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β-1,3 Glucan Sulfate, but Not β-1,3 Glucan, Induces the Salicylic Acid Signaling Pathway in Tobacco and Arabidopsis

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Sulfate substituents naturally occurring in biomolecules, such as oligosaccharides and polysaccharides, can play a critical role in major physiological functions in plants and animals. We show that laminarin, a β-1,3 glucan with elicitor activity in tobacco (*Nicotiana tabacum*), becomes, after chemical sulfation, an inducer of the salicylic acid (SA) signaling pathway in tobacco and *Arabidopsis thaliana*. In tobacco cell suspensions, the oxidative burst induced by the laminarin sulfate PS3 was Ca^{2+} dependent but partially kinase independent, whereas laminarin triggered a strickly kinase-dependent oxidative burst. Cells treated with PS3 or laminarin remained fully responsive to a second application of laminarin or PS3, respectively, suggesting two distinct perception systems. In tobacco leaves, PS3, but not laminarin, caused electrolyte leakage and triggered scopoletin and SA accumulation. Expression of different families of Pathogenesis-Related (PR) proteins was analyzed in wild-type and mutant tobacco as well as in Arabidopsis. Laminarin induced expression of ethylene-dependent PR proteins, whereas PS3 triggered expression of ethylene- and SA-dependent PR proteins. In Arabidopsis, PS3-induced PR1 expression was also NPR1 (for nonexpressor of PR genes1) dependent. Structure-activity analysis revealed that (1) a minimum chain length is essential for biological activity of unsulfated as well as sulfated laminarin, (2) the sulfate residues are essential and cannot be replaced by other anionic groups, and (3) moderately sulfated β-1,3 glucans are active. In tobacco, PS3 and curdlan sulfate induced immunity against *Tobacco mosaic virus* infection, whereas laminarin induced only a weak resistance. The results open new routes to work out new molecules suitable for crop protection.

INTRODUCTION

Oligosaccharins are naturally occurring complex carbohydrates with biological regulatory functions (Albersheim et al., 1983). In plants and animals, they act as molecular signals that regulate growth, development, and survival in the environment (Côté and Hahn, 1994; Bakkers et al., 1999; Alban and Franz, 2001; Shibuya and Minami, 2001). Oligosaccharins active in plants derive from microbe, plant, or marine macroalgae cell wall structures. Only a few are fully characterized in terms of their structure and spectrum of biological activities. Different structural motifs have been described, including microbial β-1,6-1,3 glucans and chitin-derived oligomers, plant oligogalacturonides and xyloglucans, as well as marine macroalgae laminarin and fucans (Côté and Hahn, 1994; Klarzynski et al., 2000, 2003).

The relevance of some of these oligosaccharins as in vivo actors in defense systems is supported by their possible natural occurrence during plant–microbe interactions (Fritig et al., 1998). Evidence has also been provided that oligosaccharins enhance plant resistance against pathogens (Joubert et al., 1998), and it is thought that mimicking pathogen attack with such nonspecific elicitors might prove useful in the development of alternative strategies for crop protection.

Marine macroalgae constitute an inexpensive possible source of oligosaccharins because they contain a diversity of unique polysaccharides (Kloareg and Quatrano, 1988). For example, laminarin, a reserve carbohydrate of the brown alga *Laminaria digitata*, is a water-soluble β-1,3 glucan with an average degree of polymerization (DP) of 25 glucose units and with up to three single β-glucose branches at position 6 (Read et al., 1996). Laminarin stimulates defense responses in cell suspensions of tobacco (*Nicotiana tabacum*) (Klarzynski et al., 2000), grapevine (*Vitis vinifera*) (Aziz et al., 2003), alfalfa (*Medicago sativa*) (Cardinale et al., 2000), and rice (*Oryza sativa*) (Inui et al., 1997). The defense responses include activation of mitogen-activated protein kinases, Ca^{2+} influx, oxidative burst, and alkalinization of the extracellular medium. Applied to tobacco or grapevine plants, laminarin induces the accumulation of phytoalexins and expression of a set of Pathogenesis-Related (PR) proteins (Klarzynski et al., 2000; Aziz et al., 2003).

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However, laminarin does not trigger the hypersensitive response (HR).

The biological activity of β-glucans is thought to result from their binding to receptors. A putative receptor for a β-1,3, β-1,6 heptaglucan (β-1,6 glucose backbone with two β-1,3 glucose branches) has been cloned in soybean (*Glycine max*) and bean (*Phaseolus vulgaris*) (Umemoto et al., 1997; Mithofer et al., 2000). Furthermore, plant cells can discriminate between closely related glucans. For example, rice responds to a *Pyricularia oryzae* pentaglucan (β-1,3 glucose backbone with one β-1,6 branch) but not to a hexaglucan (β-1,6 glucose backbone with two β-1,3 branches) (Yamaguchi et al., 2000), and tobacco reacts to laminarin but not to the β-1,3, β-1,6 heptaglucan (Klarzynski et al., 2000).

Oligosaccharins can carry decorations that are important for their biological function. It is well established that the sulfate groups of some oligosaccharins mediate recognition in biochemical and physiological processes occurring in algae, land plants, and animals. The pattern of sulfate substitution in carrageenans is crucial for recognition between *Chondrus crispus*, a marine red algae, and its green algal pathogenic endophyte, *Acrochaete operculata* (Bouarab et al., 1999). The sulfate decoration of Nod factors, which are signaling compounds for the symbiosis between legumes and rhizobacteria, is a determinant of host specificity in the *Sinorhizobium meliloti*–alfalfa interaction (Roche et al., 1991). In mammals, basic fibroblast growth factor, a well-characterized signal molecule, is only biologically active and binds to its receptor in the presence of a specific sulfated oligosaccharide fragment of heparan sulfate that binds to both the growth factor and its receptor (Pye et al., 1998). Thus, there is growing evidence that biomolecules substituted with sulfate groups are involved in major physiological functions in plants and animals.

Because sulfate groups can modulate the biological activities of oligosaccharides, we chemically sulfated laminarin from *L. digitata.* The elicitor activities of sulfated and unmodified laminarin were investigated in tobacco and *Arabidopsis thaliana* both in tissue culture and in whole plants. We also report on studies of structure-activity relationships to evaluate the importance of the sulfation pattern, of the chain length, and of the sugar backbone on elicitor activity. Finally, using the tobacco–*Tobacco mosaic virus* (TMV) interaction as a model system, we compared the resistance inducing activity of sulfated and unmodified β-1,3 glucans. Our results indicate that naturally occurring carbohydrates can be chemically modified to alter their biological activities, thereby opening new routes for crop protection strategies.

