Plant species-specific recognition of long and short β-1,3linked glucans is mediated by different receptor systems

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SUMMARY

Plants survey their environment for the presence of potentially harmful or beneficial microbes. During colonization, cell surface receptors perceive microbe-derived or modified-self ligands and initiate appropriate responses. The recognition of fungal chitin oligomers and the subsequent activation of plant immunity are well described. In contrast, the mechanisms underlying β -glucan recognition and signaling activation remain largely unexplored. Here, we systematically tested immune responses towards different β -glucan structures and show that responses vary between plant species. While leaves of the monocots *Hordeum vulgare* and *Brachypodium distachyon* can recognize longer (laminarin) and shorter (laminarihexaose) β -1,3-glucans with responses of varying intensity, duration and timing, leaves of the dicot *Nicotiana benthamiana* activate immunity in response to long β -1,3-glucans, whereas *Arabidopsis thaliana* and *Capsella rubella* perceive short β -1,3-glucans. Hydrolysis of the β -1,6 side-branches of laminarin demonstrated that not the glycosidic decoration but rather the degree of polymerization plays a pivotal role in the recognition of long β -1,3-glucans in *N. benthamiana* and rice is independent of CERK1, indicating that β -glucan recognition may be mediated by multiple β -glucan receptor systems.

Keywords: glycan ligand, fungal cell wall, plant immunity, cell surface receptors, reactive oxygen species, calcium influx, mitogen-activated protein kinase, CERK1, BAK1.

INTRODUCTION

Plants establish intimate relationships with a broad range of microorganisms, most of which do not affect or are beneficial to their health. Some microbial invaders, however, are pathogens that pose a serious threat to plant survival. In order to identify beneficial and potentially harmful microbes, plants employ surface-localized receptor proteins. Such receptors recognize either modified-self or microbe-derived molecules (Cook *et al.*, 2015). Depending on the type of molecule, ligand recognition by corresponding receptors leads to a series of cellular events that either promote or restrict microbial colonization (Zipfel and Oldroyd, 2017).

The microbial cell wall plays an important role in the interaction with plants as it represents the site of first physical contact with plant host cells. One structural building block of fungal cell walls is chitin, a polymer consisting of β -1,4-linked *N*-acetylglucosamine monosaccharides (Latgé, 2007). In plants, recognition of chitin oligosaccharides results in the activation of immunity (Antolín-Llovera *et al.*, 2014; Sánchez-Vallet *et al.*, 2015). The first responses include an increase in cytosolic calcium (Ca²⁺) concentrations, the generation of extracellular reactive oxygen species (ROS), and the phosphorylation of intracellular receptor-like cytoplasmic kinases (Boller and Felix, 2009; Couto and Zipfel, 2016). The subsequent activation of calcium-dependent protein kinase and mitogen-activated protein kinase (MAPK) cascades ultimately result in gene expression changes to protect the plant from invasion by potential pathogens (Boller and Felix, 2009; Seybold *et al.*, 2014; Lee et al., 2015). Several lysin motif (LysM)-containing receptors involved in chitin recognition have been identified and functionally characterized (Antolín-Llovera et al., 2014; Sánchez-Vallet et al., 2015). Both in rice (Oryza sativa) and Arabidopsis thaliana, the LysM receptor kinase CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) plays a central role in the activation of immune signaling upon chitin recognition (Miya et al., 2007; Petutschnig et al., 2010; Shimizu et al., 2010; Liu et al., 2012b; Cao et al., 2014; Hayafune et al., 2014; Erwig et al., 2017). Additionally, CERK1 is required for the activation of immune signaling following the perception of bacterial peptidoglycan (Willmann et al., 2011; Liu et al., 2012a; Ao et al., 2014; Gust, 2015). Chitin derivatives such as lipochitooligosaccharides produced by rhizobacteria and arbuscular mycorrhizal fungi are similarly perceived by LysM receptors, and require close homologs of CERK1 to initiate signaling events leading to the establishment of beneficial symbiotic relationships (Oldroyd, 2013; Limpens et al., 2015). Interestingly, chitooligosaccharides and lipochitooligosaccharides together enhance symbiosis signaling while suppressing plant immunity, which is mediated by host receptors involved in the perception of both types of ligands during mycorrhizal colonization (Feng et al., 2019). In contrast to chitin and its derivatives, proteinaceous ligands are recognized by receptors with extracellular leucine-rich repeat (LRR) domains. Equivalent to LysM receptor proteins, LRR receptors form complexes with the co-receptor BRASSI-NOSTEROID INSENSITIVE 1 (BAK1/SERK3) to activate immune signaling (Monaghan and Zipfel, 2012; Macho and Zipfel, 2015; Couto and Zipfel, 2016). Thus, the recruitment of co-receptor kinases seems to be determined by the type of ligand-receptor pairs.

Despite the strong immune responses triggered by chitin, it only accounts for a small fraction of cell wall glycans in fungi and several comvcete genera including Saprolegnia and Aphanomyces (Mélida et al., 2013). Depending on the species of filamentous microbes, 50-60% of the cell wall's dry weight consists of glucan (Bowman and Free, 2006). Glucans are composed of glucose subunits that are assembled into polymers through various chemical linkages. By far the most abundant polymer is β-1,3-linked glucan (Fesel and Zuccaro, 2016). The linear β -1,3-glucan backbones are frequently modified with β -1,6linked glucose, forming branched polysaccharides that are unique to filamentous microbes. These polymers can be tightly associated to the cell wall or form a gelatinous and diffuse β -glucan matrix, as has recently been shown for the hyphae of some root associated-fungi during root colonization (Wawra et al., 2019). The most widely studied oomycete-derived heptaglucoside (a β -1,6-linked and β -1,3branched glucan) was shown to induce phytoalexin biosynthesis after partial purification in several legumes and in potato (Cline et al., 1978; Sharp et al., 1984b; Cosio

et al., 1996; Côté et al., 2000). Laminarin, a long-chain β -1,3-glucan backbone with side-branches of one or more glucose units linked via β -1,6 linkage from the marine brown algae Laminaria digitata, triggers defense responses in planta (Klarzynski et al., 2000; Aziz et al., 2003; Wawra et al., 2016). Similarly, the short linear β -1,3-glucan laminarihexaose induces changes in cytosolic Ca²⁺ levels, MAPK activation and defense-related gene expression in A. thaliana (Mélida et al., 2018). So far, only a single putative β -glucan receptor (GBP) with high affinity for the oomycete-derived heptaglucoside was identified from soybean (Glycine max) (Mithöfer et al., 1996, 2000; Umemoto et al., 1997; Fliegmann et al., 2004). Its role in β-glucan-triggered defense signaling remains unclear, and it has been proposed that GBP alone is not sufficient for the activation of β-glucan immunity (Fliegmann et al., 2004). This hypothesis is in line with the recent finding that laminarihexaoseinduced immune responses in A. thaliana are dependent on CERK1 (Mélida et al., 2018), indicating that CERK1 may additionally act as co-receptor of β -glucan receptor(s) in this plant.

