µmol m^{-2} s^{-1}, at 22°C and 60% relative humidity in a climate chamber (York Industriekälte, Mannheim, Germany). The Arabidopsis mutants *bxl1* (Ws ecotype; CS16299, Feldmann, 1991) and *mpk3*-DG (Li et al., 2002) and *B. cinerea* strain B05-10 (Staats and van Kan, 2012) were used for this work. The *Botrytis* spores were cultured on Potato dextrose broth (Sigma-Aldrich) plus agar, grown at RT for 10 days before harvesting by washing the spores off the plates using ¼ PDB and sieving through Miracloth to collect the conidoispores. Conidiospores were counted using a hemacytometer and stocks in 25% glycerol were made and stored at -80°C. T-DNA mutant lines of *bxl4-1* (SALK_071629) and *bxl4-2* (SAIL_331_B06) were sourced from Nottingham Arabidopsis Stock Centre and homozygous mutants where confirmed through genotyping PCR on genomic DNA using REDTaq® ReadyMix™ (Sigma-Aldrich) following their protocol. Primers used are listed in table S1.

Gene expression analysis (qRT-PCR)

RNA was extracted from leaves using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). cDNA from DNaseI (Thermo Scientific, Waltham, MA, USA) treated RNA was made using Revert Aid™ H minus Reverse Transcriptase (Thermo Scientific). cDNA derived from leaf RNA was used for qRT-PCR using Takyon™ No Rox SYBR® MasterMix dTTP Blue (Eurogentec, Lüttich, Belgium). Quantification of expression was done relative to ACTIN8. The primers used are found in table S1.

Molecular cloning and Arabidopsis transformation

The R4 Gateway Binary Vectors (R4pGWB; Nakagawa et al., 2008) were employed to make the constructs used in this work. The *TBA2* constructs with and without a citrine tag were made by first amplifying the *TBA2* promoters using PCR and cloning into entry vector pDONRP4-P1R. The cDNA constructs were made by amplifying the cDNA with PCR (primers used in Table S1) and cloning into entry vector pDONR207. A tripartite LR reaction was performed to incorporate the *TBA2* promoter and cDNA into R4pGWB501 (modified vector) with and without citrine tag (Nakagawa et al., 2008). Arabidopsis plants were transformed by floral dipping as described (Clough and Bent, 1998) and the T1 seeds grown on MS medium supplemented with hygromycin for selection.

Confocal microscopy

The transformed Arabidopsis T2 seeds were visualised under a confocal microscope for localisation of BXL proteins. Confocal images were recorded using confocal microscope Zeiss LSM 780 (Carl Zeiss Inc., Jena, Germany). Citrine was excited at 488 nm through a 488 nm major beam splitter (MBS). Detection of fluorophore was done at a wavelength of 514 – 530 nm.

Botrytis cinerea infection assay

B. cinerea spores were diluted to 5x 10^5 spores per millilitre in ¼ Potato dextrose broth (Sigma-Aldrich) for drop infection assay or Vogels (Vogel, 1956) media for spray infection assay used for qRT-PCR analysis. The spores were pre-germinated for 4 h before infections were carried out. For drop infections, 6 µL of spore suspension in ¼ PDB was carefully placed on the adaxial side (away from the midrib) of a fully expanded rosette leaf of 6-7 weeks old Arabidopsis (at least 30 leaves were used from 10 independent plants). For spray infection, plants were sprayed until spray droplets began to run off the leaves. Plants were then covered and grown under high humidity conditions for 72 h. Lesion diameters were measured using a veneer calliper. For spray infections, whole rosettes were harvested at day zero (d0 immediately after spraying) and at 72 h post infection (d3). For fungal genome quantification, fungal DNA was extracted using Plant/Fungi DNA isolation Kit (Norgen Biotek Corp) following manufacturer's protocol. The fungal β-ACTIN genomic DNA was quantified by qPCR (Ettenauer et al., 2014) and primers used are listed in Table S1.

Mucilage staining with ruthenium red

5 mg of Arabidopsis seeds were placed in 500 µL $_{dd}H_2O$ in an Eppendorf tube before being gently shaken for 1 h on a rotary shaker. Water was gently removed and 500 µL 0.02% ruthenium red was added. Seeds were put back on the shaker for another 15 min before the ruthenium red was removed and seeds resuspended in 500 µL $_{dd}H_2O$ added again. A droplet with stained seeds was placed on a microscopic slide and viewed under a light microscope.

Monsaccharide analysis-mucilage

Mucilage was extracted by agitating 5 mg of seeds in water for two h on a rotary shaker. Seeds were then allowed to settle for a few minutes before 1 mL mucilage solution was removed and placed in a Duran culture tube. Mucilage solution was evaporated in a water bath at 40°C under nitrogen stream. Dry samples were hydrolysed for 1 h at 121°C using 2 M trifluoroacetic acid, before being evaporated again. 100 µL *allo*-inositol and 500 µL ddH$_2$O were added to resuspend the hydrolysed mucilage. 20 µL sample was evaporated under a nitrogen stream before overnight derivatisation in 15 µL methoxyamide (MOX, 30mg/mL in anhydrous pyridine). The next day, 30 µL MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide) were added and samples were analysed GC-MS 1-6 h after MSTFA addition.

GC-MS analysis

Samples were analysed with a 7890B GC-System coupled to a 5977B MSD quadrupole set-up from Agilent Technologies. GC-separation was achieved on a HP-5 column (Agilent Technologies) using the following temperature gradient: 150°C for 2 min, 5 K/min gradient for 20 min, 15 K/min to a final temperature of 320°C, which was held for 3 min. For each run, 1 µL of the derivatised sample was injected. Identification of compounds was done by a combination of retention times compared to external standards and MS-spectra. Prepared mucilage samples were quantified relative to the internal standard *allo*-inositol. In parallel runs, monosaccharide standards of different concentration were used to determine response factors for area-to-molar amount conversion allowing absolute quantification.

Monsaccharide analysis of the alcohol insoluble residue of leaves

The alcohol insoluble residue (AIR) was extracted from plant leaves (6-7 weeks old plants) grown in the dark for 48 h. The AIR was extracted as described (Gille et al., 2009). The leaves were flash frozen in liquid nitrogen before they were pulverised using mortar and pestle. The ground material was washed 2 times with 70% (v/v) ethanol, washed thrice with a chloroform:ethanol mixture (1:1 [v/v]), and lastly with acetone, before being air dried. Hot water pectin extraction (Yeoh et al., 2008) was used by shaking 2 mg of AIR in 1.4 mL ddH$_2$O at 90°C for 2 h. Monosaccharide analysis was then carried out on AIR using the same GC-MS method used on mucilage as described above.

Phytohormone measurements

Extraction of phytohormones was carried out as described by Kusch et al., 2019. Reversed phase separation of constituents was performed as previously described (Kusch et al. 2019) using an ACQUITY UPLC® system (Waters Corp., Milford, MA, USA) equipped with an ACQUITY UPLC® HSS T3 column (100 mm x 1 mm, 1.8 µm; Waters Corp., Milford, MA, USA). Nanoelectrospray (nanoESI) analysis was carried out as described in Kusch *et al.*, (2019) and phytohormones were ionized in a negative mode and determined in a scheduled multiple reaction monitoring mode with an AB Sciex 4000 QTRAP® tandem mass spectrometer (AB Sciex, Framingham, MA, USA). Mass transitions were used as described by Iven et al., (2012), with some modifications specified in Table S2.

RESULTS

BXL4 localises to the apoplast

The BXL4 protein possesses a predicted signal peptide for secretion to the apoplast (Goujon et al., 2003). To test if the protein has cell wall localisation, a construct of *BXL4* with a C-terminal Citrine fusion (*BXL4*-Citrine) driven by the seed coat specific *TBA2* (*TESTA ABUNDANT2*) promoter (Tsai et al., 2017) was generated and transformed into Arabidopsis *bxl1* mutant and Col-0 to generate stable expression lines. Arabidopsis seed coat epidermal cells of T2 seeds expressing p*TBA2:BXL4-CITRINE* were visualised under a confocal microscope 7 d post anthesis. The BXL4-CITRINE fluorescence could be strongly detected in the mucilage pocket of seed coat epidermal cells (Figure 1A and 1B) similar to the control line p*TBA2:BXL1-CITRINE* (Supplementary Figure1).

BXL4 rescues the mucilage phenotype of bxl1

While in the Arabidopsis wild type (ecotype *Wassilevskia*, Ws), mucilage is extruded from the seed coat, the *bxl1* knockout mutant has a defect in mucilage extrusion after hydration of the seeds (Figure 2A) (Arsovski et al., 2009). To test if the putative betaxylosidase BXL4 could perform a similar enzymatic activity and therefore rescue the mucilage defect phenotype, the *bxl1* knockout mutant was complemented with the p*TBA2*: *BXL4* construct with and without a Citrine tag. The T2 seeds were placed in

water and the mucilage was stained with ruthenium red. The results indicated that the *bxl1* mutant line could be complemented with p*TBA2*:*AtBXL4* (Figure 2C) with and without the Citrine tag, in a similar way as with p*TBA2*: *BXL1* (Figure 2D), as these lines could extrude their mucilage in the same manner as wild type Ws.
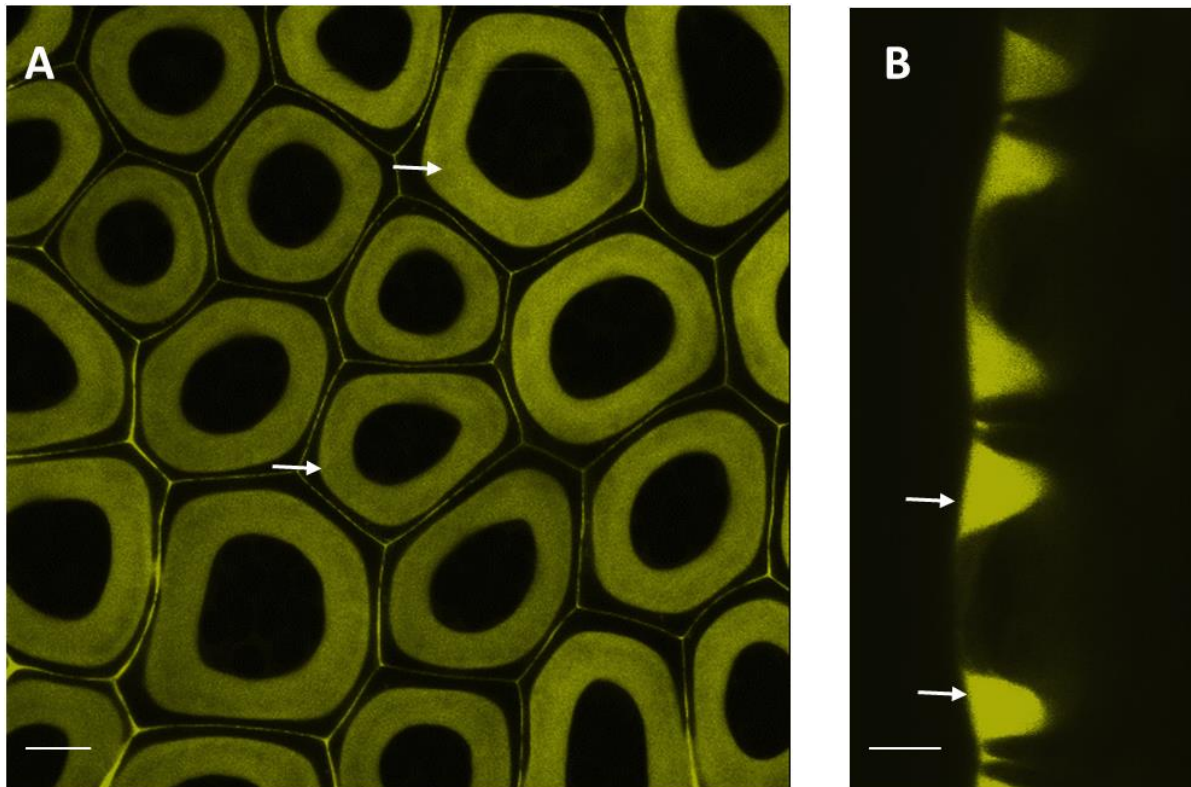

Figure 1: BXL4-CITRINE localises to the apoplast in Arabidopsis seed coat epidermal cells

(A) BXL4-CITRINE stably expressed under the TBA2 promoter localises to the apoplast (deposited predominantly in the mucilage pocket) of Arabidopsis seed coat epidermal cells 7 days post anthesis (dpa). Single plane images of the seed coats were obtained by confocal microscopy. The apopolast of the of seed coat epidermal cells appears like a doughnut ring that surrounds the cytoplasm when imaged from the top. **(B)** Visualisation of the seed coat epidermal cells from the side at 7 dpa shows the apoplast as two pockets in the apical corners of the cells (arrow). Scale bars, 10 µm

The Arabidopsis *bxl1* knockout mutant is known to produce mucilage pectin with higher levels of arabinans (Arsovski et al., 2009). To investigate if the p*TBA2*:*BXL4* complemented *bxl1* lines had mucilage with a monosaccharide composition similar to wild type Ws, analysis was done on the water extracted mucilage from the T2 seeds. Extracted mucilage was hydrolysed with TFA before derivatisation with MOX and

MSTFA to enable monosaccharide composition analysis by GC-MS. Mucilage is composed mainly of pectin RG-I (Dean et al., 2007; Arsovski et al., 2009; Arsovski et al, 2010; Haughn & Western, 2012), and consistent to that, monosaccharide analysis showed that rhamnose and galacturonic acid were the most abundant sugars. The *bxl1* mutant exhibited a 4-fold increase in abundance of arabinans in comparison to the wild type Ws, while the *bxl1* lines complemented with p*TBA2*:*BXL1* and p*TBA2*:*BXL4* showed arabinose levels reverted to wild type levels (Figure 2E). The *bxl1* mutant line complemented with p*TBA2*:*BXL4* also showed a greater reduction in xylose compared to the wild type (Figure 2E). Arabidopsis lines overexpressing *BXL4* were made by transforming wild type Col-0 with p*TBA2*:*BXL4*. The mucilage from the T2 seeds was extracted and analysed for monosaccharide composition analysis using GC-MS. The overexpression lines showed arabinose levels similar to wild type, whilst the xylose composition was further depleted (Figure 2F).