RESULTS

Synthesis and Characterization of the Laminarin Sulfate PS3

The structure, size, and purity of the laminarin used for chemical sulfation were previously characterized by ^{13}C NMR spectroscopy and high-pressure anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Lepagnol-Descamps et al., 1998). Laminarin consists of a β-1,3 glucan backbone with an average DP of 25 and one or two C6 glucose branches.

The chemical sulfation of laminarin was performed according to the method described by Alban et al. (1992), which had been optimized to obtain regular β-1,3 glucan sulfation without any polysaccharide degradation and to control the resulting degree of sulfation (DS). A DS is mainly dependent on the molar ratio of SO_3/pyridine complex to glucose but is also influenced by other parameters, such as temperature and reaction time. Methylation analysis revealed that the primary OH group in position 6 is ~10 times more accessible to sulfation than the secondary OH groups in positions 2 and 4 (Alban and Franz, 1994). One of the resulting laminarin sulfates was called PS3. PS3 has a DS of 2.4, as determined by elemental analysis, by conductivity detected ion chromatography of the sulfate ions released by trifluoroacetic acid hydrolysis, and by conductimetric NaOH titration of PS3 transformed into the acid form by ion exchange. The latter method also demonstrated that the sodium salt of PS3 is stable for several years. HPAEC-PAD analysis of PS3 showed no degradation of the glucan chain (i.e., the DP and the polydispersity remained unchanged). This was confirmed by gel permeation chromatography. The apparent molecular mass of PS3 (i.e., its hydrodynamic volume) is 18 kD as determined by gel permeation chromatography using neutral pullulans as standards. According to the methylation analysis, the C6-OH of PS3 is nearly completely sulfated, and the substitution of both C2-OH and C4-OH amounts to ~70%.

Various analyses proved the purity of PS3. The absence of nitrogen according to elemental analysis indicated that the product is free of pyridine and *N,N*-dimethylformamide (DMF) used for the chemical sulfation. This was confirmed by ^{13}C NMR spectroscopy and UV spectroscopy. As expected, after dialysis for 7 d against flowing deionized water, no contamination with salts or other low molecular weight compounds, such as oligosaccharides, was observed in gel permeation chromatograms.

Perception of Laminarin and PS3 by Cultured Tobacco Cells

Cultured plant cells have been widely used as model systems to rapidly investigate the perception of molecules. To answer the question whether sulfate substitution of laminarin modifies the perception of this glucan by plant cells, we analyzed the extracellular oxidative burst induced in tobacco cell suspensions upon treatment with laminarin or PS3. Because laminarin showed maximal activity at 200 μg/mL (Klarzynski et al., 2000), we compared the effects of laminarin and PS3 at this concentration. Compared with laminarin, PS3 induced a fourfold to sixfold higher oxidative burst (Figure 1A, closed circles). Pretreatment with staurosporine, a kinase inhibitor, inhibited the laminarin and PS3-induced oxidative bursts (Figure 1A, open circles). Of note, the PS3-induced oxidative burst was inhibited by only 50% as observed in four independent experiments using different concentrations of PS3 (20, 40, 100, and 200 μg/mL). This partial inhibition was not because of the application of not enough staurosporine. Indeed, the same concentrations of staurosporine were able to totally suppress an elicitin-induced oxidative burst of similar extent (data not shown).

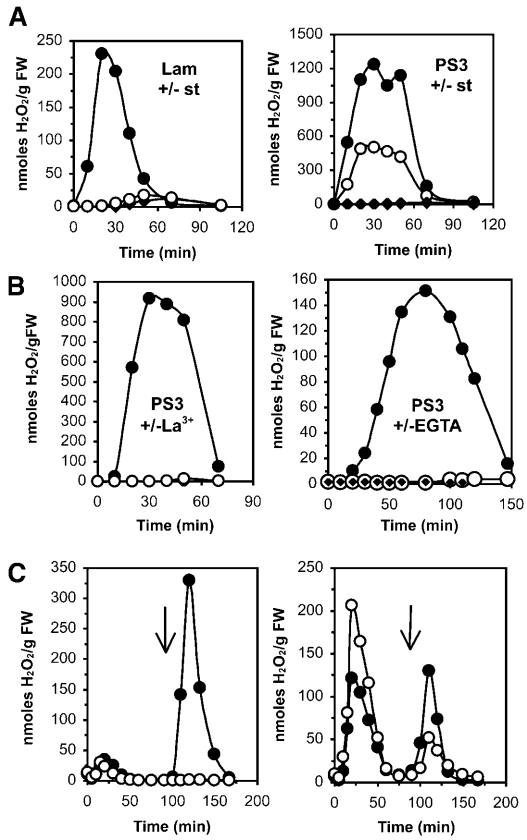

Figure 1. Oxidative Burst Induced by Laminarin and PS3 in Tobacco Cell Suspensions.

Laminarin (Lam) and PS3 were applied to 1 g of tobacco cell suspensions. The oxidative burst was measured using luminol. Values are expressed as H_2O_2 equivalents calculated from a standard H_2O_2 curve. Each experiment was repeated up to four times. The graphs illustrate the results from a representative experiment. FW, fresh weight.

(A) Effect of staurosporine on the Lam- and PS3-induced oxidative burst. Staurosporine (10 μM) was applied 10 min before addition of Lam (200 μg/mL) or PS3 (200 μg/mL). Closed circles, elicitor alone; open circles, elicitor + staurosporine (st); closed diamonds, untreated control.

(B) Effect of La^{3+} and EGTA on the PS3-induced oxidative burst. $LaCl_3$ (2 mM) was applied 10 min before addition of PS3 (200 μg/mL). EGTA (2 mM) was applied 10 min before addition of PS3 (50 μg/mL). Closed circles, PS3 alone; open circles, PS3 + La^{3+} or EGTA; closed diamonds, untreated control.

(C) Oxidative burst induced by successive addition of Lam or PS3. Lam (200 μg/mL) or PS3 (200 μg/mL) were applied at time 0, and the oxidative burst was measured. When the oxidative burst returned to basal values, the same or the second elicitor was added (arrows). The left graph shows the treatments Lam/Lam (open circles) and Lam/PS3 (closed circles). The right graph shows the treatments PS3/PS3 (open circles) and PS3/Lam (closed circles).