To clarify the commonalities and differences in β -glucan perception between plant species, we systematically analyzed immune responses in monocot (Hordeum vulgare and Brachypodium distachyon) and dicot species (Nicotiana benthamiana and A. thaliana) upon treatment with short (laminarihexaose consisting of six glucose subunits) and long (laminarin with a backbone of 20-25 glucose subunits; Nelson and Lewis, 1974; Alderkamp et al., 2007) β-1.3-glucans. We show that the perception of the short and the long β -1,3-glucans differs between plant species, and that recognition of the long β-1,3-glucan is not determined by the presence of the β -1,6-glycosidic sidebranches but rather depends on the length of the β -1. 3-linked polymer in N. benthamiana. Moreover, in contrast to short non-branched B-1.3-glucan, immune responses triggered by long-chained β-1,3-glucan do not depend on CERK1 in N. benthamiana and rice. Our findings emphasize the diversity of β -glucan perception systems among plant species and their specialized role in glycan-triggered immunity.

RESULTS

Recognition of β-glucan structures is plant species-specific

In order to test whether different β -1,3-glucans activate early immune reactions in monocots, ROS production was measured upon addition of laminarihexaose and laminarin to leaf discs of 2- to 3-week-old barley (*H. vulgare*) or 4- to 5-week-old *B. distachyon*, when plants were at comparable developmental stages under our growth conditions. In these grasses both sugars triggered ROS production (Figure 1a,c). While laminarihexaose evoked a distinct ROS peak within the first 10 min, laminarin generally led to a

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Figure 1. β-Glucan-triggered immune responses in *Hordeum vulgare* and *Brachypodium distachyon*.

Leaf discs were collected from 2- to 3-week-old barley (*H. vulgare*) and 4- to 5-week-old *B. distachyon* plants. Production of reactive oxygen species (ROS) was measured using luminol-based chemiluminescence for 180 min after treatment of (a) barley and (c) *B. distachyon* leaf discs with 100 nm flg22, 4 mg ml⁻¹ (1 mM) laminarin and 250 μ M laminarihexaose. Water (mock) served as negative control. ROS response intensity was measured in relative luminescence units (RLU). The order of the graph legend is organized according to total ROS production (integral). Colored ticks on the *x*-axis indicate the timing of the first ROS peak for each treatment. Values represent means \pm SE of 16–24 leaf discs from four different plants. Experiments were performed at least three times with similar results. MAPK activation in (b) barley and (d) *B. distachyon* leaf discs was detected using phospho-p44/42 MAPK (ERK1/2) antibody following elicitor treatment at the indicated time points. Membranes were stained with PonceauS to confirm equal loading.

later but long-lasting phase of ROS production with up to three peaks. To further corroborate the activation of immune signaling by these glucans, we tested the activation of MAPKs, which represents a prominent molecular link between ligand perception and immune reprogramming (Meng and Zhang, 2013). In barley, treatment with the bacterial flagellin derivate flg22 and laminarin prompted stronger phosphorylation of MAPKs after 15 and 30 min (Figure 1b), compared with MAPK activation after the addition of laminarihexaose. Treatment with flg22 resulted in strong MAPK phosphorylation after 15 min in B. distachyon, whereas weaker but distinct MAPK signals were detected in response to both β -glucans (Figure 1d). To confirm the activation of downstream defense responses, we tested whether the expression of the defense-regulatory transcription factor genes HvWRKY1 and *HvWRKY2* would be activated by the different β -glucans in barley (Shen et al., 2007; Method S1; Table S1). Confirming previously published work, flg22 treatment resulted in a significant upregulation of the expression of both transcription factor genes at 1 h (Figure S1a). Similarly, HvWRKY1 and HvWRKY2 were strongly induced 1 h

after laminarin and laminarihexaose addition (Figure S1a). Together with the generation of ROS and the activation of MAPKs, the changes in barley defense-related gene expression show that both β -glucans display immunomodulatory activities in the tested monocots.

To monitor the activation of common B-glucan-triggered responses among dicots, a similar set of experiments was performed using leaf discs of 3- to 4-week-old A. thaliana and N. benthamiana. All assays were carried out in lines expressing the Ca²⁺ reporter aeguorin to test cytosolic Ca²⁺ influx upon β-glucan treatment in addition to ROS production and MAPK activation (Segonzac et al., 2011; Choi et al., 2014). In contrast to the tested monocots, N. benthamiana SLJR15 and A. thaliana Col-0^{AEQ} displayed substrate-specific perception of β -glucans (Figure 2). In N. benthamiana SLJR15 plants, treatment with the longchained, branched β-glucan laminarin resulted in the generation of extracellular ROS, an increase in cytosolic Ca²⁺, and MAPK activation (Figure 2a-c). Similarly, expression of the two defense-related genes NbACRE31 and NbWRKY4 (Kanzaki et al., 2003; Heese et al., 2007; Segonzac et al., 2011) was significantly induced 1 and 3 h after laminarin