Disruption of BXL4 does not affect plant growth

Alteration of the cell wall composition may result in the disruption of normal growth and development of Arabidopsis plants (Noguchi et al., 1997). To check if knocking out *BXL4* affects the normal growth of Arabidopsis, two Arabidopsis T-DNA insertion lines *bxl4-1* and *bxl4-2* that carry insertions in exon 4 and 5 (Figure 3A) were obtained from the stock centre. To confirm the position of the insert, the genomic DNA of the mutants was sequenced. To check if the two lines were knockouts, the expression of *BXL4* in the T-DNA insertion lines by quantitative reverse transcription polymerase chain reaction (qRT-PCR) was done on RNA extracted from 4 weeks old plants grown on soil. The *bxl4-1* line appears to be a knockout line whilst *bxl4-2* showed a small amount of transcript (Figure 3B). Gene expression was normalised to reference gene ACTIN8 and relative to Col-0. To check if knocking out of *BXL4* had effects on the monosaccharide composition of the pectin, water extracted pectin from alcohol insoluble residues (Gille et al., 2009) of leaf material from 6 weeks old Arabidopsis plants was analysed for monosaccharide composition using GC-MS. The monosaccharides were normalised to rhamnose. The *bxl4* mutants showed an increased abundance of arabinose compared to Col-0. There was no significant difference in the xylose content (Figure 3C) or any other pectin monosaccharide

measured. Arabidopsis *bxl4* mutants did not exhibit any obvious growth defects and were comparable to Col-0 (Figure 3D).
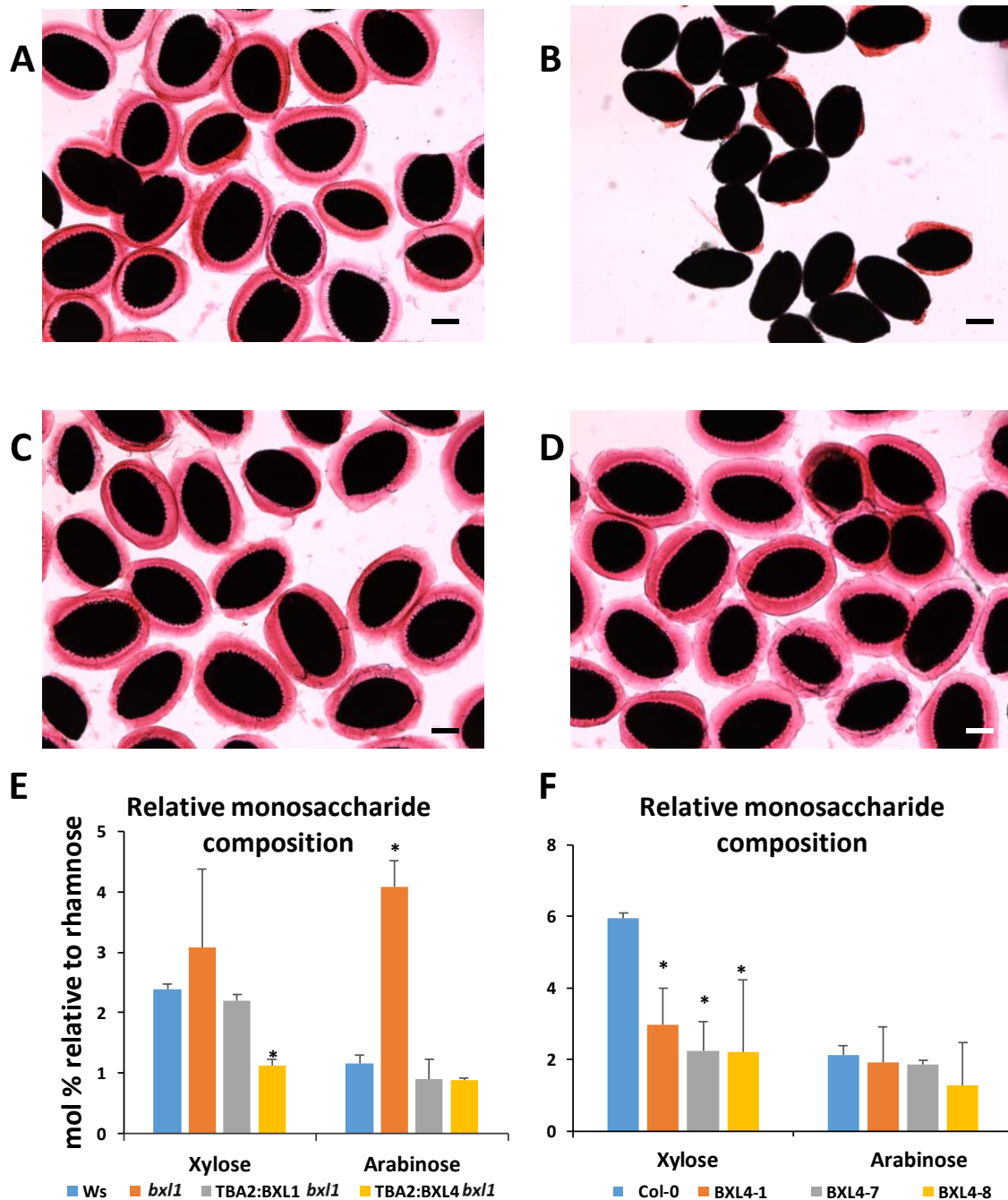

Figure 2: BXL4 can complement the mucilage phenotype of bxl1.

(A) In the wild type (ecotype Ws), the extrusion of the ruthenium red-stained mucilage forms a halo around the seeds. (B) *bxl1* seeds extrude their mucilage in a patchy manner. (C-D)This phenotype can be complemented by introducing p*TBA2:BXL1* (C) or p*TBA2:BXL4* (D) into the *bxl1* background. Bars, 100 µm. (E) Relative monosaccharide composition of mucilage extracted from Ws, *bxl1*, *bxl1* p*TBA2:BXL1* and *bxl1* p p*TBA2:BXL4*. (F) Monosaccharide composition of mucilage extracted from wild type Col-0 and three BXL4 overexpression lines (p*TBA2:BXL4* line 1, 7 and 8). Monosaccharide composition was determined by GC-MS and

normalised to rhamnose. Error bars show SD (n=3 biological replicates), Statistical difference to WT (Student's t-test),* indicates p<0.05.
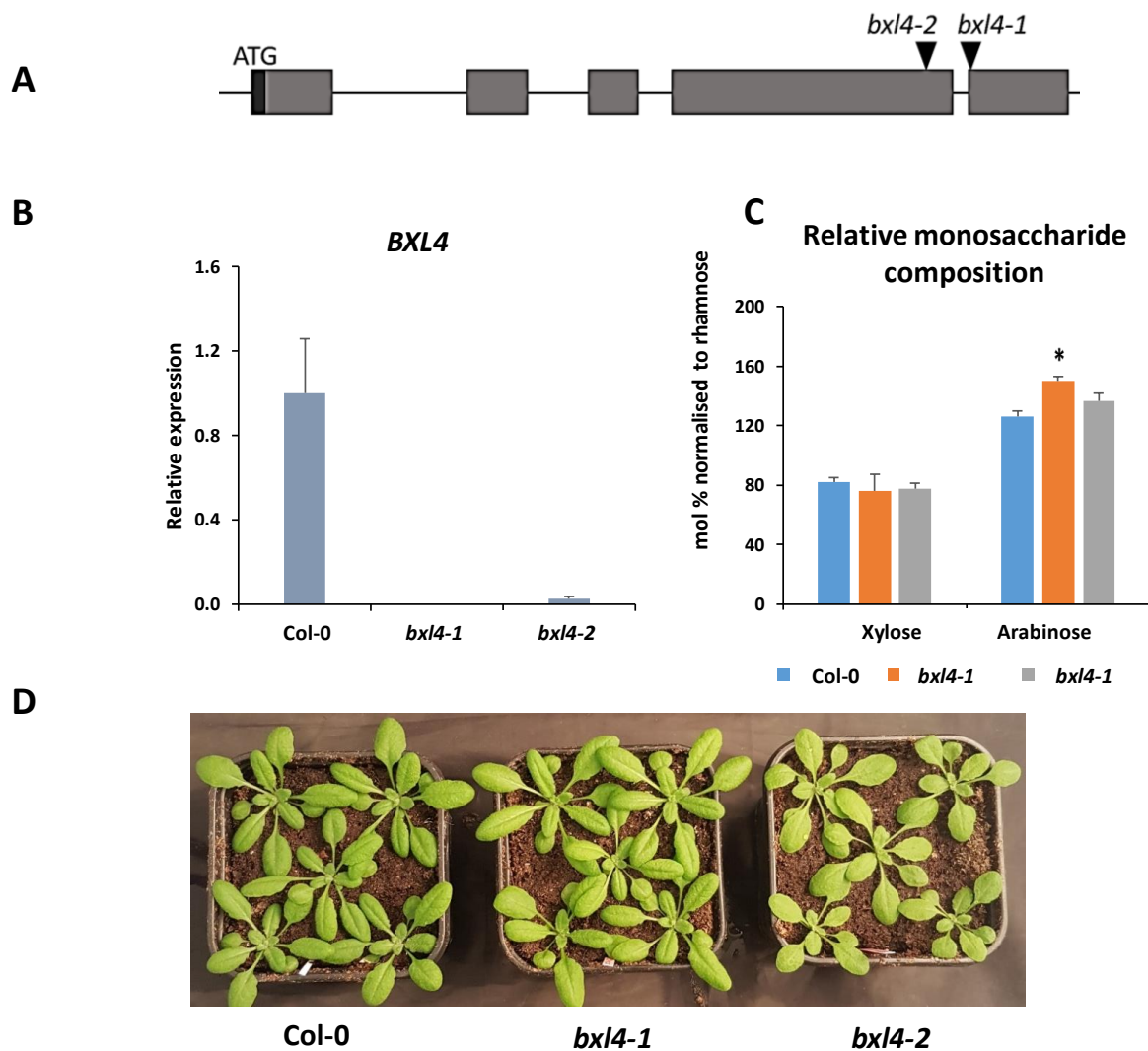

Figure 3: The disruption of BXL4 has mild effects on the cell wall composition but does not alter plant growth.

(A) The intron-exon structure of BXL4 and the T-DNA insertion positions of *bxl4-1* and *bxl4-1*. The exons are represented by grey boxes, introns black lines, black triangles show positions of the T-DNA insertions and the UTR black box. (B) Relative expression of BXL4 from RNA extracted from leaves of WT and *bxl4* mutant lines determined by qRT-PCR using primers shown in Supplementary table 1. Expression values were normalised to the reference gene ACTIN 8 and are presented relative to the wild type levels. Error bars represent standard error of three biological replicates. (C) Monosaccharide composition of water extracted pectin from Arabidopsis leaves of wild type, *bxl4-1* and *bxl4-2* mutant lines. The monosaccharides were normalised to rhamnose. Water extracted pectin was analysed by GC-MS. Error bars represent SD (n=4 biological replicates), Statistical difference to WT (Student's t-test), *P<0.05.

BXL4 expression is wound and Botrytis infection induced and jasmonoyl-isoleucine-dependent

According to publicly available databases, *BXL4* is upregulated by infection with various pathogens. To confirm that *BXL4* is a stress-induced gene, its expression pattern after mechanical wounding and infection with *B. cinerea* was analysed. Generally, *BXL4* gene expression is relatively low in Col-0 grown under normal conditions. However, the gene was induced 15-fold upon wounding of the rosettes (Fig. 4A). Induction of *BXL4* was also investigated in the jasmonoyl-isoleucine (JA-Ile) deficient mutant line *dde2-2* (von Malek et al., 2002), because JA-Ile regulates the expression of many wounding responsive genes (Turner et al., 2002). Relative to wild type expression, upregulation of BXL4 transcript after wounding was greatly reduced in the *dde2-2* mutant (Figure 4A). To confirm that *BXL4* is a *B. cinerea* infection associated gene, accumulation of *BXL4* transcript after infection with *B. cinerea* was checked and showed significant induction in Arabidopsis after infection. Mechanical wounding and attack from necrotrophic pathogens in Arabidopsis is known to trigger defence responses regulated via JA-Ile signalling (Turner et al., 2002). To check if the *bxl4* mutants had impaired JA-Ile signalling, the transcript abundance of JA-Ile reporter genes *JAZ10* (Yan et al., 2007; Chung et al., 2008) and *PDF1.2* (Penninckx, 1998; Zarei et al., 2011) after wounding was tested in wounded Col-0 and *bxl4* mutants. The *bxl4* mutants had a decreased transcript accumulation of *JAZ10* (Figure 4B) and *PDF1.2* (Figure 4C) at 2 h post wounding compared to Col-0. Furthermore, the transcript accumulation of the JA-Ile associated marker genes *JAZ10* and *PDF1.2* after necrotrophic pathogen infection was also assessed. Transcript accumulation of *JAZ10* did not show a significant difference between the wild type and the *bxl4* mutants (Figure 4E) whilst *PDF1.2* was higher in Col-0 compared to *bxl4* mutants and expression increased with increased disease development (Figure 4F). Finally, the relative expression of *PAD3*, a gene involved in camalexin biosynthesis that is induced after attack from necrotrophic pathogens (Zhou et al., 1999), was measured in Col-0 and *bxl4* mutant lines after infection with *B. cinerea*. *PAD3* and camalexin are known to play a defensive role upon infection of Arabidopsis by both biotrophic and necrotrophic pathogens (Ferrari, 2003; Schuhegger et al., 2006). The transcript accumulation of *PAD3* in RNA extracted from *bxl4* knockout mutant lines after infection