We further investigated whether the PS3-induced oxidative burst is Ca^{2+} dependent. Pretreatment of the cells with $LaCl_3$ (2 mM), a calcium surrogate, or with EGTA (2 mM), a calcium chelator, prevented the oxidative burst (Figure 1B). The observation that the PS3-induced oxidative burst was partially kinase independent suggested a different mode of perception of laminarin and PS3 by the tobacco cells. To confirm this hypothesis, we analyzed whether successive addition of laminarin and PS3 would result in a refractory state. The establishment of a refractory state (i.e., the failure of cells to respond to a second dose of the same elicitor) is a convenient method to differentiate between different chemical stimuli (Felix et al., 1993; Rouet-Mayer et al., 1997; Binet et al., 1998). Cells pretreated with laminarin were refractory to a second application of laminarin, which confirms previous results measuring medium alkalinization (Klarzynski et al., 2000). However, they were not refractory to an application of PS3 (Figure 1C). After initial treatment with PS3, a second application of PS3 induced a very low oxidative burst, whereas the cells remained fully responsive to a second laminarin exposure (Figure 1C). This points to a different mode of perception of PS3 and laminarin by tobacco cells.

Symptoms Induced in Tobacco Plants by PS3

Next, we analyzed the eliciting activities of PS3 and laminarin on whole plants. Tobacco leaves infiltrated with laminarin (200 μg/mL) did not develop any symptoms. In contrast with this, tissues treated with PS3 (200 μg/mL) became shiny after a few hours followed by the development of a slight chlorosis after 2 d. These symptoms were strictly limited to the infiltrated tissue areas and never evolved to visible necrosis. Trypan blue and Evans blue staining revealed no dead cells in PS3-treated tissues. Furthermore, microscopic observations showed no cell alterations in tissues treated with either laminarin or PS3.

We also measured electrolyte leakage (Figure 2) to check the possibility of membrane damage that did not lead to cell death. Treatment with laminarin or water resulted in identical kinetics of electrolyte leakage. PS3 induced a significantly higher release of electrolytes. Compared with a treatment with the elicitin β-megaspermin, which induces a typical HR (Baillieul et al., 2003), electrolyte leakage induced by PS3 was comparable at early stages but still significantly lower at the end of the experiment. Thus, PS3, but not laminarin, induced electrolyte leakage.

Induction of Defense Responses in Tobacco and Arabidopsis Plants by PS3

Two days after treatment, PS3-treated tobacco leaves revealed under UV light a blue fluorescence, which was limited to the infiltrated tissues. No fluorescence was detected in laminarin-treated plants. Tissues surrounding a HR lesion caused by elicitin infiltration or TMV infection showed a similar fluorescence as a result of scopoletin accumulation (Costet et al., 2002). As shown in Figure 3, scopoletin accumulated only in PS3-treated and not in laminarin-treated tissues, reaching 20 μg/g fresh weight 7 d after PS3 treatment.

By protein gel blotting, we monitored the expression of acidic and basic PR1, PR2, PR3, and PR5 proteins in tobacco tissues after treatment with laminarin or PS3 (Figure 4). The basic isoforms of PR2, PR3, and PR5 accumulated in tissues treated with laminarin or PS3. By contrast, expression of the acidic isoforms of PR1, PR2, PR3, and PR5 occurred only in PS3-infiltrated tissues. Among the acidic isoforms, PR1 represents

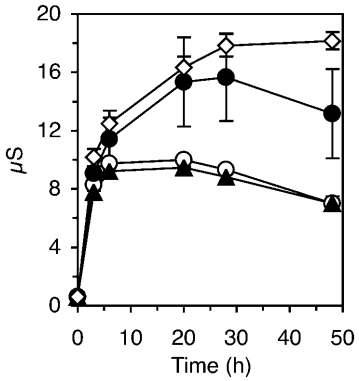

Figure 2. Monitoring of Electrolyte Leakage after Treatment of Tobacco Leaf Tissue with PS3 or Laminarin.

Leaves of tobacco plants were infiltrated with 200 μg/mL of PS3 (closed circles), laminarin (open circles), 50 nM β-megaspermin (open diamonds), water (closed triangles), or left untreated. Electrolyte leakage was measured from leaf discs punched out 3 h after treatment and placed on water. Results were expressed as the means ± SD calculated from three independent experiments. Because the untreated tissues gave the same values of conductivity as the water control, only the latter curve is shown.

a typical marker of defense response in tobacco. Measuring acidic PR1 expression in seven independent experiments performed over a period of 36 months, we never observed PR1 protein accumulation in tissues treated with laminarin, but always in PS3-treated tissues. Increasing the concentration of laminarin to 1000 μg/mL also did not result in acidic PR1 accumulation (Figure 4B).

In tobacco, salicylic acid (SA) induces the expression of the acidic isoforms of PR1, PR2, PR3, and PR5 proteins (Ward et al., 1991; Yalpani et al., 1991; Cordelier et al., 2003), and ethylene induces the expression of the basic counterparts (Brederode et al., 1991; Knoester et al., 1998; Ohtsubo et al., 1999; Cordelier et al., 2003). Thus, our results suggest that laminarin induces an ethylene-dependent signaling pathway, whereas PS3 triggers both an ethylene-dependent and an SA-dependent signaling pathway. To test this hypothesis, we analyzed the expression of the acidic and basic PR proteins in NahG and ETR tobacco plants as well as SA levels. NahG plants catabolize SA into the inactive catechol (Delaney et al., 1994), whereas ETR plants are ethylene insensitive (Knoester et al., 1998). As shown in Figure 4C, expression of the acidic PR1 and of the basic PR3 proteins was suppressed in PS3-treated NahG and ETR plants, respectively. Expression of the basic PR3 protein was also suppressed in ETR tissues treated with laminarin (data not shown). SA accumulated in wild-type tobacco tissues treated with PS3, whereas no significant SA increase occurred in tissues treated with laminarin (Figure 5). These results support the hypothesis of a differential activation of signaling pathways by laminarin and PS3.

Because such a differential activation profile of the two compounds may be specific to tobacco, we additionally analyzed the induction of SA- and ethylene-dependent responses in Arabidopsis. *PR1* and *PDF1.2* are well-recognized markers for the SA and ethylene signaling pathways, respectively, in Arabidopsis (Penninckx et al., 1996, 1998). Expression of both genes was analyzed by semiquantitative RT-PCR (Figure 6). As a control of the method and to confirm the results of protein analysis, we first analyzed the expression of acidic *PR1* and of basic *PR3* genes in tobacco leaves. The results were similar to the protein gel blotting analysis. The acidic *PR1* PCR product was detected after treatment with PS3, whereas the basic *PR3* PCR product was detected after treatment with PS3 or laminarin. In Arabidopsis, *PR* gene expression corresponded to that observed in tobacco. In wild-type Columbia-0 (Col-0) plants, the *PR1* gene was induced after PS3 treatment but not after laminarin treatment, whereas *PDF1.2* was induced after both treatments. Furthermore, *PR1* expression was suppressed in Arabidopsis NahG plants and strongly reduced in the *nonexpressor of PR genes1* (*npr1*) mutant, whereas *PDF1.2* expression was suppressed in the *ein2* mutant. Thus, the differences between laminarin and PS3 in the induction of signaling pathways in Arabidopsis were similar to those found in tobacco.