Figure 2. β-Glucan-triggered immune responses in Arabidopsis thaliana and Nicotiana benthamiana. Leaf discs were collected from 3- to 4-week-old A. thaliana Col-0^{AEQ} and N. benthamiana SLJR15 plants. Production of reactive oxygen species (ROS) was mea-

treatment (Figure S1b). In contrast, the addition of shortchain laminarihexaose did not elicit early immune responses, and only had a weak and transient effect on defense gene expression (Figures 2a-c and S1b). In the aequorin-expressing A. thaliana accession Col-0, laminarihexaose but not laminarin triggered a weak but consistent cytosolic Ca^{2+} influx and production of ROS (Figure 2d,e). Despite these responses, no activation of MAPKs was observed under the conditions tested (Figure 2f). This is in contrast to previously published work where the perception of laminarihexaose prompted Ca²⁺ influx and MAPK phosphorylation in A. thaliana Col-0 seedlings (Mélida et al., 2018). To test whether these apparently contradicting observations were due to the different plant tissues tested, we repeated our experiments using A. thaliana Col-0^{AEQ} seedlings. Indeed, addition of laminarihexaose to

seedlings resulted in MAPK phosphorylation after 15 min (Figure S2), indicating that the different immune responses of A. thaliana to laminarihexaose could be age- and/or tissue-dependent. As for leaf discs from 3- to 4-week-old plants, we did not observe ROS production or elevations of cytosolic Ca²⁺ in A. thaliana Col-0^{AEQ} seedlings upon laminarin treatment despite the high elicitor concentrations used. This is in contrast to the laminarin-responsive species barley and *N. benthamiana* where ROS generation was concentration dependent (Figure S3). It has previously been reported that different batches of laminarin exhibit considerable variability in activity (Smith et al., 2018). Therefore, we have tested various batches of laminarin, but have not observed differences in responsiveness. Additionally, the characterization of two laminarin batches used in this study by high-performance liquid chromatography

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did not show differences in glucan composition (Figure S4; Method S2), suggesting that laminarin is, if at all, only weakly immunogenic in *A. thaliana* Col-0. To further investigate whether the lack of laminarin-triggered immune response activation in *A. thaliana* Col-0 is a conserved trait in *Brassicacea*, we additionally tested ROS production in leaf discs of *Capsella rubella*, a phylogenetically close relative of *A. thaliana* (Koch *et al.*, 1999). Similar to *A. thaliana* Col-0^{*AEQ}, <i>C. rubella* responded with a ROS burst to laminarinexaose but not to laminarin (Figure S5), suggesting that the inability to mount a ROS response after addition of long-chain β -1,3-glucan may represent a common feature among *Brassicacea*.</sup>

Presence of $\beta\mbox{-1,6-linkages}$ does not contribute to recognition of laminarin

Laminarihexaose and laminarin consist of glucose monomers, but display different degrees of polymerization (DP) and branching (DB). While laminarihexaose is a defined linear hexamer of glucose subunits solely linked by β -1,3linkages, laminarin is composed of a mix of β-1,3-linked glucose polymers of varying length that additionally contain β -1,6-glycosidic side-branches. To clarify if the β -1,6 side-chains of laminarin are required for recognition, we produced a laminarin substrate that was debranched with the enzyme *Fb*GH30 from the bacterium *Formosa* sp. strain B and purified by size exclusion chromatography (SEC; Becker et al., 2017; Unfried et al., 2018; Wawra et al., 2019). *Fb*GH30 specifically removes the β -1,6-linked glucose sidechains of laminarin producing long, linear β-1,3-glucan polymers. The specific release of β -1,6-linked glucose from laminarin following hydrolysis with FbGH30 could be visualized on thin-layer chromatography (TLC; Figure S6a). Additionally, the absence of β -1,6 side-chains after hydrolysis was confirmed by microscale thermophoresis showing the lack of binding between debranched laminarin and the lectin FGB1 (Wawra et al., 2019). This lectin specifically and efficiently binds to B-1.6-linked alucose attached to linear β -1,3-glucan (Wawra *et al.*, 2016). We then tested the immunogenic activity of laminarin and debranched laminarin in ROS production assays. In N. benthamiana SLJR15, both substrates elicited similar ROS responses (Figure 3a). Comparable results were obtained in N. benthamiana SLJR15 (Figure 3b) and H. vulgare (Figure S6b) using debranched laminarin that was not purified by SEC, indicating that not the β -1,6-glycosidic side-branches but rather the polymer length is essential for β -glucan recognition in these plant species. To test this hypothesis, we treated laminarin with a mixture of commercially available β-1,3 exo- and endoglucanases, which resulted in the complete hydrolysis of long β-glucan chains yielding mainly laminaribiose and glucose (Figures 3c and S4). This hydrolyzed laminarin was then tested for its activity in N. benthamiana SLJR15 plants. Unlike debranched

laminarin, hydrolyzed laminarin did not elicit ROS production in *N. benthamiana* SLJR15 (Figure 3d). This demonstrates that *N. benthamiana* is not able to mount early immune responses upon treatment with short β -glucan fragments with a DP smaller than six, but requires longer β -1,3-linked glucan chains for recognition (Figure 3). In contrast, the ability of *H. vulgare* to respond to laminarihexaose led us to speculate that hydrolyzed laminarin would still be able to elicit ROS responses in this plant species. We therefore incubated *H. vulgare* leaf discs with laminarin, hydrolyzed laminarin and various short β -glucan fragments with a DP smaller than six (Figure S6c,d). While *H. vulgare* could respond to the shorter fragments as well as the hydrolyzed laminarin, the level of ROS production was clearly decreased compared with untreated laminarin.