was reduced compared to Col-0. Expression of *PAD3* was normalised to *ACTIN8* and is shown relative to Col-0 at 0 h (Figure 4G).
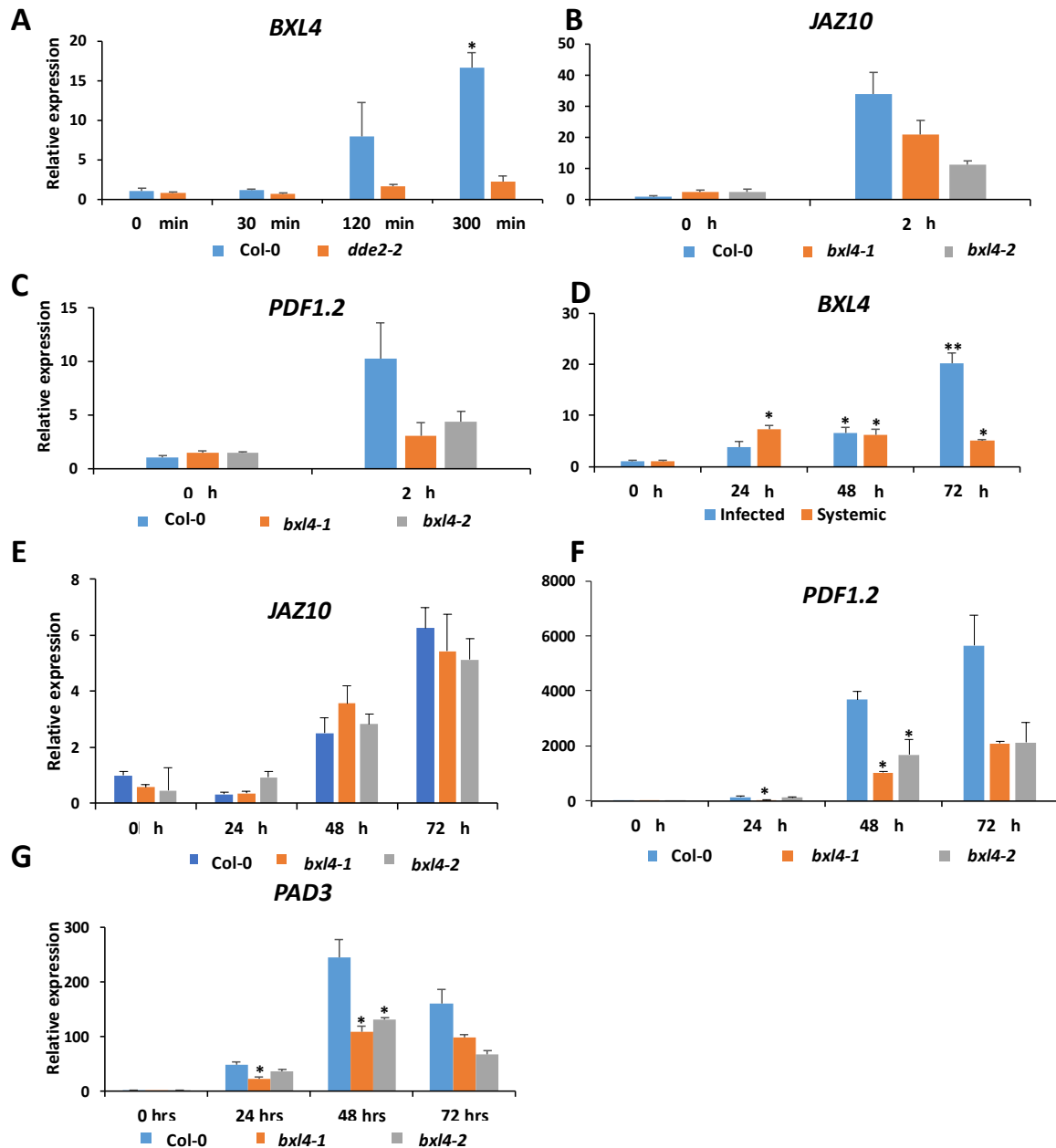

Figure 4: Expression of BXL4 is induced by wounding and *B.* infection and acts upstream of jasmonoyl-isoleucine signalling.

(A) Expression pattern of *BXL4* in Col-0 and jasmonoyl-isoleucine (JA-Ile)-deficient mutant *dde2-2*. RNA for qRT-PCR was extracted from 6 weeks old plants harvested before wounding (0 mins) and at 30, 120 and 300 min after wounding. (B-C) Relative expression of JA-Ile marker genes *JAZ10* (B) and *PDF1.2* (C) in Col-0, *bxl4-1*, and *bxl4-2* at 0 and 2 hours post wounding. (D) Relative expression of *BXL4* in 6 week old Col-0 plants drop inoculated with *B. cinerea* conidiospores. Samples were collected from infected and systemic leaves at 0 (before

infection), 24, 48 and 72 h after infection. (E-F) Relative expression of JA-Ile marker gene *JAZ10* (E) and *PDF1.2* (F) in Col-0, *bxl4-1* and *bxl4-2* at 0, 24, 48 and 72 h after infection with B. cinerea. Relative expression was measured by qRT-PCR, normalised to reference gene *ACTIN8*, and relative to wild type 0. Error bars represent standard error of 3 biological replicates. Statistical difference to WT (Student's t-test), *P<0.05, **P<0.01. (G) Relative expression of camalexin marker gene *PAD3* in *B. cinerea* infected Col-0 and *bxl4* mutant lines. RNA extracted from samples collected at 0, 24, 48, 72 h after infection and normalised to *ACTIN8*, relative to Col-0 at 0 hrs. Error bars show standard error of 3 biological replicates.

bxl4 mutant plants have a reduced production of JA and camalexin upon infection with

B. cinerea

To confirm if the observed reduction in expression of *PDF1.2* and *PAD3* in *bxl4* mutants correlated with a reduction in accumulation of JA and camalexin respectively, the levels of the JA and camalexin which are triggered by infection with *B. cinerea* (Nie et al., 2017; Ferrari et al., 2003, 2007; Scalschi et al., 2015) were measured. The *bxl4* mutants showed a reduction in JA production at 72 h post infection compared to Col-0 (Figure 5A). There was also a reduction in the accumulation of the JA active derivative JA-Ile (Thines et al., 2007; Yan et al., 2009) in the *bxl4* mutants at 72 h post infection (Figure 5B). The abundance of camalexin after *B. cinerea* infection was reduced at 72 h post infection in the *bxl4* mutants compared to Col-0 (Figure 5C). The wounded *bxl4* mutants also had a slightly reduced accumulation of JA and JA-Ile compared to Col-0 (Supplementary Figure 3).

BXL4 contributes to resistance against B. cinerea

After confirming that *BXL4* is a stress induced gene, we went on to check if the *bxl4* mutants had a compromised resistance to *B. cinerea*, and the plants were drop inoculated with 5×10^5 conidiospores/mL and lesion diameter measured 72 h post inoculation. The *bxl4* mutants developed significantly larger lesions compared to Col-0. *mpk3* was used as a susceptible control (Galletti et al., 2011) (Figure 6A & 6C). An additional infection method was used to confirm susceptibility to *B. cinerea*, which involved spraying the plants and quantifying the fungal β ACTIN genomic DNA (Ettenauer et al., 2014) using qPCR. Fungal DNA was quantified immediately after spraying and after 3 days. The *bxl4* mutants showed a significantly higher accumulation of fungal genomic DNA as compared to Col-0 (Figure 6B).
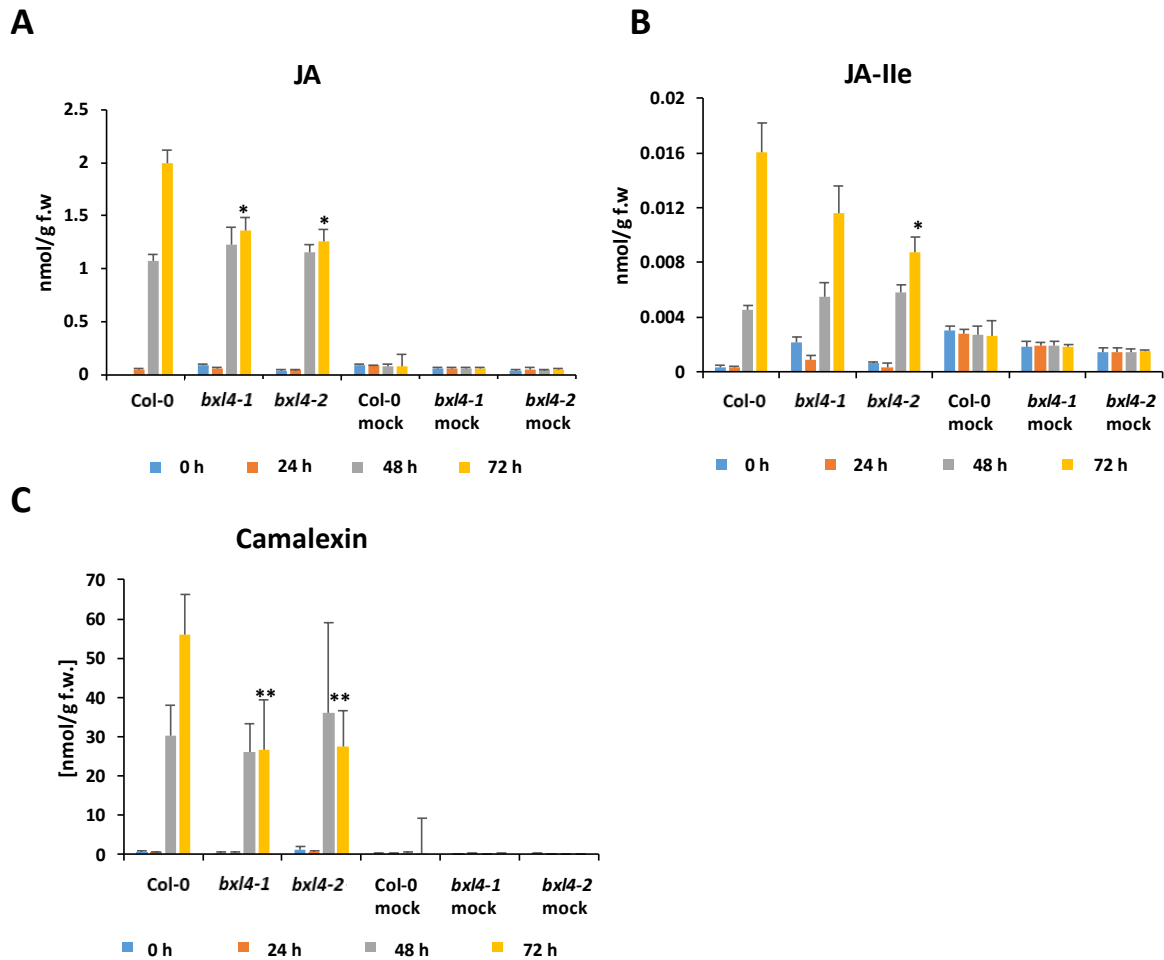
A

JA

B

JA-Ile

C

Camalexin

Figure 5: Levels of *B. cinerea* infection-induced JA, JA-Ile and camalexin are reduced in *bxl4* mutant lines

Col-0 and *bxl4* mutant lines were spray-infected with *B. cinerea* and leaves were sampled at 0, 24, 48 and 72 h after infection. Extracted levels of JA **(A)**, JA-Ile **(B)** and camalexin **(C)** were analysed using nanoESI. Error bars represent standard deviation of 6 biological replicates, statistical difference to WT (Student's t-test), *$P<0.05$, **$P<0.01$.

DISCUSSION

The importance of pectin biosynthesis and modification in plant defences has been elucidated in many previous publications. Here, we provide evidence that BXL4 modifies cell wall polysaccharides contributing to immunity against *B. cinerea.*

BXL4 acts on arabinans present in the apoplast

Our *BXL4*-Citrine fusion protein driven by a seed coat specific promoter *TBA2*, localised in the mucilage pocket of Arabidopsis seed coat epidermal cells at seven
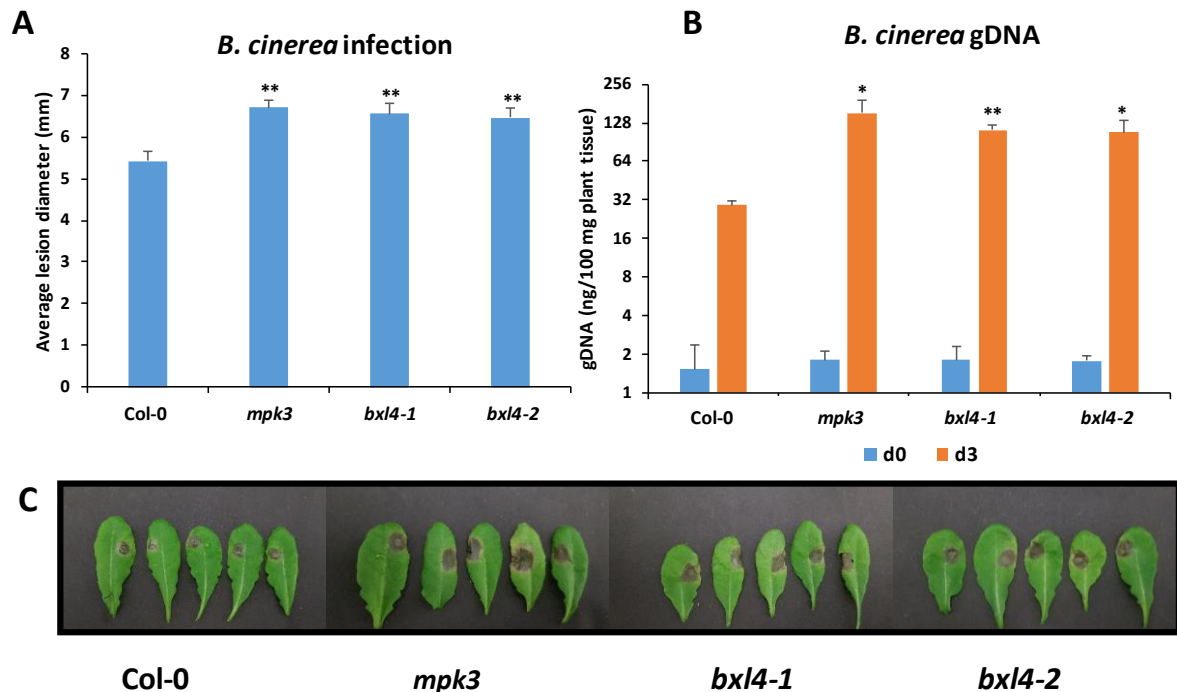
Figure 6: *bxl4* mutant lines are more susceptible to *B. cinerea* infection

(A) Infection phenotype of Col-0, *bxl4-1* and *bxl4-2* after *B. cinerea* infection. A minimum of 30 leaves from 5 independent plants were drop inoculated with 6 µL *B. cinerea* conidiopores (5×10^5 spores/mL), and lesion diameter measured 3 days after infection. *mpk3* was used as the susceptible control. Error bars show SE, Statistical difference to WT (Student's t-test), **$P<0.01$. **(B)** Infection phenotype measured after spraying plants with *B. cinerea* conidiospores and quantifying fungal genomic DNA with qPCR. Fungal genomic DNA was quantified immediately after infection (d0) and 3 days after infection (d3). Error bars show SE (n=3 biological replicates), Statistical difference to WT (Student's t-test), *$P<0.05$, **$P<0.01$. **(C)** Lesion phenotype of detached leaves 3 days post infection with *B. cinerea.*

days post anthesis. The apoplastic localisation was consistent with work by Goujon *et al.*, (2003) and data obtained using SignalP-5.0, which showed that BXL4 has a predicted signal peptide for localisation in the extracellular matrix. It is known that mucilage and RG-I modifying proteins, including BXL1 are deposited in the mucilage pocket between four and eight days post anthesis (Western et al., 2000; Arsovski et al., 2009; Haughn and Western, 2012; Breitenbach et al., 2014). Our observation of *BXL4* localisation in the apoplast means it is in the right compartment to perform cell wall remodelling.

The Arabidopsis *bxl1* knockout mutant shows a distinct phenotype as the mucilage from hydrated seeds extrudes in a delayed and uneven manner (Arsovski et al., 2009).