Structure-Activity Analysis

To establish structure-activity relationships, we analyzed the PR protein inducing activity of several oligosaccharides and polysaccharides in tobacco (Table 1). The activities of laminarin sulfates with DS of 0.4, 0.7, 1.5, 1.8, or 2.4 clearly demonstrated the dependency of the activities on the DS (Figure 7A). As shown by LamS(0.4) and LamS(0.7), a DS higher than 0.4 is required to trigger acidic and basic PR protein expression. The activity increases with increasing DS, but a DS of 1.5 seems to be sufficient to achieve maximal activity because LamS(1.5) and LamS(1.8) were as active as PS3 with a DS of 2.4. At first view, the lack of basic PR protein accumulation in tissues treated with LamS(0.4) observed in three independent experiments was

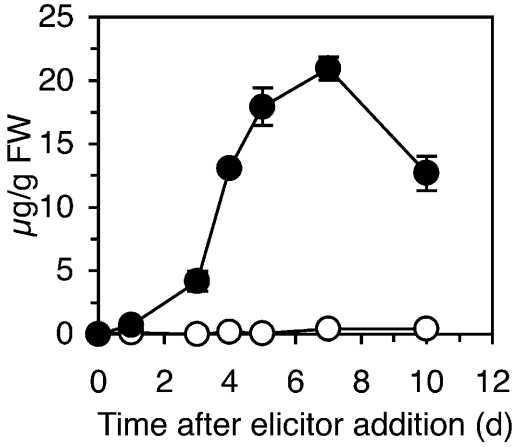

Figure 3. Scopoletin Accumulation in Tobacco Leaves Infiltrated with PS3 or Laminarin.

Leaves of tobacco plants were infiltrated with 200 μg/mL of PS3 (closed circles) or laminarin (open circles). Scopoletin levels were quantified by HPLC. Results are representative of a typical experiment and are expressed as the means ± SD calculated from two HPLC runs.

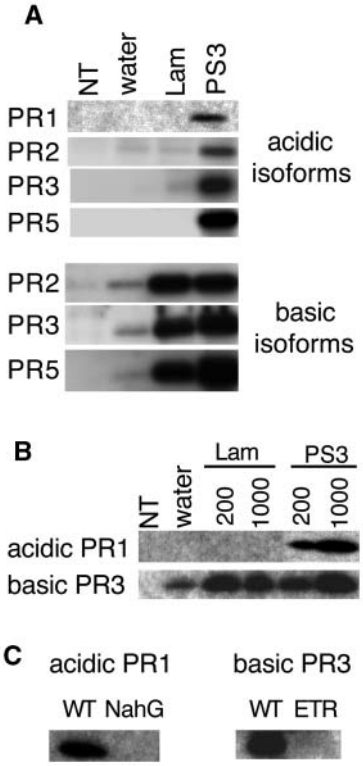

Figure 4. Expression of PR Proteins in Tobacco upon Treatment with PS3 or Laminarin.

Leaves of tobacco plants were infiltrated with PS3 or laminarin (Lam), with water, or left untreated (NT). Proteins were extracted 3 d after treatments and submitted to protein gel blotting with specific antibodies.
(A) Analysis of acidic PR1, PR2, PR3, and PR5 proteins and basic PR2, PR3, and PR5 proteins in plants treated with 200 μg/mL of test compounds.
(B) Analysis of acidic PR1 and of basic PR3 in plants treated with 200 μg/mL and 1000 μg/mL of test compounds.
(C) Analysis of acidic PR1 in NahG tobacco plants and wild-type tobacco and of basic PR3 in ETR tobacco plants and wild-type tobacco treated with 200 μg/mL of PS3.

surprising because laminarin (DS = 0) triggered the expression of these PR proteins. The same result, however, was observed with laminarin or curdlan substituted with other functional groups (see below).

Next, we analyzed the PR-inducing activity of linear β-1,3 glucan sulfate molecules to evaluate the importance of β-1,6 side chains. Curdlan is a linear β-1,3 glucan isolated from the soil bacterium *Alcaligenes faecalis*. Curdlan was sulfated (Alban and Franz, 2001) to obtain CurS(1.7) with a DS of 1.7 and CurS(1.0) with a DS of 1.0, respectively. CurS(1.7), CurS(1.0), and PS3 induced the same symptoms on tobacco leaves (i.e., a slight chlorosis and, under UV light, a blue fluorescence limited to the infiltrated tissues). The three compounds exhibited the same PR protein–inducing activity (Figure 7B), indicating that the β-1,6 glucose side chains are not important for the biological activity.

The molecular weight of CurS(1.7) and of CurS(1.0) is about 2 and 10 times, respectively, higher than that of PS3 (Table 1). Although the lower DS of the two compounds allows no final conclusion yet, prolongation of the carbohydrate chain obviously does not modify the biological activity. To evaluate the influence of shortened chain length, we tested dLamS(2.4) with a DS of 2.4 and a DP of 15 to 25. PS3 and dLamS(2.4) displayed the same PR protein–inducing activity (Figure 7C), so that the activity seems to be independent of the chain length ranging from a DP of 15 to 25. In contrast with this, laminaripentaose sulfate (Lam5S) with a DS of 2.6 remained inactive (Figure 7C), indicating the requirement of a certain minimum chain length for any biological activity. This was confirmed by Lam5, the unsulfated control, which was also completely inactive, whereas laminarin triggered basic PR protein expression. Thus, above a certain minimum chain length, β-1,3 glucans trigger the basic PR protein expression in tobacco plants. For the additional acidic PR protein expression by such molecules, less than one sulfate per glucose unit appears to be sufficient, and for maximal activity 1 to 1.5 sulfate groups per glucose unit are apparently required.