Long-chain $\beta\mbox{-glucan}$ recognition is not dependent on CERK1 or BAK1

It was recently shown that CERK1 plays a crucial role in the recognition of laminarihexaose in A. thaliana Col-0. In contrast, mutation of AtBAK1 did not alter laminarihexaose perception (Mélida et al., 2018). However, whether CERK1 is a general component involved in the recognition of different β-glucans and β-glucan-triggered immunity across plant species remains unanswered. To address this guestion, we silenced the homologs of AtCERK1 and AtBAK1 in N. benthamiana by tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS; Figure 4a; Liu et al., 2002; Senthil-Kumar and Mysore, 2014). Plants treated with TRV:NbCERK1 displayed significantly reduced NbCERK1 mRNA levels, whereas those of NbBAK1 were not affected (Figure S7; Gimenez-Ibanez et al., 2009). Similarly, by using primers targeting the two homologs of NbBAK1 (NbSERK3a and NbSERK3b) present in the N. benthamiana genome, we confirmed that the TRV construct for NbBAK1 efficiently silenced both homologs but not NbCERK1 (Figure S7). The same construct was previously shown to not target the closely related NbSERK2 highlighting construct specificity (Heese et al., 2007; Chaparro-Garcia et al., 2011). Plants treated with silencing constructs against the green fluorescent protein gene (GFP) from Aequorea victoria (TRV:GFP) were used as negative controls (Saur et al., 2016). As shown previously, plants silenced for NbCERK1 and NbBAK1 but not those silenced for GFP showed reduced ROS production and Ca²⁺ influx upon addition of chitohexaose and flg22 in N. benthamiana SLJR15, respectively (Figures 4 and 5; Heese et al., 2007; Gimenez-Ibanez et al., 2009; Segonzac et al., 2011). However, *NbBAK1* silencing did not affect immune activation following laminarin treatment, confirming that BAK1 is not required for β-glucan-triggered immunity (Figures 4c,g and 5b,f). Similarly, silencing of NbCERK1 did not result in altered laminarin-triggered immunity (Figures 4b,g and 5a,f), suggesting the presence of a CERK1-independent perception pathway for long-chain β -glucans in *N. benthamiana*.

To test whether the perception of long-chain β -glucans is similarly independent of CERK1 in monocots, we made use of two independent rice lines in which the first exon of *OsCERK1* was replaced with a hygromycin resistance cassette resulting in the disruption of the *OsCERK1* gene (#53-KO and #117-KO; Kouzai *et al.*, 2014). These mutants were previously shown to have lost their ability to perceive

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chitooligomers and to display reduced responsiveness to peptidoglycan. Segregated wild-type plants (#53-Rev and #117-Rev) were used as respective controls. As expected, *oscerk1* lines were still responsive to flg22, while ROS production following chitohexaose treatment was completely abolished in the KO mutant lines (Figure S8). In contrast, laminarin-triggered ROS production was not affected in



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Figure 3. Reactive oxygen species (ROS) production is dependent on the length of the β -glucan backbone but not on the β -1,6 side-branches of laminarin in *Nicotiana benthamiana*.

Leaf discs were collected from 3- to 4-week-old N. benthamiana SLJR15.

(a) Laminarin was debranched using the β -1,6-exoglucanase *Fb*GH30 (Becker et al., 2017) and purified via size exclusion chromatography (SEC; debranched laminarin). Laminarin without glucanase treatment was used as positive control. Production of ROS was measured using luminol-based chemiluminescence for 60 min after treatment with 4 mg ml⁻¹ laminarin or 4 mg ml⁻¹ debranched laminarin. Water (mock) served as negative control. ROS response intensity was measured in relative luminescence units (RLU).

(b) Integration of ROS kinetics (total ROS production) following elicitor treatment. Significant differences between treatments were determined with a non-parametric Kruskal–Wallis analysis (P < 0.05) followed by Dunn's *post hoc* test. The values shown are from two independent experiments, in which either purified debranched laminarin (squares) or crude debranched laminarin (circles) was used. As positive control treatments, laminarin solutions were treated like their respective debranched counterparts.

(c, d) Laminarin was completely hydrolyzed using an enzyme mix consisting of 0.1 mg ml⁻¹ endo- β -1,3-glucanase from *Helix pomatia* (Sigma), 0.1 mg ml⁻¹ exo- β -1,3-glucanase from *Hordeum vulgare* (Megazyme). The complete digest (hydrolyzed laminarin) was analysed by thin-layer chromatography (TLC) (c) using glucose, laminaribiose, gentiobiose, laminaritriose, laminaripentaose and laminarin (each 2 mg ml⁻¹) as standards. ROS assays (d) were performed on leaf discs of *N. benthamiana* SLJR15 with treated (T) and untreated (UT) laminarin (2 mg ml⁻¹, 0.5 mm) as well as glucose, laminaribiose, laminarithexaose (each 250 μ M). Water and the enzyme mix alone were used as controls. Values represent means \pm SE of 8–16 leaf discs from four different plants. Experiments were performed three times with similar results.

oscerk1 lines, confirming that the activation of immune responses upon laminarin perception does not require CERK1.

DISCUSSION

Cell walls of filamentous microbes mainly consist of chitin and β -glucans (Latgé, 2007). During plant host colonization these polysaccharides are targeted by plant hydrolases that release soluble fragments and interfere with cell wall integrity (Kombrink *et al.*, 2011; Sánchez-Vallet *et al.*, 2015; Fesel and Zuccaro, 2016). Recognition of chitooligomers results in the activation of plant immune responses, including ROS production, accumulation of cytosolic Ca²⁺, MAPK activation and changes in gene expression (Antolín-Llovera *et al.*, 2014; Sánchez-Vallet *et al.*, 2015; Rovenich *et al.*, 2016). Similarly, β -glucans are well-characterized elicitors of immunity in animals (Romani, 2011). Moreover, recent evidence indicates that β -glucans also play an important role during interactions between plants and filamentous microbes (Wawra *et al.*, 2016; Mélida *et al.*, 2018).