To check if *BXL4* was functionally similar to its homologue *BXL1*, we expressed p*TBA2:BXL4* in the mucilage of the *bxl1* mutant. Examination of the T2 seeds of p*TBA2:BXL4 bxl1* lines revealed that they extruded mucilage normally. *BXL4* has a predicted glycosyl hydrolase domain (Goujon et al., 2003), and thus should be capable of carrying out similar enzymatic functions as *BXL1*. The TBA2 protein is one of the most abundant and highly expressed seed coat proteins (Tsai et al., 2017). Therefore, the TBA2 promoter was used for ectopic expression in the seed coat upon which rescue of the mucilage phenotype could be observed for *BXL4*.

BXL1 is a known bifunctional β-D-xylosidase/α-L-arabinofuranosidase and the *bxl1* knockout mutant has an abundance of (1-5)-linked arabinans which can form one of the side chain present in mucilage RG-I (Arsovski et al., 2009). Expression of p*TBA2:BXL4* in the *bxl1* mutant background resulted in the production of mucilage with a reduced arabinose content similar to wild type. The arabinosidase activity of BXL4 was also suggested by the increased abundance of arabinans in the leaf cell walls of *bxl4* mutants. The generation of *BXL4* overexpression lines under the *TBA2* promoter in wild type Col-0 showed a further decrease in the xylose content compared to Col-0, whilst the arabinan levels were comparable to Col-0. The xylosidase activity was also suggested in the p*TBA2:BXL4 bxl1*, which exhibited a further reduction in xylose levels compared to the wild type or control line (*pTBA2:BXL1 bxl1*). The greater reduction in xylose in p*TBA2:BXL4* complemented *bxl1* compared to p*TBA2:BXL1* complemented line suggests the stronger xylosidase activity of BXL4 when expressed in mucilage. Recent findings by Ralet et al., (2016) suggest the presence of xylan side chains on RG-I that mediate the interaction with cellulose through non-covalent linkages. It is therefore possible that BXL4 acts on these xylan side chains. Mucilage is also thought to contain small quantities of the hemicelluloses xyloglucan and xylans with a β-1,4-linked D-xylose backbone (Ebringerová and Heinze, 2000; Voiniciuc et al., 2015; Hu et al., 2016). BXL4 could also act on these xylans whilst xyloglucan would not make a suitable substrate because of the α-D-Xyl-(1→6)-β-D-Glc (Zabotina, 2012) linkage which betaxylosidases cannot cleave. It is a well-known phenomenon that enzymes acting on polysaccharides show dual functions (Watt et al., 1998), and BXL4 exhibits this dual function acting on chains of the two diastereomers L-arabinose and D-xylose.

BXL4 acts upstream and downstream of JA signalling and contributes to camalexin production.

According to publicly available databases, *BXL4* is induced by infection with various plant pathogens. Our research findings concur with this, as *BXL4* was upregulated upon infection with *B. cinerea.* It is known that *B. cinerea* infection in Arabidopsis results in transcriptional reprogramming of many genes (Sham et al., 2014). We further checked if *BXL4* was a tissue damage associated protein by mechanically wounding leaves of Arabidopsis. Mechanical wounding resulted in upregulation of *BXL4*, in a JA-Ile-dependent manner as the *dde2-2* mutant, which is deficient in JA-Ile (von Malek et al., 2002), did not reveal any significant *BXL4* upregulation upon wounding. Wounding is known to activate JA-Ile production which in turn triggers expression of downstream genes involved in mitigatory responses (Turner et al., 2002). Pectin methylesterases are induced upon wounding in a JA-Ile-dependant manner resulting in the formation of oligogalacturonides in dimer form (crosslinked with Ca^{2+}) which elicit strong biological responses (Kohorn and Kohorn, 2012; Reymond et al., 2000; Bethke et al., 2014). It is plausible that *BXL4* is triggered by JA-Ile after wounding to possibly render cell walls more recalcitrant to further damage as evidenced by the reduced induction of *BXL4* in the JA-Ile-deficient mutant.

Our data shows that *bxl4* mutant Arabidopsis lines challenged with *B. cinerea* had a reduced expression of the JA-Ile marker gene *PDF1.2* together with decreased tissue abundance of JA and its biologically active derivative JA-Ile. Our data suggest that *bxl4* knockout mutants are compromised in JA-Ile signalling upon infection. Oligogalacturonides regulate the expression of many genes involved in the JA-Ile biosynthesis pathway such as *LOX3* and *LOX4* (Denoux et al., 2008). The nature and quantity of the oligogalacturonides derived from *bxl4* mutants could impair their ability to activate the expression of the JA-Ile biosynthetic genes. Predicted functional partners of BXL4 using STRING (version 11.0) (Szklarczyk et al., 2015) show its interaction with various pectate lyases and a polygalacturonase, which could be involved in the generation of elicitor active oligosaccharides. It is known that the modifications on oligosaccharides such as methylation and acetylation can influence the biological activity of oligosaccharides (Randoux et al., 2010) and that abundant arabinans can hinder polygalacturonase activity (Yoshioka et al., 1995). The *bxl4* mutants could be producing fewer elicitor active oligogalacturonides. It is also known

that many mutations involving cell wall related genes result in the alteration of phytohormone production or reprogramming of their signalling pathways (Ellis, 2002; Vega-Sanchez et al., 2012; Wang et al., 2017) which could also be the case with *bxl4* mutants. Our *B. cinerea* infection data together with the wounding data suggests that *BXL4* is JA-Ile dependent, and probably induces a positive feedback loop resulting in increased production of JA and JA-Ile. It is known that JA-Ile triggers the expression of its biosynthetic genes causing a positive feedback loop that promotes further JA-Ile accumulation and subsequent activation of defence responses (Browse, 2009).

We also observe a reduced production of camalexin together with decreased expression of *PAD3* in the *bxl4* mutants after *B. cinerea* infection. This indicates that the oligosaccharides produced by the fungus when it breaches the cell wall or the plant itself might have a lower biological activity. Oligogalacturonides trigger downstream signalling pathways upon perception by wall associated kinases resulting in *PAD3* induction and phytoalexin accumulation (Davis et al., 1986; Ferrari et al., 2007). It has also been revealed that camalexin biosynthesis is under the control of JA-Ile signalling. Rowe et al., (2010) demonstrated that Arabidopsis mutants impaired in JA biosynthesis or perception show a reduced accumulation of camalexin after *B. cinerea* infection compared to wild type plants. Therefore, the reduced JA-Ile production that is characteristic of our mutant after *B. cinerea* infection could also result in reduced camalexin accumulation.

BXL4 provides resistance to B. cinerea

The *bxl4* mutants exhibited a compromised resistance to *B. cinerea* infection. Various mechanisms could be responsible for the compromised immunity such as the observed reduction in JA and JA-Ile biosynthesis after infection. The production of JA upon cell wall degradation is a known defence response to necrotrophic pathogen attack (Turner et al., 2002; Laluk and Mengiste, 2010). JA-Ile alters the pectin structure in response to wounding or attack from necrotrophic pathogens. Taurino et al., (2014) showed that JA-Ile reduced the methylesterification of pectin in potato plants, which contributed to resistance to the necrotrophic pathogen *Dickeya dadantii.* Reduced methylesterification results in crosslinking of homogalacturonan with Ca^{2+}. JA-Ile also regulates the expression of *BXL4* as indicated in our wounding assays. Our *bxl4* mutants show an increased abundance of arabinans. Arabinans play a role in cell wall

reinforcement, and in Arabidopsis, the interspacing of homogalacturonan with arabinan rich RG-I reduces crosslinking with Ca^{2+}, making cell walls more flexible (Jones et al., 2003; Moore et al., 2008; Merced and Renzaglia, 2019). Reduction in arabinan levels in wild type plants could result in greater pectin crosslinking as compared to the arabinan rich *bxl4* mutants, thus making the walls more recalcitrant reducing the penetration of fungal hyphae.

The *bxl4* mutants also reveal a reduction in expression of *PAD3* and accumulation of the antimicrobial phytoalexin camalexin. Camalexin is known to play an important role in resistance to *B. cinerea* (Ferrari et al., 2007). Camalexin triggers fungal programmed cell death in *B. cinerea* minimising lesion development during early *B. cinerea* infection (Shlezinger et al., 2011). The reduction in the accumulation of camalexin in *bxl4* mutants contributes to enhanced susceptibility to the pathogen.

CONCLUSION

The results of this study reveal that *BXL4* is a putative cell wall localised bifunctional xylosidase/arabinofuranosidase that modifies cell wall polysaccharides and contributes to immunity against *B. cinerea.*

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AUTHOR CONTRIBUTIONS

A.G., G.H., and T.I. designed the research, A.G., R.M., D.H., P.S., and K.Z. performed the experiments. M.W., G.H., and T.I. provided the tools and materials. A.G., and T.I wrote the manuscript with contributions from the other authors.

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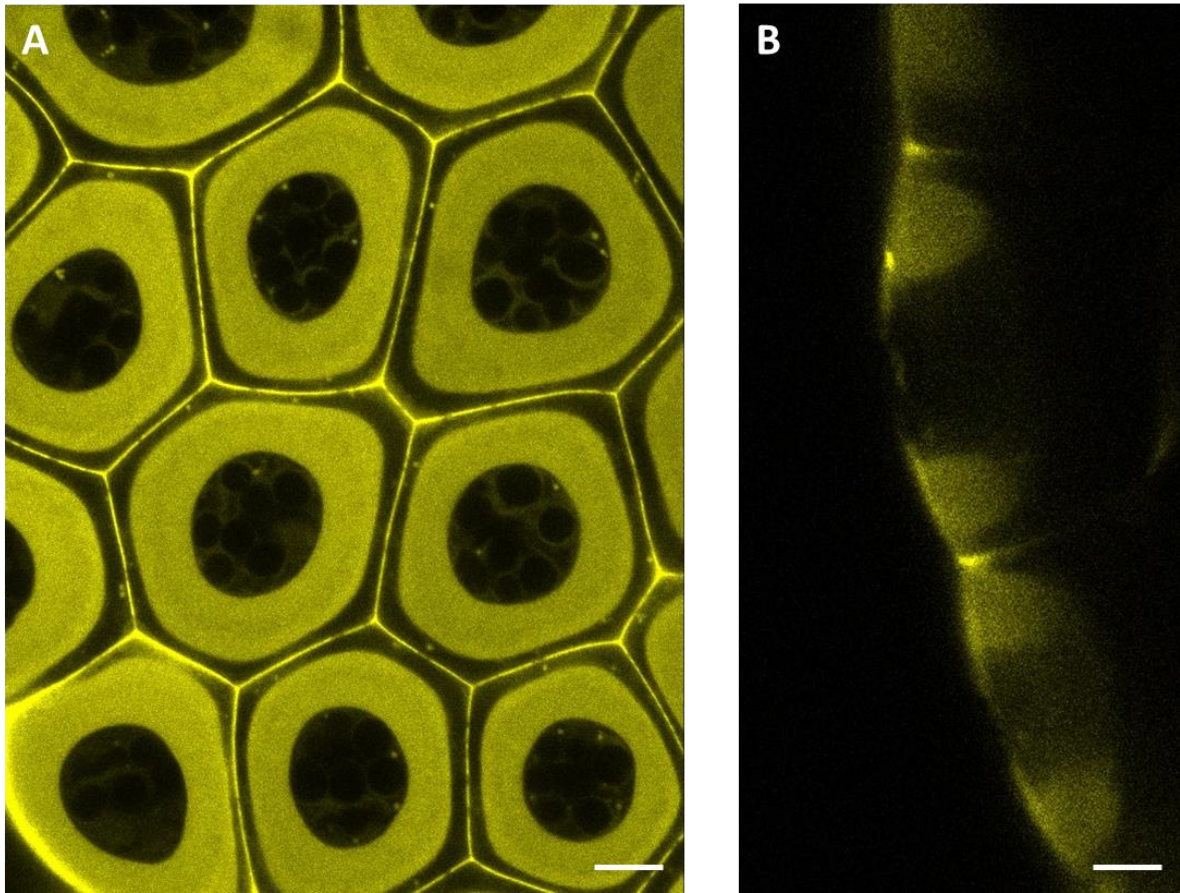
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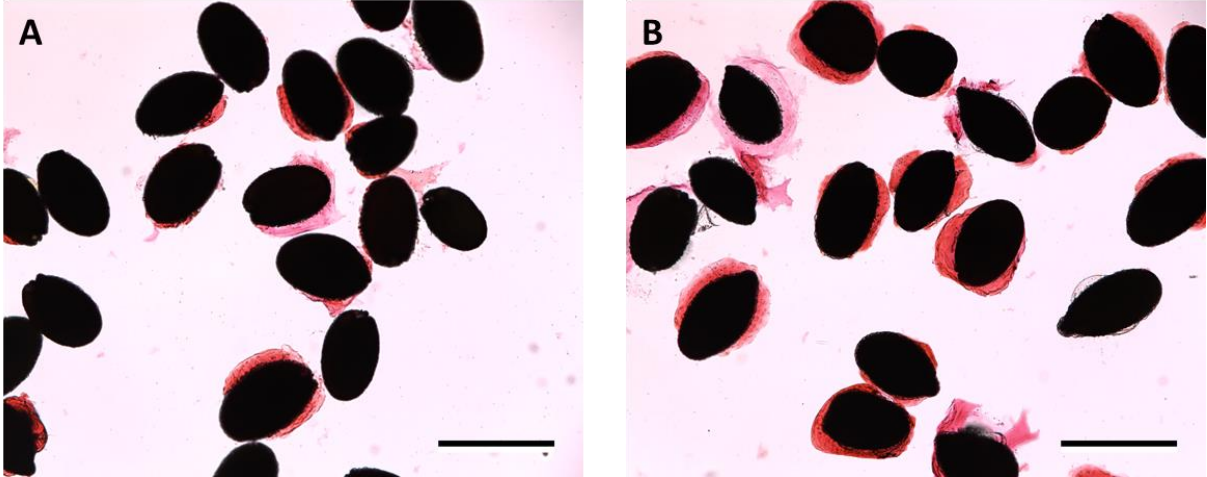
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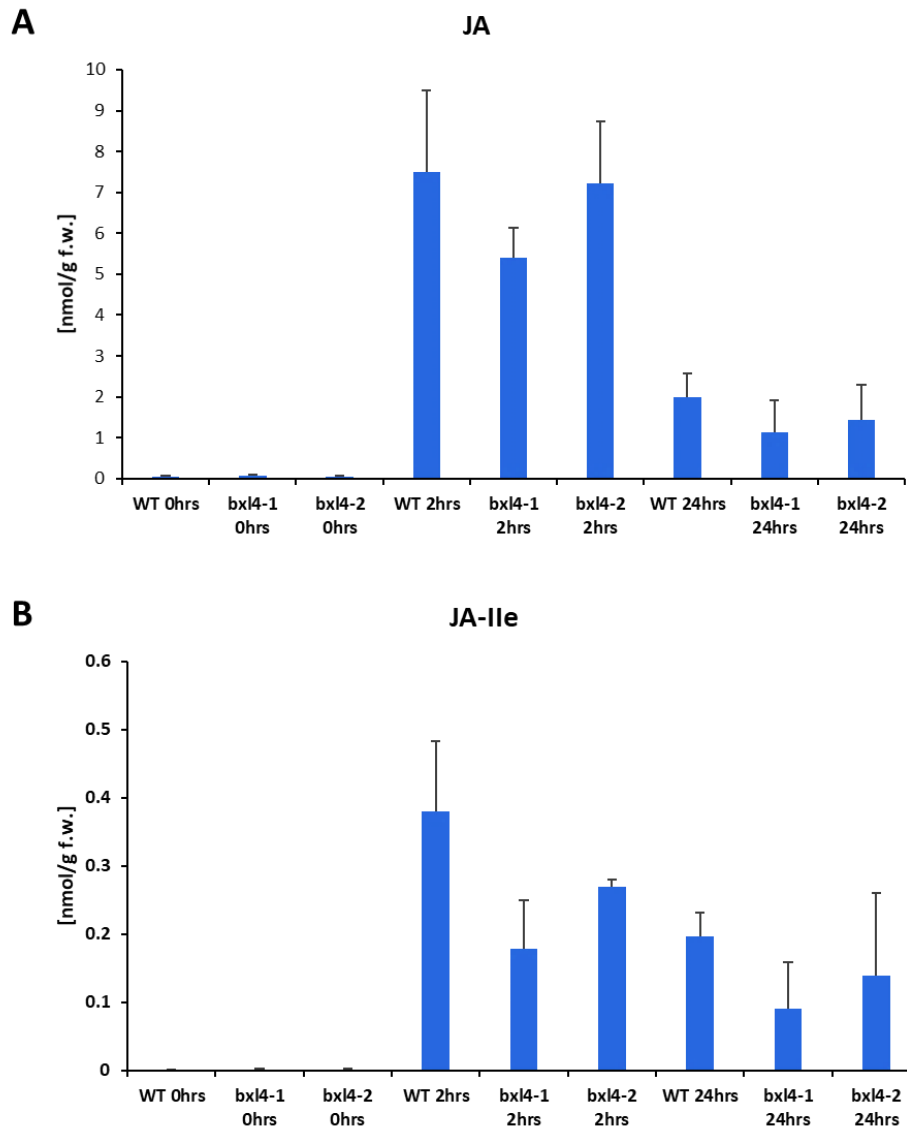
SUPPLEMENTARY FIGURES