To answer the question of whether sulfate residues can be replaced by other types of negatively charged groups, we examined carboxylmethylated laminarin (CM-Lam) and the three different galacturonans, pectin, polygalacturonic acid, and oligogalacturonic acid with a DP of 14. None of these molecules caused significant acidic and/or basic PR protein expression (Figures 7D and 7E). Because carboxylic acids are weaker acids than sulfuric acids and the degree of substitution of these compounds is lower than the DS of PS3, we also tested curdlan phosphate (CurP) with a degree of substitution of 2.3, which was similar to the DS of PS3. Because CurP was inactive as well (Figure 7E), we concluded that sulfate groups are essential for the induction of the acidic PR protein expression. However, in view of the induction of basic, but not acidic, PR protein expression by fucan (Figure 7D), sulfate groups do not seem to be sufficient for this activity. The tested fucan is a decasaccharide consisting of

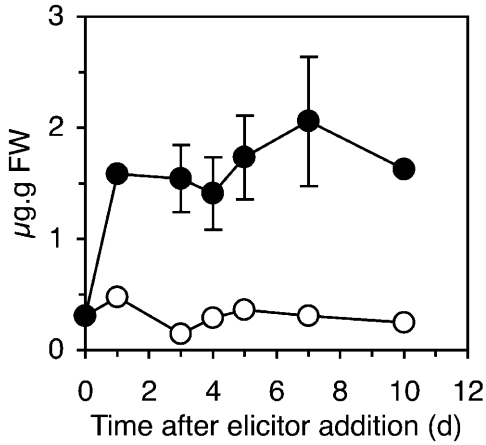

Figure 5. SA Quantification in Tobacco Leaves Infiltrated with PS3 or Laminarin.

Leaves of tobacco plants were infiltrated with 200 μg/mL of PS3 (closed circles) or laminarin (open circles). SA was quantified by HPLC. Results are representative of a typical experiment and are expressed as the means ± SD calculated from two HPLC runs.

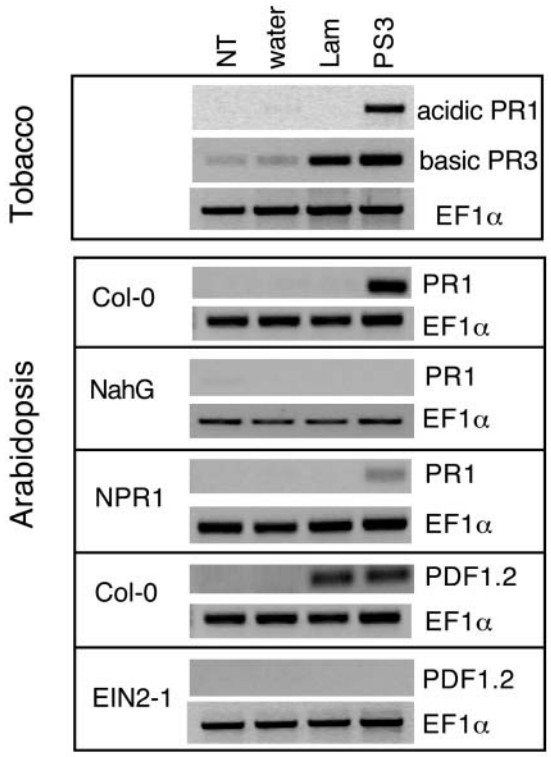

Figure 6. Expression of PR Transcripts in Tobacco and Arabidospis Tissues Treated with PS3 or Laminarin.

Tobacco plants were treated with 200 μg/mL of PS3 or laminarin (Lam), with water, or left untreated (NT). Tissues were harvested 24 h after treatments, and total RNA was isolated and used for semiquantitative RT-PCR analysis. Equal loading was monitored by amplification of EF1α PCR product. Expression of acidic PR1 and basic PR3 transcripts was analyzed in tobacco plants. Expression of PR1 and PDF1.2 transcripts was analyzed in Arabidopsis Col-0 (wild type), *npr1* and *ein2* mutants, and *nahg* transgenic plants.

fucose units with a DS of 1.5. Its basic structure considerably differs from that of β-1,3 glucans. The activity profile found for fucan (induction of basic, but not acidic, PR protein expression) suggests that both a β-1,3 glucan backbone and sulfate residues are necessary to trigger acidic PR protein expression.

Induction of Resistance against TMV Infection in Tobacco by PS3

Because PS3 induced a larger spectrum of defense responses compared with laminarin, we tested whether PS3 would induce increased resistance against pathogen infection. Tobacco leaves were infiltrated at four to six spots per leaf with laminarin, PS3, CurS(1.7), or water. Three days later, the treated leaves were challenge inoculated with TMV. Figure 8 shows the symptoms after a period of another 7 d. PS3 and CurS(1.7) induced total immunity against TMV infection (i.e., no lesions developed in the infiltrated tissues). Total immunity was also observed when TMV was inoculated 8 d after PS3 treatment. The absence of lesions in PS3-treated tissues was observed in four independent experiments representing 58 infiltrated spots. In contrast with this, TMV lesions developed in tissues infiltrated with laminarin. The number of the lesions was lower, but lesions were not significantly smaller than those occurring in water-treated tissues. For a representative experiment, we measured 154 lesions in laminarin-infiltrated tissues and 446 lesions in water-infiltrated tissues. The mean lesion diameters were 2.43 ± 0.53 mm and 2.45 ± 0.50 mm, respectively.

DISCUSSION

Naturally occurring carbohydrates can be sulfated, and these sulfate groups often play a key role in major physiological processes in plants and animals. Here, we show that chemical sulfation of a β-1,3 glucan leads to a molecule with new and improved biological activities in tobacco and Arabidopsis.

The semisynthetic β-1,3 glucan sulfate PS3 triggered defense and resistance responses, which were not induced by the native unsulfated molecule. In tobacco, PS3, but not laminarin, caused accumulation of the phytoalexin scopoletin and of the acidic PR1, PR2, PR3, and PR5 proteins as well as induction of total immunity to TMV infection. The lack of acidic PR1, PR2, PR3, and PR5 protein induction by laminarin was at first unexpected. Indeed, Klarzynski et al. (2000) reported an accumulation of PR proteins of family 1, 2, 3, and 5 in tobacco leaves infiltrated with the same laminarin fraction. However, the acidic and basic isoforms were not discriminated. As for other tobacco/elicitor (Cordelier et al., 2003) and tobacco/pathogen (Friedrich et al., 1995) systems, induction of the acidic PR proteins by PS3 occurred via the SA signaling pathway and that of the basic counterparts by PS3 or laminarin via the ethylene signaling pathway. Upon PS3 treatment, NahG and ETR plants failed to produce acidic PR1 and basic PR3, respectively, and SA accumulated in response to PS3 but not to laminarin. In tobacco, SA and scopoletin are derived from L-Phe via the phenylpropanoid pathway (Murray et al., 1982; Dorey et al., 1997; Schalk et al., 1998; Chong et al., 2001). Accumulation of these two metabolites in tobacco upon PS3 but not laminarin treatment further highlights the specific activity of PS3. To our knowledge, this is the first report showing that by chemical sulfation a polysaccharidic elicitor becomes an inducer of the SA signaling pathway in tobacco.