Using mycelial cell wall fractions of the fungal phytopathogen Plectosphaerella cucumerina, Mélida et al. (2018) have recently identified the linear β -1,3-glucan hexamer laminarihexaose as major P. cucumerina cell wall component and elicitor of immunity in seedlings of A. thaliana Col-0. Here we show that laminarihexaose also activates immunity in the form of ROS production and MAPK activation in leaf tissue of the monocots barley and B. distachyon (Figure 1). However, the leaves of N. benthamiana did not mount immune responses upon treatment with laminarihexaose (Figure 2a-c), suggesting that this short β-1,3-glucan hexamer is not a universal elicitor of immunity in plants. Depending on the species, plant cell walls may also contain β-1,3-linked glucan. Grasses, including barley, rice, sorghum (Sorghum bicolor) and wheat (Triticum aestivum), as well as the herbaceous perennial Equisetum arvense, harbor unbranched glucose polysaccharides with alternating β -1,3 and β -1,4 linkages

that are absent from dicots (Trethewey et al., 2005; Burton et al., 2006; Sørensen et al., 2008). In contrast, N. alata cell walls were found to contain large amounts of β -1,3-linked glucan polysaccharides (Rae et al., 1985). Moreover, plants may form papillae that consist of β -1,3-glucan-rich callose to reinforce their cell walls at sites of filamentous microbe penetrations (Hückelhoven, 2007; Albersheim et al., 2011; Chowdhury et al., 2014; Hückelhoven, 2014). Thus, like fungal-derived β -1,3-glucan structures, short β -1,3-glucans could be released from plant cell walls during growth, physical injury, and through the activity of plant or microbial hydrolytic enzymes during colonization by filamentous microbes. It has previously been shown that genomes of plant-associated fungal endophytes, as well as hemibiotrophic and necrotrophic pathogens, are enriched for genes encoding cell-wall-degrading enzymes required for host cell invasion (Lahrmann et al., 2015). Because plantderived β-1,3-glucan fragments would be virtually indistinguishable from β -1,3-glucans of microbial origin, these molecules defy the classical categorization into self and non-self. This is in contrast to oligogalacturonides and cellobiose, which are almost solely derived from plant-specific pectin and cellulose carbohydrates, respectively, and activate plant immune responses (Kohorn et al., 2009; Brutus et al., 2010; de Azevedo Souza et al., 2017).

In contrast to β -1,3-linked glucans, β -1,6-glucosidic linkages do not occur in plants but are present in cell walls of filamentous microbes (Bowman and Free, 2006; Latgé, 2007). To date, β -1,6-glucan backbones have been reported in cell walls of several yeasts, lichenized fungi, and are common among oomycetes (Pereyra *et al.*, 2003; Carbonero *et al.*, 2006; Latgé and Calderone, 2006). Oomycetes, like diatoms and brown algae, from which laminarin is isolated, belong to the phylum Heterokonta. This evolutionary relatedness explains the similarity in β -1,6-linkagecontaining glucan structures between these classes. More recently, β -1,6-glucosidic linkages were shown to frequently occur in the extrahyphal matrix surrounding the





Figure 4. Virus-induced gene silencing (VIGS) of *NbCERK1* and *NbBAK1* in *Nicotiana benthamiana* does not reduce β-glucan-triggered reactive oxygen species (ROS) accumulation.

Young, fully-developed leaves of 2-week-old *N. benthamiana* SLJR15 (*n* = 5–8) were infiltrated with *Agrobacterium tumefaciens* suspensions for tobacco rattle virus (TRV)-based silencing of *NbCERK1* or *NbBAK1*. TRV constructs targeting *GFP* were used as negative control.

(a) Phenotypes of TRV-treated plants ~3.5 weeks following infiltration. Green marker: 6 cm.

(b, c) Generation of ROS was measured using luminol-based chemiluminescence for 60 min after treatment with 100 nm flg22, 10 μ m chitohexaose or 2 mg ml⁻¹ (0.5 mm) laminarin of TRV:*NbCERK1*- (TRV:*CERK1*) (b) or TRV:*NbBAK1*-treated (TRV:*BAK1*) (c) plants. Water (mock) served as negative control. Values represent means \pm SE of 24–32 leaf discs. Results are representative of four biological replicate experiments. ROS response intensity was measured in relative luminescence units (RLU).

(d–g) Integrals of ROS kinetics following elicitor treatment were normalized to TRV:*GFP* control (total ROS production). Significant differences in ROS production between *GFP-*, *NbCERK1-* and *NbBAK1-*silenced plants are depicted with letters (*P* < 0.05, Kruskal–Wallis analysis with Dunn's *post hoc* test). Each independent experiment is represented by a different shape.

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Figure 5. Glucan-triggered cytosolic Ca^{2+} fluxes are not affected by virus-induced gene silencing (VIGS) of *NbCERK1* and *NbBAK1*. Young, fully-developed leaves of 2-week-old *Nicotiana benthamiana* SLJR15 (n = 5-8) were infiltrated with *Agrobacterium tumefaciens* suspensions for tobacco rattle virus (TRV)-based silencing of *NbCERK1* or *NbBAK1*.

(a, b) TRV constructs targeting *GFP* were used as negative control. Elevations of cytosolic calcium concentrations (Ca^{2+} influx) were measured as relative luminescence units (RLU) following treatment with 100 nm flg22, 10 μ m chitohexaose or 2 mg ml⁻¹ (0.5 mm) laminarin of TRV:*NbCERK1*- (TRV:*CERK1*) (a) or TRV: *NbBAK1*-treated (TRV:*BAK1*) (b) plants. Water (mock) served as negative control. Remaining aequorin was discharged by adding CaCl₂. Discharge kinetics were integrated and normalized to the maximum Ca²⁺ level. The discharge integral was then used to normalize Ca²⁺ kinetics in response to elicitor treatment. Values represent means \pm SE of 24–32 leaf discs.

(c-f) Integrals of Ca^{2+} kinetics following elicitor treatment (total Ca^{2+} influx) were normalized to TRV:*GFP* control. Significant differences in Ca^{2+} influx between *GFP-*, *NbCERK1-* and *NbBAK1-*silenced plants are depicted with letters (P < 0.05, Kruskal–Wallis analysis with Dunn's *post hoc* test). Each independent experiment is represented by a different shape.

cells of plant-associated fungi during host colonization (Wawra *et al.*, 2019). The best-studied β -1,6-glucan in this context is a heptaglucoside from cell walls of pathogenic oomycetes (Ayers *et al.*, 1976; Ebel *et al.*, 1976; Albersheim and Valent, 1978; Sharp *et al.*, 1984b). This oomycete-derived heptaglucoside consists of a β -1,6-linked glucose pentamer backbone with two β -1,3-linked glucosyl branches (Sharp *et al.*, 1984a). It triggers phytoalexin production in several leguminous plant species and in potato (Cline *et al.*, 1978; Sharp *et al.*, 1984b; Cosio *et al.*, 1996;