Supplementary Figure 1: BXL4-CITRINE localises to the apoplast in Arabidopsis seed coat epidermal cells

(A) *BXL1-CITRINE* stably expressed under the *TBA2* promoter localises to the apoplast of Arabidopsis seed coat epidermal cells 7 days post anthesis (dpa). Single plane images of the seed coats were obtained by confocal microscopy. The apopolast of the of seed coat epidermal cells appears like a doughnut ring that surrounds the cytoplast when imaged from the top. **(B)** Visualisation of the seed coat epidermal cells from the side at 7 dpa shows the apoplast as two pockets in the apical corners of the cells (arrow). Scale bars, 10 µm

Supplementary Figure 2: p*TBA2:BXL6* expressed in *bxl1* does not rescue mucilage extrusion phenotype.

p*TBA2:BXL6* when expressed in *bxl1* fails to rescue mucilage extrusion as shown by complemented line 567 (A) and line 572 (B). Scale bar 300 µm

A

B

Supplementary Figure 3: JA and JA-Ile accumulation after wounding in Arabidopsis

Col-0 and *bxl4* mutant lines were mechanically wounded and leaves were sampled at 0, 2 and 24 h after infection. Extracted levels of JA **(A),** JA-Ile **(B)** were analysed using nanoelectrospray coupled to a tandem mass spectrometer. Error bars represent standard deviation of 3 biological replicates

Supplementary Table 1: List of primers

Cloning primers	Forward 5'-3'	Reverse 5'-3'
BXL1	GGGGACAAGTTTGTACAAAAAAGCAGGC TATGTCTTGTTATAATAAAGCACTATTG	GGGGACCACTTTGTACAAGAAAGCTGGGT AAAGTTGCGGTTGGACCAA
BXL4	GGGGACAAGTTTGTACAAAAAAGCAGGC TTGGGCTCTTCTTCTCCATTA	GGGGACCACTTTGTACAAGAAAGCTGGGT AGATTCTAATGCTTAAGGAATGTTTTA
BXL6	GGGGACAAGTTTGTACAAAAAAGCAGGC T ATGAATCTTCAGTTGACTCTAATC	GGGGACCACTTTGTACAAGAAAGCTGGGT AGAATTCAACAGAGAGAGAATGT
Genotyping primers	**LB**	**RB**
bxl1	ATTTTGCCGATTTCGGAAC	AACCGTCGCGTCGGCTTCAC
bxl4-1	ATTTTGCCGATTTCGGAAC	ATCTCCGACATGAAGAAGATGC
bxl4-2	TAGCATCTGAATTTCATAACCAATCTCGAT AC	ATCTCCGACATGAAGAAGATGC
bxl6	ATTTTGCCGATTTCGGAAC	TACCACAGCATTGAAGTCGTATC
qRT-PCR primers	**Forward 5'-3'**	**Reverse 5'-3'**
BXL4	ATACACAACACCACTACAAGGAC	ATCTCCGACATGAAGAAGATGC
PDF1.2	TTGCTGCTTTCGACGCA	TGTCCCACTTGGCTTCTCG
JAZ10	ATCCCGATTTCTCCGGTCCA	ACTTTCTCCTTGCGATGGGAAGA (Benthke et al., 2016)
PAD3	TGCTCTCAAGTTCACCACT	CGAATCTCGTCTTGCACTT (Benthke et al., 2016)
ACTIN8	GGTTTTCCCCAGTGTTGTTG	CTCCATGTCATCCCAGTTGC
Botrytis ACTIN	TGGAGATGAAGCGCAATCCA	AAGCGTAAAGGGAGAGGACG
Botrytis Tubulin	CCGTCATGTCCGGTGTTAC	CGACCGTTACGGAAATCGG

Supplementary Table 2: Mass transitions and corresponding conditions for determination of the phytohormones.

MRM Transitions		Analyte	DP [declustering potential]	EP [entrance potential]	CE [collision energy]
Q1	Q3				
209	59	JA	-30	-4.5	-24
225	59	11,12-OH-JA	-35	-9	-28
263	165	dinor-oPDA	-40	-5	-20
296	170.2	D5-oPDA	-65	-4	-28
305	97	12-HSO$_4$-JA	-30	-4	-32
308	116	JA-Val	-45	-5	-28
322	130	JA-Ile/Leu	-45	-5	-28
325	133	D4-JA-Leu	-80	-4	-30
324	116	12OH-JA-Val	-45	-10	-30
338	130	12OH-JA-Ile	-45	-10	-30
352	130	12COOH-JA-Ile	-45	-10	-30
387	59	12-O-Gluc-JA	-85	-9	-59

4 Discussion

4.1 Pectin monosaccharide analysis using GC-MS

Plant cell wall composition varies during development and in response to environmental changes. Plant attack by pathogens induces the differential regulation of many genes involved in remodelling of cell wall pectin. The effect of many proteins acting on pectin are not well understood given the complexity in cell wall analytics and the close interlinkages existing amongst cell wall polysaccharides. One objective of this project was to design a fast and easy method to determine pectin composition. Our protein of interest, BXL4, putatively acts on the pectin rhamnogalacturonan I therefore, we developed a method that could give a credible monosaccharide composition of pectin from various tissues.

Cell wall pectin analysis has been based on various strategies including immunolabeling using antibodies specific to different pectins. The limitation however is that most of these antibodies are large probes and consequently the cell walls have to be pre-processed to reduce masking of cell wall components and allow sufficient binding of the probes (Voiniciuc et al., 2018). Pre-processing leads to loss of some cell wall components and loss of valuable information. Immunolabeling may also lead to misleading conclusions for instance pectin labelling with 2F4 which recognises demethylesterified, Ca^{2+} cross-linked homogalacturonan (Liners et al., 1989), requires addition of calcium ions (Liners and Van Cutsem, 1992), at levels higher than physiological level resulting in overestimation of presence of calcium cross linked homogalacturonan (Hocq et al., 2017). Click chemistry, which involves joining small sugar units to form a substance (DeBolt and Estevez, 2012), has been used for pectin analytics but if fails to give a monosaccharide composition profile of various pectins. Dyes have also been developed that stain certain pectin domains such as ruthenium red (Hanke and Northcote, 1975) and propidium iodide (Rounds et al., 2011) when used at low concentrations. However, ruthenium red staining of mucilage for instance can be affected by other external factors such as presence of calcium ions (Voiniciuc et al., 2013), whilst propidium iodide is only useful in living cells (Rounds et al., 2011). Other methods that have been used in cell wall analytics involve GC-MS and HPLC based methods (Biswal et al., 2017; Bethke and Glazebrook, 2019). The GC-MS

based method involves either derivatisation of the sugars to alditol acetates which makes it impossible to detect uronic acids, or methanolysis which is a very time-consuming process. Chromotograms derived from methanolysis of sugars are very difficult to analyse because one monosaccharide can generate several signals due to production of several analytes (Biswal et al., 2017).

Our method employs water extraction of pectin from various sources including mucilage, and the alcohol insoluble residue (AIR) derived from various tissues. The putative target pectin of our protein of interest BXL4 is RG-I, which is very abundant in Arabidopsis seed mucilage (Western et al., 2000). Hydration of Arabidopsis seeds leads to the extrusion of this mucilage, which can be easily extracted for analysis. Likewise, the agitation in hot water of an alcohol insoluble residue obtained from Arabidopsis leaves leads to the extraction of water-soluble pectin, as has been described previously (Vriesmann et al., 2011). In our protocol, the extracted pectin is hydrolysed with trifluoroacetic acid (TFA) followed by evaporation of the TFA (Li et al., 2018), eliminating the need for a neutralisation step, which precipitates most of the galacturonic acid. The hydrolysed sugars are sequentially derivatised using MOX and MSTFA, thus giving a reduction in preparation time. The method also allows for quantification of galacturonic acids, which is not possible when sugars are derivatised to alditol acetates (Merkle and Poppe, 1994). Our method gives another advantage of being able to simultaneously detect and quantify neutral sugars and galacturonic acid. The advantage of using mucilage model with our method is that the predominantly RG-I composition of mucilage allows for detection of subtle changes that might occur in the RG-I sugars. Data obtained using our method show that we can accurately quantify the relative composition of mucilage from Arabidopsis mutant *bxl1,* Ws and Col-0 to levels that have been published. The arabinose composition in the *bxl1* mutant shows a two-fold increase compared to wild type Ws, as described in previous work (Arsovski et al., 2009). As expected, rhamnose and galacturonic acid are the most abundant sugars, given that RG-I backbone consists of alternating galacturonic acid and rhamnose residues (Harholt et al., 2010). Our method also gives a reliable quantification of galacturonic acid measured in citrus peel pectin sourced from Sigma-Aldrich, thus providing further evidence of robustness. Analysis of pectin extracted from leaf AIR of Col-0 showed an abundance of galacturonic acid of more than 70% which is consistent to what has already been published (Mohnen, 2008). Our method

is able to detect all the expected sugar residues, hence making it a useful tool for comparative analytics of all the various pectin moieties.

Overall, we were able to develop a robust GC-MS based method, which is reliable and time-saving to generate monosaccharide profiles of pectin from various sources. These monosaccharide profiles can be used for comparative purposes and provide an insight to the link between pectin composition and function.

4.2 BXL4 could act on arabinans in the apoplast and its disruption does not affect plant morphology

Previous work (Breitenbach et al., 2014; Goujon et al., 2003) and generated prediction using SignalP-5.0 (Almagro Armenteros et al., 2019) indicate that *BXL4* has a predicted signal peptide for localisation in the extracellular matrix. We constructed a p*TBA2*:*BXL4*-CITRINE construct for expression in Arabidopsis seed coat epidermal cells. We expressed *BXL4* using a seed coat specific promoter *TBA2* (*TESTA ABUNDANT2*), which had been identified in previous screens to be seed coat specific and highly expressed in Arabidopsis seed coats (Tsai et al., 2017). The Citrine-tagged BXL4 localised in the mucilage pocket at seven days post anthesis (Figure 1A &B, Manuscript B). As the secretory pathway is likely conserved, our results indicate that BXL4 is likely also secreted to the apoplast in other tissues such as leaves. These results are therefore consistent with the finding of BXL4 in the cell wall of Arabidopsis plants expressing a *P. syringae* effector protein AvrRpm1 (Breitenbach et al., 2014).

As mucilage is easy to investigate, the generated plant lines also gave us the opportunity to analyse a potential influence of BXL4 on cell wall composition. Mucilage is a highly pectinaceous substance made by the seed coat epidermal cells consisting predominantly of RG-I, and small amounts of homogalacturonan, cellulose and hemicellulose (Arsovski et al., 2010; Haughn and Western, 2012). It is known that Arabidopsis seed coat epidermal cells secrete mucilage together with mucilage modifying proteins to the apoplast between four and eight days post anthesis (Arsovski et al., 2010; Dean et al., 2007; Haughn and Western, 2012; Western et al., 2000).

Various proteins acting on mucilage components have already been confirmed to localise to the mucilage pocket. The beta-galactosidase MUM2, and xylosidase/arabinofuranosidase BXL1 acting on mucilage are deposited

simultaneously with mucilage into the apoplast were they are thought to act on the side chains of RG-I (Dean et al., 2007; Arsovski et al., 2009). Knocking out either of the two genes results in mucilage that does not extrude properly, as it is lacking the correct chemical balance. Work by Arsovski et al., (2009) showed that mucilage from *bxl1* knockout mutants has a higher abundance of (1-5)-linked arabinans relative to the wild type, thus indicating the arabinofuranosidase activity of BXL1. *BXL4* is expressed at a low level in many organs, but it has not been identified in mucilage. Analysis of the protein structure indicates that it possesses glycosyl hydrolase domains (InterPRO-EMBL-EBI). Earlier work by Goujon et al., (2003) indicated that *BXL4* clustered together with *BXL1* in a small gene family of Arabidopsis betaxylosidases (BXLs) with seven members. The betaxylosidases are similar to those characterised in fungal and bacterial pathogens and involved in cell wall degradation (de Vries et al., 2000). The presence of glycosyl hydrolase domains and the localisation of BXL4 all point to a putative role in hydrolysing some polysaccharide component of the cell wall. Expression of *TBA2* driven *BXL4* in the mucilage of the *bxl1* mutant was able to rescue the mucilage phenotype suggesting that BXL4 and BXL1 are functionally similar (Figure 2D, Manuscript B). *TBA2* is a very strong promoter, as it drives the most abundant seed coat specific protein TBA2 (Tsai et al., 2017). The strong expression could have also amplified the activity of BXL4 hence the rescue of the phenotype. It is also interesting that another member of the betaxylosidase family, *BXL6* did not exhibit localisation in the mucilage and consequently it did not rescue the mucilage defect of *bxl1* (Supplementary Figure 2, Manuscript B), thus demonstrating the different functional roles within the gene family. The spacial and temporal expression of *BXL1* and *BXL4* is differentially regulated (Goujon et al., 2003) meaning that the 2 proteins are probably involved in cell wall metabolism during various processes and in different organs.