The wider spectrum of defense responses induced by PS3 correlated with an increased resistance of tobacco to TMV infection. A branched and a linear β-1,3 glucan sulfate, PS3 and CurS(1.7), induced total immunity (i.e., no lesions developed), whereas the unsulfated β-1,3 glucan laminarin caused only partial immunity. Apparently, both the unsulfated and the sulfated β-1,3 glucans were acting on virus infection initiation rather than on virus development and/or spread because the number, but not the size, of the lesions was decreased. Increased resistance to TMV was described in tobacco plants with high SA (Verberne et al., 2000) or scopoletin levels (Ahl Goy et al., 1993). However, resistance was assessed by the size of the lesions and not by the number. So far, the mechanism of virus immunity induced by β-1,3 glucan sulfate is unknown.

Table 1. Structure and Characteristics of the Oligosaccharides and Polysaccharides Used in This Study

Oligo- and Poly-β-1,3 Glucan	DP	MM (kD)	DB (%)	DS (Function)	Substituted (%)		
					C6-OH	C2-OH	C4-OH
Laminarin (Lam)	25	5.8	6–7	0.0	0	0	0
PS3	25	18.0	6–7	2.4 ($-OSO_3^-$)	100	70	70
dLamS(2.4)[a]	15–25	<18.0	n.d.	2.4 ($-OSO_3^-$)	100	70	70
Laminarin sulfate, LamS(0.4)	25	n.d.	6–7	0.4 ($-OSO_3^-$)	40	0	0
Laminarin sulfate, LamS(0.7)	25	15.4	6–7	0.7 ($-OSO_3^-$)	60	5	5
Laminarin sulfate, LamS(1.5)	25	18.0	6–7	1.5 ($-OSO_3^-$)	94	28	28
Laminarin sulfate, LamS(1.8)	25	18.0	6-7	1.8 ($-OSO_3^-$)	97	42	41
Laminaripentaose, Lam5[b]	5	n.d.	0	0.0	0	0	0
Laminaripentaose sulfate, Lam5S	5	n.d.	0	2.6 ($-OSO_3^-$)	100	80	80
CM-Lam	25	14.0	6–7	0.8 (CH_3COO^-)	100	0	0
Curdlan sulfate, CurS(1.7)	n.d.	32.0	0	1.7 ($-OSO_3^-$)	100	35	35
Curdlan sulfate, CurS(1.0)	n.d.	200.0	0	1.0 ($-OSO_3^-$)	100	0	0
CurP	n.d.	170.0	0	2.3 ($-OPO_3^{2-}$)	n.d.	n.d.	n.d.

Other Oligosaccharides and Polysaccharides	DP	Structure
Pectin[b]	n.d.	Poly-α-1,4-galacturonic acid methyl ester
Polygalacturonate[b]	n.d.	Poly-α-1,4-galacturonic acid
Oligogalacturonate[c]	14	Oligo-α-1,4-galacturonic acid
Fucan	10	Fivefold repetition of the disaccharide 1,2-α-L-Fuc*p*-2(OSO_3)-1,4-α-L-Fuc*p*-2,3(diOSO_3)

PS3 and Lam5S were synthesized for this study. LamS(0.4), LamS(0.7), LamS(1.5), LamS(1.8), dLamS(2.4), CM-Lam, CurS, CurP, and fucan were obtained and described previously (Alban and Franz, 1994, 2000, 2001; Stibich, 2000; Alban et al., 2001; Klarzynski et al., 2003). DP, mean degree of polymerization; MM, molecular mass determined by gel permeation chromatography; DB, degree of C6 branching; n.d., no data.

[a] Obtained by sulfation of laminarin under degrading conditions.

[b] Purchased from Sigma (St. Quentin Fallavier, France).

[c] Provided by the Complex Carbohydrate Research Center (University of Georgia).

Arabidopsis and tobacco responded similarly to laminarin and PS3 application. The use of Arabidopsis wild-type, transgenic, and mutant plants showed that PS3 induced an SA-dependent expression of PR1 as well as an ethylene-dependent expression of PDF1.2, whereas laminarin triggered only PDF1.2. Furthermore, PS3-induced PR1 expression was additionally NPR1 dependent. Other polysaccharides were shown to activate defense responses in Arabidopsis, such as chitin (Salinas-Mondragon et al., 1999; Ramonell et al., 2002; Zhang et al., 2002), galacturonides (Norman et al., 1999), and a Phytophthora cell wall preparation (Trezzini et al., 1993), which is known to contain highly branched β-1,3/β-1,6 glucans. Chitin signaling involved both SA- and jasmonate-dependent pathways (Ramonell et al., 2002; Zhang et al., 2002). Galacturonides induced the jasmonate signaling pathway in Arabidopsis (Norman et al., 1999) and tomato (*Lycopersicon esculentum*) (Doares et al., 1995). Our results show that β-1,3 glucans with rare β-1,6 glucosidic linkages activated the ethylene, but not the SA, signaling pathway in Arabidopsis.

One possible explanation for the differential activation of signaling pathways in Arabidopsis by sulfated and unsulfated glucans could be deduced from the recent analysis of the *pmr4* Arabidopsis mutant. PMR4 is considered to be the main callose synthase responsible for callose deposition upon biotic and abiotic stresses. This *pmr4* mutant is resistant to powdery mildew infection and is unable to produce a pathogen-induced callose response (Nishimura et al., 2003). The susceptibility to powdery mildew was restored in the *pmr4 nahg* double mutant, suggesting that callose, a linear β-1,3 glucan, or callose synthase negatively regulates the SA pathway. Because PR1 activation in Arabidopsis is regulated by the SA signaling pathway, the lack of PR1 expression in laminarin-treated Arabidopsis plants could be explained by a negative feedback of the glucan. Consequently, sulfation of the glucan would counteract the negative feedback effect. However, this remains to be demonstrated.