Côté *et al.*, 2000), but is not active in tobacco and rice cell suspensions (Klarzynski *et al.*, 2000; Yamaguchi *et al.*, 2000). Instead, tobacco cells respond to the long linear β -1,3-glucan laminarin with β -1,6 side-branches, which elicits a wide range of defense responses, including medium alkalinization, ROS production, salicylic acid accumulation and defense gene expression (Klarzynski *et al.*, 2000; Ménard *et al.*, 2004). Similar responses to laminarin were reported for grapevine (Aziz *et al.*, 2003). In addition to ROS production, we could show that laminarin triggers

MAPK activation in barley and B. distachyon leaf tissue (Figure 1). Interestingly, the kinetics of laminarin-triggered ROS production in B. distachyon and barley differed greatly from the fast and short immune responses to chitoand laminarihexaose. As has been observed upon activation of the LRR receptor kinase flagellin sensing 2 (FLS2) and the LRR receptor protein Cf4 (Robatzek et al., 2006; Salomon and Robatzek, 2006; Beck et al., 2012; Spallek et al., 2013; Postma et al., 2016), laminarin binding could result in the internalization and degradation of its corresponding receptor (complex). The resulting depletion of such a receptor at the cell surface would then cause a temporary inability of that plant to respond to laminarin causing a drop in ROS production. The replenishment of the relevant receptor at the plasma membrane would then allow the renewed recognition of remaining elicitor molecules. Conversely, considering the constitutive presence of extracellular plant glucanases (Wawra et al., 2016), the long-lasting accumulation of peaks could be caused by the gradual hydrolysis of β -glucans resulting in the production of a laminarin-derived β -glucan elicitor structure. This hypothesis should be tested following the identification of glucanases responsible for laminarin hydrolysis in barley or B. distachyon.

In N. benthamiana, laminarin treatment triggered ROS production, MAPK activation, increased intracellular Ca²⁺ concentrations and defense gene expression (Figures 2a-c and S1b). The consistent activation of immune responses following laminarin but not laminarihexaose treatment in *N. benthamiana* led us to investigate whether the β -1,6glucosidic branches in laminarin were required for its immunoactivity. Leaf discs treated with enzymatically debranched laminarin produced ROS similar to laminarin-treated controls (Figure 3a,b), suggesting that, similar to Nicotiana tabacum (Klarzynski et al., 2000), β-1,6-glucan linkages are dispensable for the recognition of long β -1.3glucan in N. benthamiana. Similar results were obtained after the addition of debranched laminarin to barley leaf discs (Figure S6). In contrast to N. tabacum and barley, however, the recognition of β -1,3-glucan appears to be determined by the DP in N. benthamiana as treatment with β -1,3-glucan fragments with a DP smaller than six did not result in the activation of early immune responses (Figure 2a-c), and laminarihexaose elicited only a weak and transient upregulation of defense-related genes (Figure S1b). Additionally, laminarin hydrolysis by β-1,3-glucanases yielded short β-glucan fragments and glucose, and abolished ROS production (Figure 3c,d). Moreover, long linear β -1,3-glucan was shown to assume a triple helical structure (Chuah et al., 1983; Kulicke et al., 1997; Young et al., 2000; Okobira et al., 2008), which is required for β -1,3-glucan recognition in insects (Mishima et al., 2009; Takahasi et al., 2009; Kanagawa et al., 2011). This triple helical quaternary structure is also crucial for effective binding by laminarin-degrading enzymes (GH81) from bacteria (Pluvinage *et al.*, 2017). Whether the assembly of long β -1,3-glucans into a triple helix plays a similar role in the activation of plant immunity remains to be investigated.

Unlike other plant species tested here, A. thaliana Col-0 leaves and seedlings did not respond to laminarin (Figures 2d-f and S2). In contrast, previous work by Mélida et al. (2018) showed that seedlings of A. thaliana Col-0 respond with a weak increase in cytosolic Ca²⁺ upon laminarin treatment. In general, the activity of laminarin, if any, appears to be weak and inconsistent in A. thaliana Col-0. It is likely that variations in growth conditions, elicitor purity, DP and resulting guaternary structures in the different laminarin batches contributed to these observations. This is in accordance with a previous report that laminarin batches from various commercial sources contained 'impurities', resulting in variations of their immunomodulatory activity in animal systems (Smith et al., 2018). These variations may also explain our finding that C. rubella does not produce ROS upon laminarin treatment (Figure S5). However, we have tested several laminarin batches in A. thaliana Col-0 and did not observe differences in responsiveness. Additionally, high-performance anion exchange chromatography-based analysis of two of these batches showed no variations in β -glucan composition (Figure S4), supporting the conclusion that laminarin is a poor elicitor of immunity in Brassicaceae at least for those batches. In contrast, laminarihexaose triggered weak but consistent ROS production and cytosolic Ca²⁺ influx in A. thaliana Col-0 leaves (Figure 2d,e), while no MAPK activation was detectable. MAPK activation and ROS production have been shown to be two independent signaling events in plant immunity (Ranf et al., 2011; Segonzac et al., 2011; Xu et al., 2014; de Azevedo Souza et al., 2017), which is in agreement with our findings. Interestingly, laminarihexaose-triggered MAPK activation was visible in seedlings (Figure S2c; Mélida et al., 2018), suggesting that the different immune responses of A. thaliana Col-0 to laminarihexaose may be age- and/or tissue-dependent.

These findings show that the structural complexity of β -glucans contributes to their differential recognition by plant species. In addition to the large number of variants in side-chain decorations and linkage patterns within β -glucan backbones, chemical modifications can influence β -glucan recognition in plants (Ménard *et al.*, 2004; Gauthier *et al.*, 2014). To solve the question whether these specific responses are the result of adaptation processes to particular microbial partners or microbe-dependent plant cell wall modifications, chemical synthesis of defined β -glucan structures as test substrates would be of high interest (Weishaupt *et al.*, 2017).