Our data provide evidence that *BXL4* is an arabinosidase as its expression in *bxl1* mucilage causes a significant reduction of arabinans to levels comparable with wild type (Figure 2E, Manuscript B). The overexpression of *BXL4* under the *TBA2* promoter in Col-0 did not induce a significant decline in arabinose levels, but there was a further reduction in xylose as compared to Col-0 (Figure 2E, Manuscript B). The non-reduction in arabinans could be explained by the BXL1 already present in Col-0 mucilage that would have already depleted all the accessible arabinans. The further

decrease in xylose could also mean that *BXL4* is a more efficient xylosidase compared to *BXL1* when expressed in mucilage. The strong xylosidase activity of BXL4 is also evident in the *bxl1* mutant complemented with p*TBA2:BXL4*, which exhibits levels of xylose that are less than in the wild type or the control line p*TBA2:BXL1* in *bxl1.* Work by Ralet *et al.,* (2016) could prove the tight proportionality that occurs in xylose-rhamnose-galacturonic acid of the outer layer of mucilage. Linkage analysis in this mucilage layer confirms the existence of xylan chains suggesting the presence of xylan side chains attached to rhamnogalacturonan I backbone. It is thus possible that BXL4 acts on these xylan side chains resulting in the reduction of xylose in the p*TBA2:BXL4* complemented *bxl1* and p*TBA2:BXL4* overexpression line in Col-0.

In addition, xylans with a backbone consisting of a β-1,4-linked D-xylose are some of the hemicelluloses found in trace amounts in mucilage (Voiniciuc et al., 2015; Hu et al., 2016; Ebringerová and Heinze, 2000), and these could also serve as substrate for BXL4, as BXL1 could act on β-xylosidase substrate *in vitro* (Goujon et al., 2003).

Work by Goujon et al., (2003) showed that in organs, where both *BXL1* and *BXL4* were expressed, like in stems, flowers and siliques, betaxylosidase activity was higher, and knocking out *BXL1* did not cause a compensatory increase in *BXL4* expression. These findings hence suggested that BXL1 and BXL4 are biologically functionaly similar but not physiologically functionaly redundant. Our research findings strongly suggest that BXL4 is a bifunctional xylosidase/arabinosidase similar to BXL1. It is known that enzymes acting on polysaccharides are capable of dual functions such as *Agrobacterium tumefaciens* derived betaglucanases also possess betaxylosidase activity (Watt et al., 1998), whilst bacterial and fungal betaxylosidases also exhibit α-L-arabinofuranosidase activity (Whitehead and Cotta, 2001).

Analysis of *BXL4* expression by qRT-PCR in the *bxl4-1* and *bxl4-2* mutant lines confirmed that *bxl4-1* is a knockout, whilst small amounts of transcript could be detected in the *bxl4-2* line (Figure 3B, Manuscript B). Both *bxl4* mutants exhibited a mild increase in abundance of arabinans (Figure 3C, Manuscript B). Our data from expression of *BXL4* in mucilage suggests that arabinans together with xylans are a substrate of BXL4 at least in the mucilage. However, there was no noticeable increase in the xylose content of the leaf cell walls of the *bxl4* mutants. Despite the increased arabinan abundance, there is no obvious morphological changes during development

compared to wild type (Figure 3D, Manuscript B). It has already been observed that *BXL1* is strongly expressed in most organs including leaves, stems and siliques compared to *BXL4*, which probably results in the non-increase of xylose in the leaves of the mutants. The betaxylosidase activity in Arabidopsis leaves is mostly attributed to BXL1 and BXL4, with the former contributing a larger portion of the activity, as the *bxl1* knockout mutants have a significantly decreased betaxylosidase activity in the apoplast (Goujon et al., 2003). The *bxl1* knockout mutants have a severe phenotype of smaller leaves that are curly, and shorter siliques with fewer seeds attributed to the increased xylose levels (Goujon et al., 2003). The increased abundance of arabinose due to knocking out *BXL4* does not cause any obvious morphological phenotype different from wild type, but it does confirm the arabinosidase activity observed after expression in mucilage.

4.3 *BXL4* is a stress induced gene and could induce JA signalling

The lack of a strong phenotype of the bxl4 mutants under optimal growth conditions is consistent with the hypothesis that BXL4 has a role especially important in stress response. Analysis of the expression of *BXL4* in *Arabidopsis* upon mechanical wounding and *B. cinerea* infection confirmed that both forms of stress trigger the upregulation of *BXL4* (Figure 3A & D Manuscript B). The induction of *BXL4* upon wounding is similar to that of other genes involved in pectin modification. Reymond et al., (2000) showed upregulation of a pectin methylesterase and a polygalacturonase inhibitor protein upon wounding, which may help reduce further cell wall damage. OGs generated from demethylesterified pectin are usually cross-linked. It is known that demethylated OGs cross-linked by calcium ions, have a greater affinity for their receptors, wall-associated kinases, and are more biologically active in triggering defence responses (Bethke et al., 2014; Kohorn and Kohorn, 2012).

Another important stress factor that induces genes is pathogen infection. For example, the infection of Arabidopsis by *B. cinerea* results in transcriptional reprogramming of many genes. Work by Birkenbihl et al., (2012) indicated that 14 hours after infection of Arabidopsis with *B. cinerea* conidiospores, 1577 genes were altered in their expression levels at least 2-fold compared to mock infected plants. Our data shows that *BXL4* is one of the many genes that is upregulated by infection of Arabidopsis with *B. cinerea*, and work by Sham et al., (2014) is consistent with our findings because

they also identified *BXL4* upregulation in their transcriptome data after *B. cinerea* infection.

Apart from an upregulation of genes that help the plants to defend against pathogens, genes that help the pathogen to invade the plant have been found to be upregulated. It is for example known that *B. cinerea* manipulates the abundance and/or activity of host proteins to aid its infection and suppress defence responses. The activation of endogenous polygalacturonases is one strategy employed by *B. cinerea* to soften the cell walls, and to aid its progression. *B. cinerea* induces the expression of endogenous polygalacturonases whose activity is not inhibited by polygalacturonase inhibitor proteins (PGIPs) as for example the tomato polygalacturonase, *SL*PG2A (Cervone et al., 1990). The activity of the polygalacturonase is known to soften the cell wall and to allow penetration of the fungus. AbuQamar *et al.*, (2006), suggested that infection of Arabidopsis also resulted in the upregulation of *PME3* (*PECTIN METHYLESTERASE3*). The activity of PME3 in plant cell walls caused the random demethylesterification of pectin, rendering it susceptible to fungal pectate lyases and polygalacturonases, whilst the mutant *pme3* was less susceptible (Raiola et al., 2011). Other pathogens also employ the strategy to activate the expression of plant genes that aid their infection. The powdery mildew pathogen *Erysiphe cichoracearum* is known to activate the expression of *PMR6* (*POWDERY MILDEW RESISTANT6*) which codes for a plant-derived pectate lyase-like protein known to alter the cell wall composition by degrading demethylesterified pectin. The *pmr6* mutant portrays reduced susceptibility to the same pathogen (Vogel, 2002; Vogel et al., 2004). The hemibiotrophic pathogen of Arabidopsis*, P. syringae* pv *maculicola (Psm)* ES4326, is known to repress the expression of genes involved in the biosynthesis of pectin. The genes *GLUCORONATE 4-EPIMERASE1* (*GAE1*) and *GLUCORONATE 4-EPIMERASE6* (*GAE6*) are involved in the conversion of UDP-D-glucuronic acid to UDP-D-galacturonic acid, which is a major component of pectin (Bethke et al., 2016). The *gae1 gae6* double knockout mutant has reduced pectin in the cell walls and exhibits compromised resistance to *B. cinerea* and *Psm.* The infection of Arabidopsis with *P. syringae* pv *tomato* (*Pst*) DC3000 upregulates the expression of *INFLORESCENCE DEFICIENT IN ABSCISSION-like 6* (*IDL6*) (*Wang* et al*., 2017*). *IDL6* triggers the expression of an endogenous polygalacturonase *ADPG2,* which normally degrades pectin during abscission of floral organs (Estornell et al., 2015).

The induced expression of *IDL6* resulted in an increased susceptibility to *Pst* DC3000*,* whilst the *idl6* knockout mutants portrayed greater resistance to the bacterial pathogen (Wang et al., 2017)*.*

On the other hand, plant infection with *B. cinerea* induces the upregulation of genes that are involved in defences against the pathogen. Infection of plants with various pathogens results in the upregulation of polygalacturonase inhibitor proteins (PGIPs) (Yao et al., 1999). PGIPs are important defence proteins belonging to a family of Leurich repeat (LRR) proteins and are present in cell walls of most plant species (Toubart et al., 1992; Ferrari, 2003). In Arabidopsis, infection with *B. cinerea* triggers the upregulation of two tandemly duplicated PGIPs via two signal transduction pathways. JA-IIe mediates the expression of *AtPGIP2*, whilst *AtPGIP1* expression is mediated by oligogalacturonides. Overexpression of the two genes results in reduced disease symptoms after infection with *B. cinerea* (Ferrari, 2003). Inhibition of polygalacturonases by PGIPs facilitates the generation of oligogalacturonides with a high degree of polymerisation that elicit various defence responses (Cervone et al., 1989). In beans infected with the pathogen *Colletotrichum lindemuthianum*, the transcripts of PGIP accumulate causing a correlated increase in hypersensitive response in incompatible interactions. In the event of a compatible interaction, there is a delay in PGIP transcript accumulation that also coincides with appearance of disease symptoms, thus emphasising the role of *PGIP* expression in resistance to fungal pathogens (Nuss et al., 1996). In sugar beet, a pectin lyase inhibitor that suppresses the activity of a *Rhizoctonia solani* pectin lyase is upregulated upon infection with the pathogen. Plant resistance to the pathogen is correlated to the expression levels of the inhibitor protein (Bugbee, 1993).

The pectin methylesterase inhibitor protein *Gh*PMEI3 found in cotton *(Gossypium hirsutum)* is unpregulated after infection with the fungus *Verticillium dahliae*. It was found out that *Gh*PMEI3 increases pectin methylesterification through the inhibition of cottons PME2 and PME31 and this reduced fungal development in cotton. Silencing of *GhPMEI1* caused an increased susceptibility to the fungal pathogen (Liu et al., 2018). In pepper (*Capsicum annuum* L.), CaPMEI1 is upregulated upon infection with *Xanthomonas campestris* pv *vesicatoria* (*Xcv*). PMEI1 inhibits the activity of PMEs and also exhibits antifungal activity against some phytopathogenic fungi. The silencing of

CaPMEI1 in pepper resulted in increased susceptibility to *Xcv* together with the reduced expression of some plant defence genes (Liu et al., 2018).

Our gene expression data showed that *BXL4* was a stress induced gene upregulated upon mechanical wounding and attack from the necrotrophic pathogen *B. cinerea*. Both forms of stress are known to trigger JA-Ile signalling which inturn induces the expression of various defence responses (Pangesti et al., 2016; Pieterse et al., 2012). To check if JA-Ile was responsible for upregulation of *BXL4* we investigated *BXL4* expression in wild type Arabidopsis versus a JA-Ile deficient mutant *delayed-dehiscence2-2* (*dde2-2)* (von Malek et al., 2002) after mechanical wounding. Wounding in Arabidopsis is known to induce various genes to restrict further damage (Reymond and Farmer, 1998) and the expression of many of these genes is JA regulated (Farmer et al., 1998; Creelman and Mullet, 1997). The production of JA and its active derivative JA-Ile resulting in activation of their downstream signalling pathway is critical for plant defence against wounding and attack from necrotrophic pathogens (Yan and Xie, 2015; Howe and Jander, 2008). Pattern recognition receptors (PRRs) located on the plasma membrane detect danger cues such as PAMPs, herbivore associated molecular patterns (HAMPs) and DAMPs, triggering a rapid accumulation of JA-Ile (Lee and Howe, 2003; Schmelz, 2003; Geng et al., 2014). The *dde2-2* mutant has a mutation in *ALLENE OXIDASE SYNTHASE* (*AOS*). AOS carries out the dehydration of 13-hydroperoxy-octadecatrienoic acid forming an unstable epoxide that is catalysed to 12-oxo-phytodienoic acid (OPDA) by ALLENE OXIDE CYCLASE (AOC) during early stages of JA biosynthesis (Figure 8) (Turner et al., 2002). Wounding of the *dde2-2* did not induce the upregulation of *BXL4* (Figure 4A, Manuscript B). This finding suggests that BXL4 is also one of the genes whose expression in response to wounding requires JA-Ile signalling suggesting a putative role in plant defences.