Structure-activity relationship analysis highlighted some features of the β-1,3 glucan sulfate relevant for its unique activation profile. Independent of the presence or absence of sulfate groups, only β-1,3 glucans above a minimum chain length (DP > 5) exhibit PR protein–inducing activity. In addition to the basic PR protein induction by the unsulfated laminarin, β-1,3 glucan sulfates with a DS above 0.4 induced acidic PR protein expression. This activity is DS dependent but did not further improve at DS ≥ 1.5. One possible explanation for the lack of Lam0.4 activity could be that the addition of a sulfate residue to about each second glucose in the chain is sufficient to change the three-dimensional structure so that the typical structure of laminarin is broken and, consequently, the affinity to the assumed receptor. But the charge density is too low to enable efficient binding to those receptor molecules that bind the higher charged laminarin sulfates. Branching of the glucan backbone did not appear to be critical for the biological activity because the linear curdlan sulfate molecules were as active as PS3 in inducing PR protein expression and resistance against TMV. A nonspecific effect

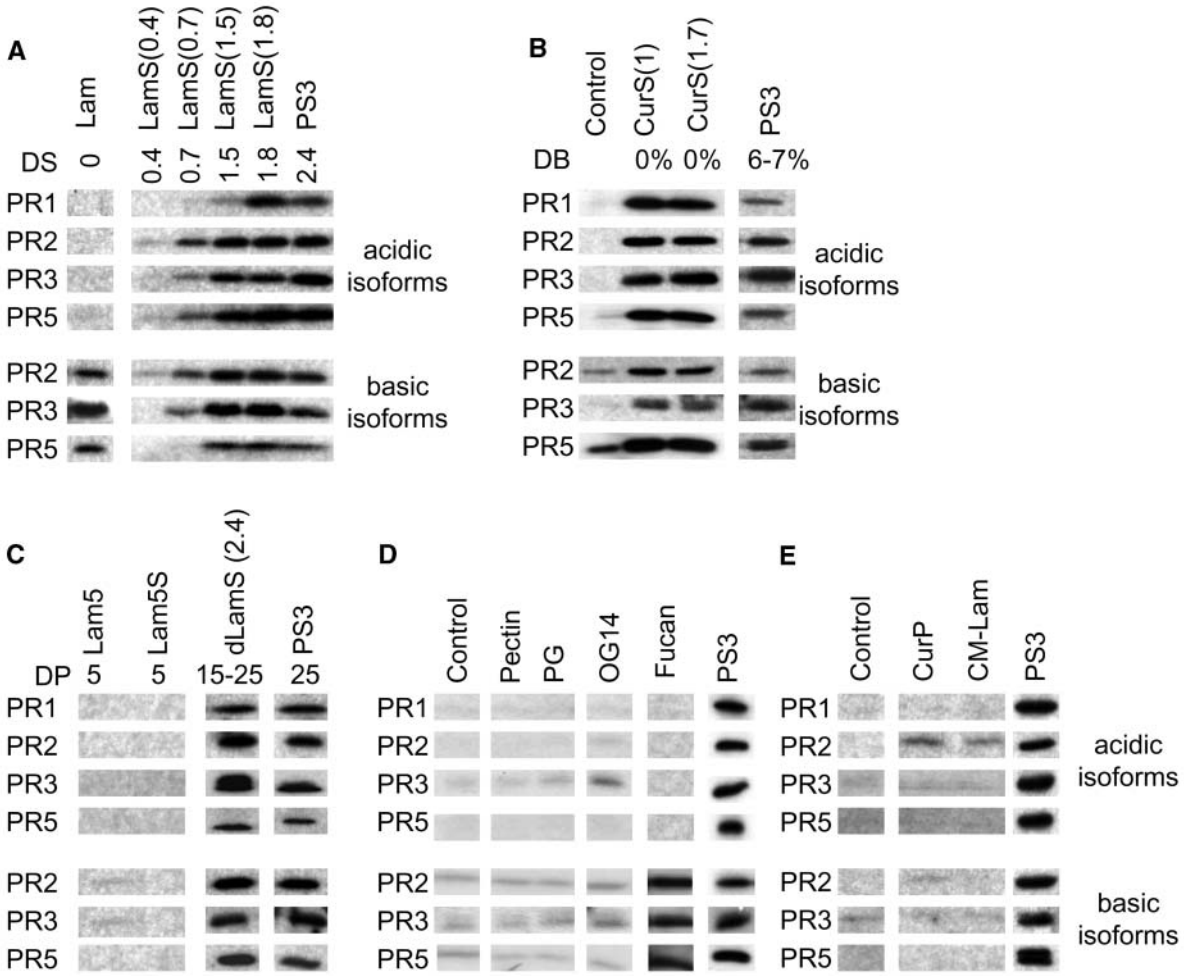

Figure 7. Structure-Activity Analysis.

The compounds used for the treatment of the tobacco plants are described in Table 1. Leaves were infiltrated with 200 μg/mL of each compound, with water, or left untreated. Proteins were extracted 3 d after treatments and submitted to protein gel blotting with specific antibodies to analyze acidic PR1, PR2, PR3, and PR5 and basic PR2, PR3, and PR5.

(A) Comparing laminarin sulfate of different DS.

(B) Comparing β-1,6 branched β-1,3 glucan sulfate (PS3) and linear β-1,3 glucan sulfate (curdlan sulfate).

(C) Comparing sulfated β-1,3 glucans of different DPs.

(D) Comparing PS3 with different oligogalacturonates and polygalacturonates and with fucan.

(E) Comparing β-1,3 glucans carrying different substitution groups.

attributable to the negative charges along the polysaccharide molecule could be excluded because CurP and CM-Lam as well as other uronic acid–containing polymers were inactive. This result underlines the important function of the sulfate residues for the biological activity. However, the presence of sulfate residues in an oligosaccharide or polysaccharide is not always associated with the same activity as that of β-1,3 glucan sulfates. Indeed, the naturally sulfated fucan (DS of 1.5) strongly induced expression of basic PR proteins but not of the acidic isoforms. This observation highlights the importance of the glucan backbone. To further clarify the role of the β-1,3 glucan backbone, experiments with other sulfated oligosaccharides and polysaccharides are necessary. It should be noted that β-1,3 glucan molecules carrying functional groups [carboxymethyl, phosphate, or even rarely occurring sulfate groups, such as LamS(0.4)] failed to induce basic PR protein expression. Hence, modification of the β-1,3 glucan backbone with negatively charged groups other than sulfate residues (at low DS for the latter) seems to suppress biological activity. This suggests that the biological activity of the β-1,3 glucan sulfates does not result from a higher eliciting efficiency but rather from a different way of sensing β-1,3 glucan sulfates by the tobacco cells.