In conclusion, the differential recognition of β -glucan structures suggests the existence of multiple receptor molecules with varying ligand specificities. To date, the

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only known putative β -glucan receptor is the β -glucan binding protein GBP of soybean, which displays high affinity for the oomycete-derived heptaglucoside (Yoshikawa et al., 1983; Mithöfer et al., 1996; Umemoto et al., 1997; Mithöfer et al., 2000; Fliegmann et al., 2004). Unlike other receptor proteins, GBP contains an enzymatically active endo-B-1,3-glucanase domain in addition to a carbohydrate-binding site (Fliegmann et al., 2004). Despite the lack of a canonical signal peptide sequence, GBP was found to localize to the apoplast as well as to the cell surface. It was, therefore, suggested that GBP represents a multicomponent receptor molecule that retains the ability to hydrolyze complex β -glucan in microbial cell walls. If and how GBP interacts with co-receptor proteins and activates immune signaling remains to be answered. In most cases, ligand binding to a receptor results in the formation of large receptor complexes that are required for the activation of downstream signaling (Couto and Zipfel, 2016). BAK1 and other members of the SERK protein family generally associate with LRR-type receptor proteins upon recognition of proteinaceous ligands, thereby mediating important processes in growth, development and immunity (Ma et al., 2016). There is currently no evidence that BAK1 is involved in the perception of carbohydrate-based ligands, which is in line with our data (Figures 4 and 5). However, based on the experiments conducted here, we cannot exclude that other SERK proteins may be required for β -glucan-triggered signaling. Recognition of chitin and chitin-derived molecules is mediated by CERK1 in A. thaliana and rice (Sánchez-Vallet et al., 2015). Similarly, laminarihexaose-triggered immune responses are mediated by CERK1 in A. thaliana Col-0 (Mélida et al., 2018). In contrast, our findings demonstrate that the recognition of long β -1.3-glucan leads to the activation of immunity via a CERK1-independent pathway in *N. benthamiana* and rice (Figures 4, 5 and S8), suggesting that there may be different receptor systems that mediate β -1,3-glucan recognition in plants.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana and Capsella rubella. All assays were performed with seeds of aequorin-expressing *A. thaliana* accessions Col-0 (Choi *et al.*, 2014) or wild-type *C. rubella* Monte Gargano. For seedling assays, seeds were surface sterilized as described previously (Nizam *et al.*, 2019). Sterile seeds were sown on ½ Murashige & Skoog (MS) medium (pH 5.7) supplemented with 0.5% sucrose and 0.4% Gelrite (Duchefa, Haarlem, the Netherlands) and stratified for 3 days. Plates were transferred to climate chambers with an 8/16 h light/dark regime (light intensity of 110 µmol m⁻² sec⁻¹) at 22/18°C. Five-day-old seedlings were transferred to fresh ½ MS agar plates or liquid medium and grown for seven additional days under the same conditions. For leaf disc assays, seeds were sterilized as before and sown on soil. Pots

were kept at 4° C for 3 days. Plants were then grown in climate chambers under the conditions described above for 3–4 weeks.

Nicotiana benthamiana. Seeds of N. benthamiana aequorinexpressing SLJR15 (Segonzac *et al.*, 2011) lines were sown on soil and grown in the greenhouse under long-day conditions (day/ night cycle of 16/8 h, 22–25°C, light intensity of ~140 µmol m⁻² sec⁻¹, maximal humidity of 60%). Plants were ~3.5 weeks old when leaf discs were collected for immunity assays.

Hordeum vulgare and *Brachypodium distachyon*. Seeds of *H. vulgare* cv. Golden Promise were sown on soil and grown in a climate chamber with a day/night cycle of 16/8 h at 22/18°C, 60% humidity and a light intensity of 108 μ mol m⁻² sec⁻¹ for 2–3 weeks. Seeds of *B. distachyon* Bd21-3 were stratified on soil for 5 days. Pots were then transferred to a climate chamber and *B. distachyon* plants were cultivated for 4–5 weeks under the same conditions as *H. vulgare*.

Oryza sativa. Seeds of *Oryza sativa* (rice) L. *japonica* cv. Nipponbare Kanto BL no. 2 control (#53-Rev and #117-Rev) and *oscerk1* KO (#53-KO and #117-KO) lines were previously characterized (Kouzai *et al.*, 2014). Seeds were surface sterilized with 3.5% bleach for 30 min, extensively washed with sterile water and germinated in jars containing water with 4 g L⁻¹ gelrite (Duchefa, Haarlem, the Netherlands). Plants were cultivated in a climate chamber (31°C, 70% humidity) before being transferred to pots with pure sand. After an acclimatization period of 2 weeks, plants were transferred onto a soil–sand–mix (8:1) and grown for a further 2–3 weeks before being used for ROS burst assays.

Elicitors

Laminarin was purchased from Sigma-Aldrich (Taufkirchen, Germany), flg22 from GenScript (Piscataway, NJ, USA), and laminarihexaose, laminaritriose, laminaribiose and chitohexaose from Megazyme (Bray, Ireland). All elicitors were dissolved in autoclaved MilliQ-water and used at indicated concentrations without additional treatment.

Calcium influx assay

Leaf discs or seedlings were placed into white 96-well plates filled with 75 μl of distilled water. To each well, 25 μl of a 40 µM coelenterazine (Roth, Karlsruhe, Germany) solution (in 100% methanol) was added, and plates were kept in the dark at room temperature overnight. Luminescence measurements were carried out using a multiwell plate reader (TECAN SPARK 10M). Following 2 min baseline measurements, 50 µl elicitor solutions [sterile water, 250 µM laminarihexaose, 100 nM flg22 or 2-4 mg ml⁻¹ laminarin (0.5-1 mM) final concentrations] were added to wells and luminescence was measured for 30 min. Remaining aequorin was discharged for 1 min with 100 µl of 2.5 M CaCl₂ (in 25% ethanol). All measurements were performed with an integration time of 300 msec. To calculate the increase of intracellular calcium concentrations, discharge curves of each well were integrated. Integrated values were normalized to the maximal discharge integral upon all wells and treatments. The values for the baseline measurement and the measurement after elicitor treatment were then normalized according to the discharge integral.