Because the *bxl4* mutants had an increased amount of arabinans, we further checked if this cell wall modification had any effect on plant defence signalling by measuring the accumulation of defence marker genes after wounding and *B. cinerea* infection. The *bxl4* mutant plants had reduced expression of JA-Ile marker genes *JAZ10* and *PDF1.2* as well as the reduction in the accumulation of JA and JA-Ile after wounding and *B. cinerea* infection. The reduction in JA-Ile production after wounding and *B. cinerea* infection suggests that BXL4 is not only upregulated by JA-Ile but plays a role

in the amplification of JA-Ile signalling as well. Pectin-derived fragments have been implicated in the regulation of enzymes involved in the biosynthetic pathway of JA-Ile. Denoux et al., (2008), demonstrated the participation of oligogalacturonides in the activation of two lipoxygenases *LOX3* and *LOX4* that take part in the biosynthetic pathway of JA-Ile. The treatment of Arabidopsis with short oligosaccharides also induces the upregulation of another gene, *AOS*, involved in JA-Ile biosynthesis (Norman et al., 1999). The possible involvement of oligosaccharides in JA-Ile signalling comes also from less direct evidence. *CYP79B2, CYP79B3* and *CYP83B1* are upregulated upon treatment of Arabidopsis with oligogalacturonides. These genes are players in the biosynthesis of indole glucosinolates (Wittstock and Halkier, 2002) and JA regulates the accumulation of indole glucosinolates after infection of Arabidopsis with *Erwinia carotovora* (Brader, 2001). Many other publications have indicated that alterations in cell wall composition caused by different mutations induce the reprogramming of various phytohormone signalling pathways (Vega-Sanchez et al., 2012; Wang et al., 2017; Ellis, 2002). It is also known that JA-Ile induces the expression of its biosynthetic genes, which in turn trigger JA-Ile accumulation through a positive feedback loop hence resulting in a robust defence response (Browse, 2009). Observations from our wounding and *B. cinerea* infection data suggests that *BXL4* is induced in a JA-Ile dependant manner, and contributes to induction of JA-Ile accumulation in a positive feedback mechanism, indicating a possible role in plant defences.
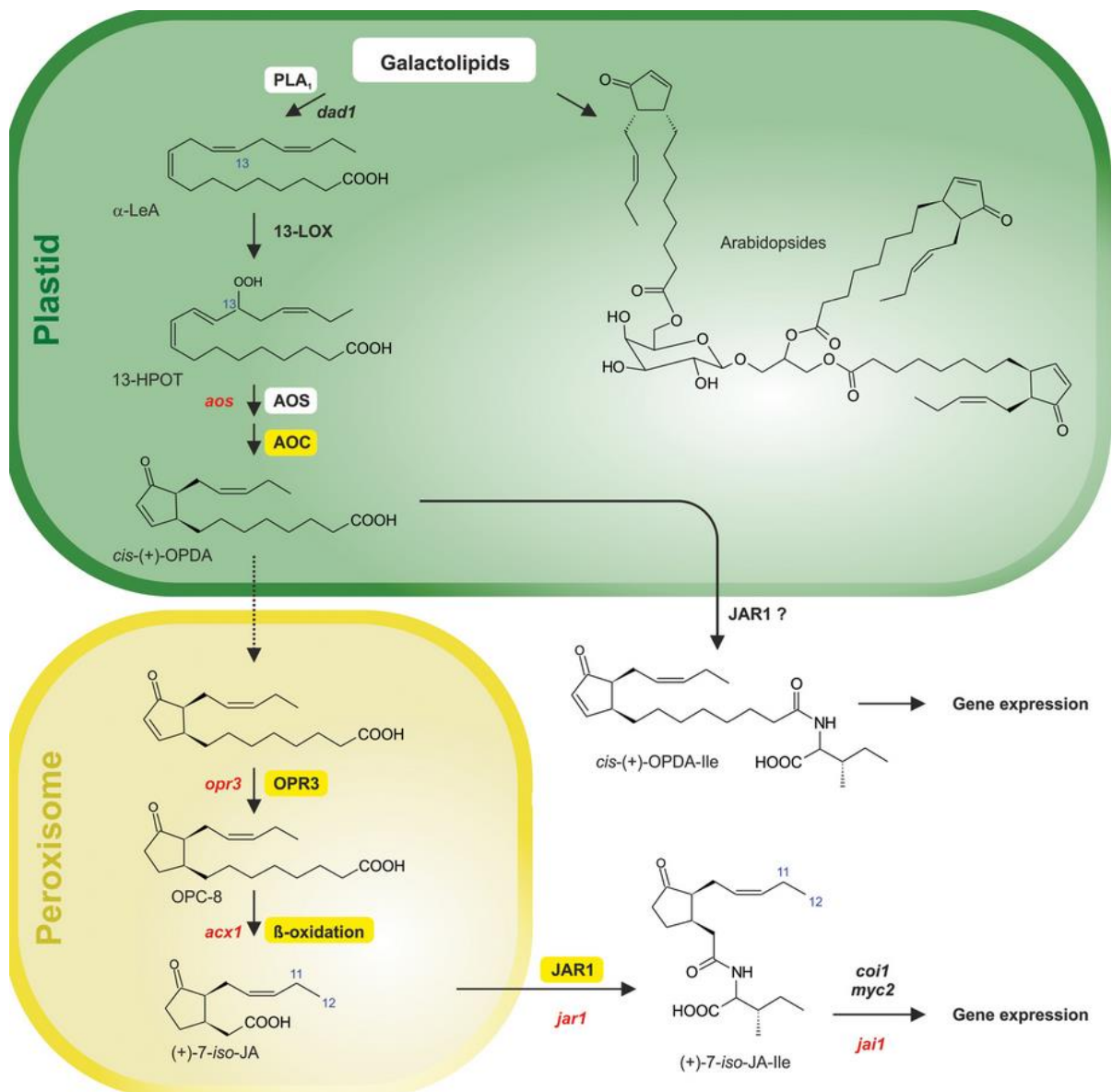
Figure 8*: **The biosynthesis of JA: Image adapted from Wasternack and Song, (2016)** Phytopathogen infection, feeding by herbivorous insects or mechanical damage, causes the release of α-linolenic acid from membranes of chloroplasts. α-linolenic acid is converted to 12-oxo-phytodiecanoic acid (OPDA) through a series of enzyme steps involving LIPOXYGENASE (LOX), followed by an ALLENE OXIDE SYNTHASE (AOS) which forms an unstable product that is quickly converted by ALLENE OXIDE CYCLASE (AOS). After transportation to the peroxisome, OPDA then undergoes several ß-oxidation cycles to form (+)-7-iso-JA (Zhang et al., 2017; Larrieu and Vernoux, 2016). (+)-7-iso-JA is then transported to the cytosol upon which the amino acid isoleucine (Ile) is conjugated to form JA-Ile, which is the most active JA derivative.

Analysis of defence marker genes after B. cinerea infection also showed that another defence related gene *PAD3* had lower expression in the *bxl4* mutants compared to wild type lines. The accumulation of the phytoalexin camalexin was reduced in the

mutant lines after *B. cinerea* infection. PAD3 is a cytochrome P450 responsible for catalysing the last step in the camalexin biosynthesis pathway (Zhou, 1999). *PAD3* is induced upon perception of oligogalacturonides by membrane bound WALL ASSOCIATED KINASE1 (WAK1), which activates downstream MAPK cascades (Ferrari, 2013). Camalexin is the most abundant phytoalexin in Arabidopsis and its biosynthesis is triggered in part by the perception of MAMPs and also oligogalacturonides (Ferrari et al., 2007). Camalexin biosynthesis is also regulated by the JA-Ile signalling pathway. Rowe et al.*,* (2010) indicated that the accumulation of camalexin after *B. cinerea* infection in Arabidopsis mutants deficient in JA-Ile signalling or JA-Ile perception was significantly reduced compared to wild type lines. It is therefore possible that the reduction in JA-Ile biosynthesis observed in the *bxl4* mutants could be responsible for the reduced production of camalexin. Observations from out data indicates that the expression of *PAD3* in the *bxl4* mutant was significantly reduced, but there was no similar reduction in camalexin accumulation, where a difference was only observed after 72 hours. Whilst *PAD3* is responsible for catalysing the last step in camalexin biosynthesis, overexpression of *PAD3* alone does not necessarily result in camalexin accumulation, and that all genes in the camalexin biosynthesis pathway have to be elicited (Schuhegger et al., 2006).

The reduction in expression of JA-Ile marker genes and PAD3 has been linked to oligosaccharides that are derived from homogalacturonan degradation (Ferrari, 2013) and not rhamnogalacturonan which BXL4 probably acts on. It has however, been demonstrated that RG-I itself and therefore RG-I-modifying proteins have an effect on the nature of homogalacturonan and consequently on the nature of the derived oligogalacturonans. The oligogalacturonide-derived responses described above in relation to JA-Ile and camalexin could also be indirectly affected by RG-I modifications induced by *BXL4.* RG-I side chain modifications affect the degree of methylesterification of homogalacturonan. Western et al., (2001), could show that the *mum2* mutant, characterised by the presence of abundant galactan side chains in mucilage RG-I, has homogalacturonan with a high degree of methylesterification. This is evidence that PME activity is altered in response to RG-I modification. Similarly, Arabidopsis plants carrying a mutation in the transcriptional regulator *LEUNIG_HOMOLOG* (*LUH*) produce mucilage with highly substituted RG-I (Walker et al., 2011). The *luh* mutant has a high abundance of galactan and arabinan chains

attached to the RG-I backbone. Homogalacturonan from this mutant also shows a high degree of methylesterification similar to what is observed in *mum2*. The increased arabinan content of RG-I associated with *bxl4* could play a similar role in inhibiting PME activity resulting in generation of methylesterified oligogalacturonans, which are known to have a low elicitor activity. Partially demethylesterified oligogalacturonans have been found to induce strong biological responses in strawberries, triggering the induction of lipoxygenases, and increasing resistance to *B. cinerea* (Osorio et al., 2008).

The predicted interaction partners of BXL4 generated using STRING version 11.0 (Szklarczyk et al., 2015a) implicate the possible role of BXL4 in the generation of oligogalacturonides (Figure 9). The prediction indicates that some polygalacturonases and pectin lyases are potential interactors. The polygalacturonase PGA4 and three-pectin lyase like proteins AT3G59870, AT5G14650 and AT3G07850 are indicated as potential interaction partners which act on homogalacturonan and might work co-operatively with BXL4 contributing in generation of oligogalacturonides with elicitor activity.

Whilst we have indicated the activity of BXL4 to be indirectly linked to defence responses by affecting the nature of homogalacturonan and its oligogalacturonides. It is also possible that the direct modifications possibly done on RG-I will have plant defence implications. The activity of BXL4 cleaves off arabinose monomers during the remodelling of cell wall polysaccharides which are unlikely to have any elicitor activity. However, it is possible that the modified RG-I left could trigger some biological effects. An interesting observation was made by Breitenbach et al., (2014), who performed comparative proteomics analysis of apoplast-enriched proteins extracts from *enhanced disease susceptibility1* (*eds1*) mutants and wild type Arabidopsis expressing AvrRpm1 an effector from *P syringae*. They identified BXL4 as one of the EDS1 dependent proteins upregulated in the apoplast upon expression of a pathogen effector, thus suggesting its possible role in plant defences. They also identified a LEGUME LECTIN LIKE PROTEIN1 (LLP1) on the plasma membrane facing the apoplast as another EDS1 dependant protein. It is thus, possible that these two proteins that are simultaneously upregulated are interactors, and modified RG-I could bind to LLP1 and trigger defence responses. In mammalian cells, lectins are known to recognise non-self, self or modified self-molecular components and activation of

trafficking in the cells together with communication between cells leading to immune signalling (Rabinovich et al., 2012). LLP1 may regulate the plant defences after recognition of the modified RG-I either in the locally *B. cinerea* infected leaf or systemic leaves leading to induction of plant defence responses.
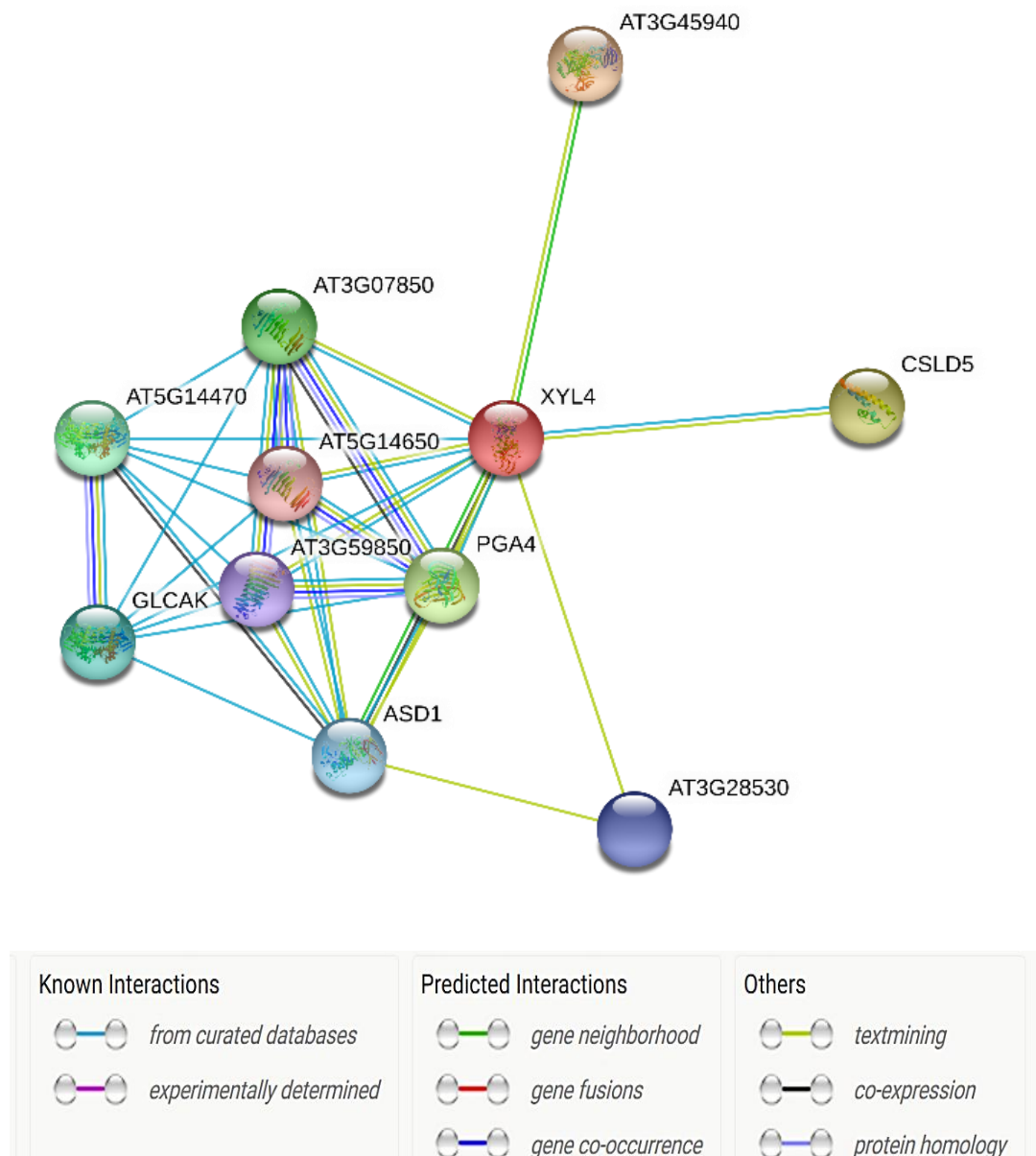

Figure 9: Predicted BXL4 functional partners. Image generated using STRING version 11.0 https://string-db.org (Szklarczyk et al., 2015a).

Table 2: Predicted functional partners of BXL4 shown in figure 9.

AT3G45940	Glycosyl hydrolase family 31 protein
CSLD5	Cellulose synthase like protein D5
PGA4	Exopolygalacturonase clone GBGE184
AT3G07850	Pectin lyase like superfamily protein
AT5G14470	GHMP kinase family protein; Sugar-1-kinase
GLCAK	Glucuronokinase 1; Sugar-1-kinase
ASD1	Alpha-L-arabinofuranosidase 1
AT3G28530	UDP-glucose 4-epimerases
AT3G59850	Pectin lyase like superfamily protein
AT5G14650	Pectin lyase like super family protein

4.4 BXL4 contributes to resistance against *B. cinerea*

In order to investigate the significance of *BXL4* induction after mechanical wounding and *B. cinerea* infection to plant immunity, a disease susceptibility assay was carried out. The *bxl4* mutant lines were significantly less resistant to *B.cinerea* compared to wild type plants. Data from our infection assays provide evidence that *BXL4* is a player contributing to resistance against the necrotrophic pathogen *B. cinerea.*

Findings from our work indicate that *BXL4* contributes to plant immunity possibly through various mechanisms including the reduction in JA production. Mechanical wounding of Arabidopsis leaves and infection with *B. cinerea* resulted in reduced expression of JA-Ile marker genes in the *bxl4* knockout lines compared to the wild type. The same observation was seen in terms of JA and JA-Ile accumulation post wounding or infection. Wounding of plant tissues together with infection of Arabidopsis with necrotrophic pathogens is known to trigger JA-Ile signalling as a defence response (Browse and Howe, 2008). The outcome of plant-pathogen interactions is determined to a large extend by the hormonal homeostasis in plants. While it is generally agreed that salicylic acid (SA) is responsible for resistance to biotrophic pathogens and JA-Ile for resistance to necrotrophs (Rahman et al., 2012), there is an overlap and cross talk between the phytohormones impacting on the various defence responses (Kachroo, 2003; Veronese, 2006). The role of JA-Ile with regards to resistance to necrotrophs has been well documented in many articles, and generally, treatment of plants with JA-Ile renders these more resistant to necrotrophic pathogens

whilst knocking out genes involved in JA-Ile biosynthesis results in increased susceptibility (Vijayan et al., 1998; Lorenzo and Solano, 2005). *CORONATINE INSENSITIVE1* (*COI1*) is a receptor of JA-Ile in Arabidopsis, and mutations in this gene lead to JA-Ile insensitivity thereby increasing susceptibility to many necrotrophic pathogens including *B. cinerea* (Thomma et al., 1998).

Jasmonates trigger the expression of their own biosynthetic genes causing a positive feedback loop that promotes JA-Ile accumulation (Browse, 2009). Jasmonates regulate the expression of cell wall proteins important for stress response. Taurino et al., (2014), showed that potato *(Solanum tuberosum)* transgenic plants lacking both *St*13–*ALLENE OXIDE SYNTHASE* genes required for JA-Ile biosynthesis had altered cell wall pectin composition compared to wild type plants. The transgenic plant named *CoAOS1/2* had a PME activity that was different from wild type, as the pectin of this mutant was highly methylated. They also indicated that the transgenic lines produced less amounts of a rhamnogalacturonan-derived oligosaccharide and were more susceptible to the necrotrophic fungal pathogen *Dickeya dadantii.* JA-Ile is known to upregulate the expression of *PGIP2*, which contributes to cell wall rigidity by inhibiting the activity of polygalacturonases derived from necrotrophic pathogens (O'Brien et al., 2012; Bellincampi et al., 2014).

The *bxl4* mutants had a reduction in the expression of *PAD3* and accumulation of camalexin after infection with *B. cinerea* compared to wild type plants. Camalexin is an indole derivative of tryptophan forming part of the defence repertoire induced in plants upon perception of various cues and in response to both necrotrophic and biotrophic pathogens (Ahuja *et al.*, 2012). Arabidopsis mutants impaired in biosynthesis of camalexin were found to be more susceptible to a variety of phytopathogens including *B. cinerea* (Denby *et al.*, 2004). Whilst the antimicrobial activity of camalexin has been confirmed *in vitro*, the exact mechanism involved is not very clear (Pedras and Khan, 2000; Stotz et al., 2011). In *B. cinerea* however, exposure to camalexin leads to a fungal programmed cell death-like response (Shlezinger et al., 2011). Hence, the production of camalexin in plant tissues upon *B. cinerea* infection could lead to this programmed cell death, limiting further development of the pathogen. In bacterial pathogens, camalexin is known to disrupt their cell membranes, and this probably explains why the *Psm* strain ES4326 is sensitive to camalexin treatment (Rogers et al., 1996). *Alternaria brassicicola* treated

with camalexin exhibits a transcriptional profiling skewed towards plasma membrane maintenance and reduced permeability of the cell wall, thus indicating the potential effects of camalexin on membranes (Sellam et al., 2007).

The *bxl4-1* mutants also exhibited mildly higher arabinan levels in the leaf cell walls compared to wild type plants. The difference is not dramatic but given that the betaxylosidase family has seven members, redundancy cannot be ruled out. The necrotrophic pathogen *B. cinerea* is known to prefer a host range that includes pectin-rich species (ten Have et al., 2002). The production of cell wall degrading proteins during infection helps in hydrolysis of the pectin component of the cell wall into its monomeric units, thus providing a source of carbon to the growing pathogen (Zhang and van Kan, 2013). The vast cell wall degrading enzyme catalogue including arabinanases (Nafisi et al., 2014) secreted by *B. cinerea* could also suggest that arabinans can also be degraded as a carbon source. It is known that degradation of cell wall components during pathogen infection, especially by *B. cinerea*, aids in the rapid development and spread of the pathogen (Battaglia et al., 2011; Zhang et al., 2011). The increased abundance of arabinans in the *bxl4* mutants could serve as an extra source of carbon for *B. cinerea* resulting in the increased disease development observed compared to the wild type.

The altered cell wall composition could also have an effect on the hyphal penetration of *B. cinerea*. Whilst *B. cinerea* invades plant tissues using a host of various cell wall degrading enzymes, it is known that softening of the cell walls through induction of endogenous polygalacturonases is important for virulence. The induction of endogenous polygalacturonases, which results in generation of oligogalacturonides that do not trigger defence responses but allow softening of cell walls (Kohorn and Kohorn, 2012), helps in fungal hyphae development. Arabinans are known to play a role in cell wall stiffening, which might impede the development of hyphae. The role of arabinans in cell wall stiffening has been articulated in various publications. For instance, the *arad1* mutant, with reduction in cell wall arabinans, has an inflorescence stem more rigid than the wild type. Jones et al., (2003; 2005) showed that arabinans are important for promoting guard cell flexibility. The mechanism in which arabinans affect cell wall stiffening is not clear, but it is thought that they hinder the close association of homogalacturonans, hence reducing the formation of calcium cross-links that promote cell wall rigidity. The possible role of arabinans in restricting the

formation of calcium cross-links could also aid in disease susceptibility observed. The generation of calcium cross-linked oligogalacturonides after cell wall hydrolysis triggers a strong defence response. Decreux and Messiaen, (2005) proved that WAK1 has a stronger affinity for calcium cross-linked oligogalacturonides triggering a stronger biological response. The contribution of arabinans to cell wall stiffness that inhibits fungal penetration is shown in the *mur8-1* mutant, which shows a reduction in cell wall RG-I (Mertz et al., 2012). Engelsdorf et al., (2017) could prove that the *mur8-1* mutant has a compromised resistance to the penetration of the fungal pathogen *Colletotrichum higginsianum.*

The possible role of BXL4 in cell wall stiffening is also suggested by its expression pattern in Arabidopsis rosette leaves. Our data shows that the expression of *BXL4* was mostly confined to mature fully developed leaves, and lower in the younger developing leaves. The fully developed leaves do not need to expand in contrast to the younger leaves, thus, they become rigid. The contribution of RG-I to cell wall stiffening is not only confined to the arabinans, but also the galactan side chains have been implicated in cell wall stiffening. Martín et al., (2011) highlighted that the chickpea betagalactosidase βI-Gal that cleaves off RG-I galactan side chain was important for modification of cell wall polymers and results in thickened cell walls. Degradation of 1,4 galactans has also been shown to induce the reinforcement of phloem cell walls (Vincken et al., 2003). Another galactosidase was shown to associate with vascular tissues of *Cicer arietinum* after the cells had ceased elongation and expansion, thus emphasising its role in cell wall fortification (Martín et al., 2008).

The trimming of RG-I side chains is an important pre-requisite for hydrolysis of both RG-I and homogalacturonan by rhamnogalacturonases and polygalacturonases, respectively (Mutter et al., 1998). The increased abundance of arabinan side chains in the mutant lines could hinder the activity of polygalacturonases that degrade HG and thereby generate elicitor active oligosaccharides. Figure 9 shows that *BXL4* is possibly linked to various proteins involved in the degradation of pectin based on STRING analysis (Szklarczyk et al., 2015b). A polygalacturonase (PGA4), three pectin lyase-like proteins AT3G59870, AT5G14650 and AT3G07850 and an alpha-L-arabinofuranosidase ASD1 are predicted functional partners of BXL4, which may act downstream, resulting in the generation of oligosaccharides required for defence responses. An overabundance of arabinans may result in increased molecular

entanglement on cell wall polysaccharides leading to reduced activity of glycosyl hydrolases acting downstream of BXL4. The synergistic activity of arabinofuranosidases and β-galactosidases to trim side chains of RG-I is an early step important for depolymerisation of pectin by polygalacturonases (Yoshioka et al., 1995).

In summary the possible contribution of BXL4 to plant defences is illustrated in the model below (Figure10).
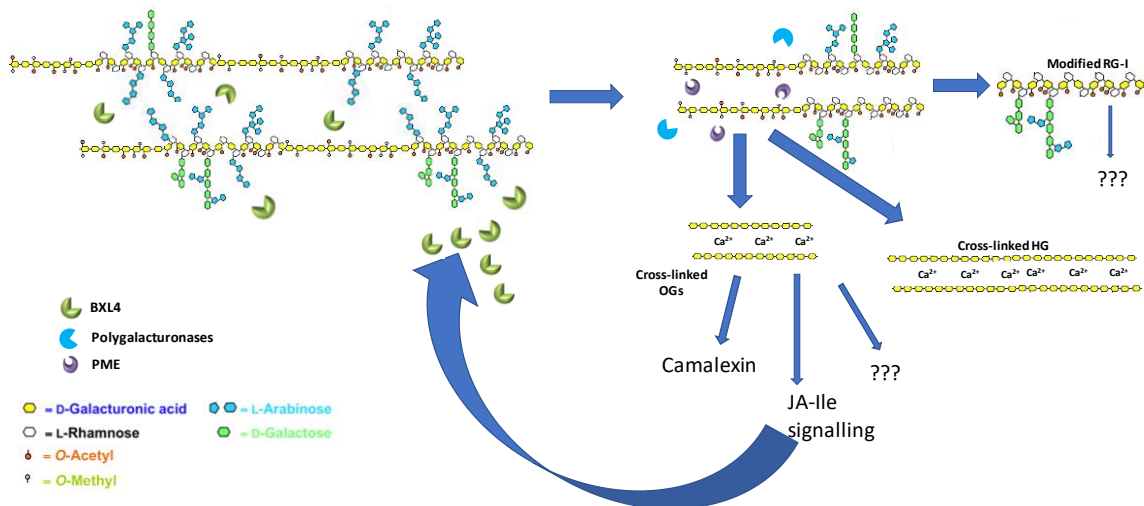
Figure 10: Predicted model showing role of BXL4 in plant defences. Plant cell wall damage due to wounding and pathogen attack triggers the induction of BXL4 in a JA-Ile dependant manner. BXL4 trims arabinans present in the apoplast (possibly RG-I side chain arabinans). The activity of BXL4 reduces the molecular entanglement in the apoplast and allows more efficient activity of PMEs which demethylesterify the homogalacturonan domains allowing for the cross-linkage of homogalacturonan with Ca^{2+}. Cross-linked homogalacturonan is rigid and limits the advancement of fungal hyphae. Polygalacturonase activity linked to BXL4 activity results in the generation of demethylesterified cross-linked oligogalacturonides, which elicit strong biological responses leading to the induction of JA-Ile and camalexin biosynthetic genes and the resultant accumulation of JA-Ile and camalexin. Ja-Ile signalling leads to the induction of JA related genes including BXL4 in a feedback loop, and consequently a stronger response reaction.

5 Conclusion

The remodelling of cell wall polysaccharides upon infection with various pathogens is still an underexplored field mainly due to the complexity associated with cell wall analytics. This project sought to contribute to the development of a robust and convenient method to use in analytics of cell wall derived from different sources. We managed to develop a GC-MS based method for analysing water extracted pectin from various tissue including mucilage, leaf cells and commercial pectin. Using our GC-MS method, we could determine the compositions of various pectin extracts to levels similar to the ones previously published. The method could detect the increased abundance of arabinose in the mucilage of *bxl1* mutant compared to wild type plants. The method offers several advantages including being able to detect all the pectic

sugar, both neutral sugars and galacturonic acid in one reaction cycle. The use of TFA employed in the protocol saves time compared to acid methanolysis. The method allows for the correct identification of monosaccharides, as it does not only rely on retention times but also uses MS-based detection.

Furthermore, we could characterise the enzyme activity of BXL4 through its expression in seed coat epidermal cells and analysis of cell walls of *bxl4* mutants. BXL4 is able to rescue the mucilage extrusion phenotype, which is altered in the *bxl1* mutant. We could observe a decrease in arabinose levels in the mucilage of *bxl1* mutant complemented with BXL4 to levels comparable to the wild type. BXL4 overexpression lines in Col-0 showed a decreased abundance of xylose compared to wild type thus confirming a bifunctional role of BXL4 as it acts as both an arabinosidase and xylosidase. The *bxl4* mutants also have a mild increase in arabinose levels, but the overall growth is not affected.

BXL4 was also shown to be a stress related gene, as both mechanical wounding of leaves and *B. cinerea* infection induced the upregulation of the gene. However, the gene is not upregulated after wounding of the JA-Ile deficient mutant *dde2-2,* whilst the *bxl4* mutants also do not have induction of JA-Ile marker genes upon mechanical wounding or *B. cinerea* infection. We therefore speculate that BXL4 is a JA-Ile dependant gene, which in a positive feedback induces the upregulation of JA-Ile marker gene, a phenomenon that occurs with JA-Ile biosynthetic genes. *B. cinerea* infection of the *bxl4* mutants results in a reduced accumulation of JA-Ile and camalexin in comparison to wild type plants. Infection of Arabidopsis plants with *B. cinerea* indicates a more susceptible phenotype in the *bxl4* mutants compared to wild type plants. We thus speculate that BXL4 is a stress induced gene that is regulated by JA-Ile signalling and is important for RG-I remodelling as a mitigatory response to pathogen infection. The pathogen defence pathway of BXL4 involves both JA-Ile signalling and camalexin production.

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