Oxidative burst assay

Oxidative burst measurements were performed on leaf discs or seedlings. Plant material was placed into white 96-well plates filled with 100 μ l of sterile distilled water. Following overnight incubation at room temperature on the bench, water was replaced with 50 μ l of fresh water containing 20 μ g ml⁻¹ horseradish peroxidase (Sigma-Aldrich) and 20 μ M L-012 (Wako Chemicals, Neuss, Germany). Following ~20 min incubation at room temperature in the dark, 50 μ l elicitor solutions [final concentrations: 250 μ M laminarihexaose, 100 nM flg22, 2–4 mg ml⁻¹ laminarin (0.5–1 mM), 10 μ M chitohexaose] or water were added. Measurements were started immediately and taken at 1 min intervals with an integration time of 300 msec using a TECAN SPARK 10M.

MAPK assay

Twenty leaf discs (3 mm) or 15 seedlings for each treatment were floated in water overnight. The plant material was treated with water as control, 250 µM laminarihexaose, 100 nM flg22 or 2-4 mg ml⁻¹ (0.5-1 mm) laminarin. Samples were collected after 0, 15 and 30 min, and snap-frozen in liquid nitrogen. Samples were ground using a tissue lyzer (Tissuelyzer II, Qiagen) and metal beads. Homogenized samples were suspended in 150 μI MAPK extraction buffer (Mine et al., 2017). Samples were vortexed and pelleted at 13 000 rpm and 4°C for 10 min. Small aliquots of supernatants were used to determine total protein concentrations using Bradford reagent (Sigma-Aldrich) according to manufacturer's instructions. Remaining supernatants were diluted with 6 \times sodium dodecyl sulfate buffer and boiled at 95°C for 5 min. Each sample (15 µg total protein) was separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes with fast Western buffer (48 mm Tris, 20 mm HEPES, 1 mm EDTA, 1.3 mm DMF). Immunoblot analysis was performed with 1:2000 anti-phospho-p44/42 (ERK1/2) MAPK (Cell Signaling Technology, Danvers, USA) as primary antibody and 1:2000 HRP-conjugated anti-rabbit-lgG (Sigma-Aldrich) as secondary antibody. Bands were detected using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific).

Virus-induced gene silencing

Virus-induced gene silencing of *NbCERK1* and *NbBAK1* in *N. benthamiana* SLJR15 was performed (Liu *et al.*, 2002; Senthil-Kumar and Mysore, 2014) using previously generated pTRV2 vectors targeting *NbCERK1* and *NbBAK1* as well as the green fluorescent protein gene (*GFP*; Heese *et al.*, 2007; Gimenez-Ibanez *et al.*, 2009; Chaparro-Garcia *et al.*, 2011; Saur *et al.*, 2016). Cultures of *Agrobacterium tumefaciens* strain GV3101 containing either pTRV1 or pTRV2 constructs were pre-cultured overnight at 28°C and 200 rpm. Bacteria were then pelleted, washed and diluted to an OD₆₀₀ = 1 with MMA buffer (10 mM MgCl₂, 10 mM MES pH 5.7, 200 μ M acetosyringone). After 3 h of incubation in the dark, bacterial suspensions with pTRV1 and pTRV2 constructs were mixed at a 1:1 ratio and infiltrated into leaves of 2-week-old *N. benthamiana* SLJR15 plants (four-leaf stage) using a 1-ml needleless syringe. Phenotypes were assessed 27 days after infiltration.

Enzymatic treatments of laminarin

The β -1,6 side-chains of laminarin were removed by hydrolysis with the β -1,6-exoglucosidase *Fb*GH30 essentially as described previously (Wawra *et al.*, 2019). To generate substrates for ROS assays, 4 mg laminarin (in water) was digested using 4 μ M *Fb*GH30 overnight (16 h, 37°C).

For the complete hydrolysis of laminarin, 20 mg ml⁻¹ laminarin (in water) was treated with a glucanase mix consisting of an endo- β -1,3-glucanase from *Helix pomatia* (Sigma, Taufkirchen, Germany), an exo- β -1,3-glucanase from *Trichoderma virens* (Megazyme, Bray, Ireland) and an endo- β -1,3-glucanase from *H. vulgare*: (Megazyme, Bray, Ireland) (each 0.1 mg ml⁻¹). After overnight incubation (16 h) at 37°C, the enzymatic reaction was stopped by boiling at 95°C for 15 min. Precipitates were removed by centrifugation at 17 000 **g** for 20 min. The supernatant was used for TLC analysis and ROS assays.

Thin-layer chromatography

A glass chamber was equilibrated with 8:4:1:1:1 ethyl acetate/ acetic acid/methanol/formic acid/water. Twice, three spots of 0.4 µl per sample were spotted on a line 2 cm above the lower edge of a plate of silica gel 60 F254 (Merck-Millipore, Darmstadt, Germany). After drying, the plate was placed into the equilibrated glass chamber. The solvent reached the top of the plate after approximately 2 h. Plates were dried under the fume hood and then sprayed with developer solution [82.6% ethanol (v/v), 10.7% H₂SO₄ (v/v), 7.6% water, 1 mg ml⁻¹ 1-naphthol]. To visualize carbohydrate fragments, plates were developed in an oven at 99.9°C for 5–10 min.

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CONFLICTS OF INTEREST

All authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

AW, HR, JHH, SW and AZ conceived and planned experiments. AW, HR, FS, SV and SB carried out experiments and analyzed results. AW, HR and AZ wrote the manuscript.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. β -Glucan triggered defense gene expression in *H. vulgare* and *N. benthamiana*.

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Figure S2. Laminarihexaose but not laminarin activates immune responses in *A. thaliana* Col-0 seedlings.

Figure S3. Laminarin-triggered ROS production is concentration dependent.

Figure S4. Qualitative characterization of different laminarin preparations.

Figure S5. Laminarihexaose triggers ROS production in *C. rubella*. Figure S6. *H. vulgare* produces ROS upon elicitation with short and long unbranched β -glucans.

Figure S7. Efficiency and specificity of *NbBAK1* and *NbCERK1* silencing in *N. benthamiana*.

Figure S8. OsCERK1 is not involved in the recognition of laminarin in O. sativa.

Table S1. Primers used for gene expression analysis.

Method S1. Defense gene expression analysis and VIGS efficiency test.

Method S2. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

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