Analysis of the oxidative burst in tobacco cell suspensions provides two arguments for a different mode of perception of PS3 and laminarin by tobacco cells. The oxidative burst as well as Ca^{2+} influx, phosphorylation events, and extracellular

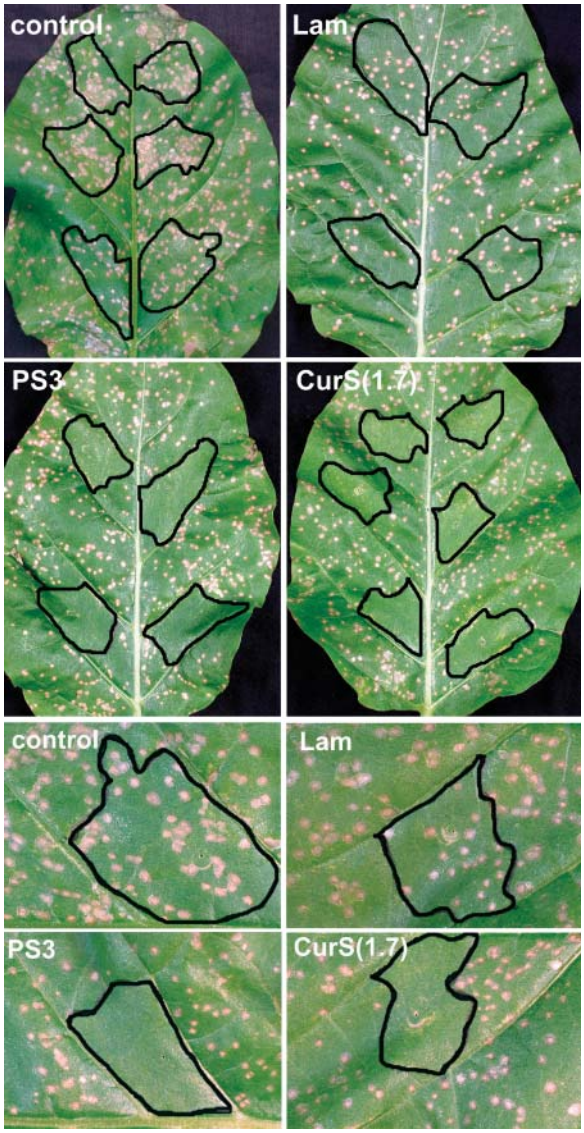

Figure 8. Induction of Resistance against TMV in Tobacco Treated with PS3, Curdlan Sulfate, or Laminarin.

Leaves of tobacco plants were infiltrated with PS3, curdlan sulfate [CurS(1.7)], or laminarin (Lam) at 200 μg/mL for each compound. Three days later, the treated leaves were challenge inoculated with TMV, and the symptoms were photographed 7 d later. Controls were leaves infiltrated with water.

alkalinization have been described as events occurring after elicitor treatments and preceding defense response activation (Dixon and Lamb, 1990). Staurosporine, a kinase inhibitor, blocked the oxidative burst induced by either PS3 or laminarin, indicating the recruitment of a signaling pathway involving protein phosphorylation for both elicitors. However, the 50% suppression of the PS3-induced oxidative burst points to the involvement of a kinase-independent mechanism. Both the kinase-dependent and -independent mechanisms were shown to be Ca^{2+} dependent because the oxidative burst was blocked by either a Ca^{2+} surrogate or a Ca^{2+} chelator. Therefore, PS3 is suggested to activate another signaling pathway in addition to that triggered by the unsulfated laminarin. A possible kinase-independent mechanism of action would be the direct interaction of PS3 with plasma membranes. Yeast elicitor, a mixture of glucan, mannan, and chitin, was shown to exhibit pore-forming properties resulting in ion fluxes (Klusener and Weiler, 1999). This pore-forming activity would apply to PS3 but not to laminarin, and it could explain the electrolyte leakage caused by PS3. However, no pore formation by PS3 was observed in human cells, as no haemolysis was observed (S. Alban, unpublished data).

A second argument is the fact that cells pretreated with laminarin or PS3 were not refractory to a second addition of PS3 or laminarin, respectively. The establishment of a refractory state has been used as a convenient method to differentiate between different qualities of chemical stimuli (Felix et al., 1993; Rouet-Mayer et al., 1997; Binet et al., 1998). Klarzynski et al. (2000) have shown that tobacco cells first treated with laminarin failed to produce an alkalinization response after a second application of laminarin. This refractory state was confirmed here by measuring the oxidative burst. PS3-treated cells, however, responded only partially to a second PS3. The biological significance of this observation remains to be elucidated. It could be compared with the kinase-independent mechanism discussed above.

Our results further highlight the observation that different plants have developed the ability to react to structurally different, but related, β-glucans: soybean and rice recognize branched β-glucans, and tobacco reacts to linear β-1,3 glucans. The well-known β-1,3 β-1,6 heptaglucoside (β-1,6 backbone with β-1,3 side chains) elicits phytoalexin accumulation in soybean (Sharp et al., 1984) and other plants in Fabacae (Cosio et al., 1996), but it is inactive in tobacco (Klarzynski et al., 2000). A β-1,6 β-1,3 glucan (β-1,3 backbone with a β-1,6 side chain) induces phytoalexin production in rice but not in soybean (Yamaguchi et al., 2000). In rice, linear β-1,3 glucans are inactive (Yamaguchi et al., 2000), and in soybean, a linear β-1,6 glucan is inactive as well (Cheong et al., 1991), emphasizing the importance of the side chains for biological activity in these plants. In soybean, laminaribiose (DP of 2) shows very poor activity at a very high concentration (>7 mM, the β-1,3 β-1,6 heptaglucoside shows activity at 8 nM), whereas linear β-1,3 glucans of higher DP were not tested (Cheong et al., 1991). Plasma membrane–located proteins binding with high affinity to the β-1,3 β-1,6 heptaglucoside have been purified (Cheong et al., 1993; Mithofer et al., 1996, 1999) and cloned from soybean (Umemoto et al., 1997) and bean (Mithofer et al., 2000). Membranes prepared from Arabidopsis or tobacco plants do not bind this β-1,3-1,6 heptaglucoside (Côté et al., 2000).

In conclusion, our results show that chemical sulfation of naturally occurring polysaccharides can dramatically change their biological activities in plants to increase their defense and resistance eliciting activities. This opens new routes for the development of new compounds suitable for crop protection. Interestingly, in animal and human cell test systems, PS3 exhibits several biological effects, such as strong anti-inflammatory and antimetastatic activities (Alban and Franz, 2001; Becker et al.,

2003). Thus, PS3 and more generally sulfated β-1,3 glucans seem to have a distinct structure that enables interactions with molecules involved in both plant and animal/human biological processes.

METHODS

Synthesis and Characterization of Sulfated β-1,3 Glucans

Synthesis

Laminarin was extracted and purified from the marine brown algae *Laminaria digitata* as described (Klarzynski et al., 2000). Laminaripentaose (Lam5), a purified pentasaccharide consisting of five β-1,3 linked glucose units, was purchased from Sigma. Sulfation of laminarin and Lam5 resulted in a laminarin sulfate fraction called PS3 and Lam5S. To produce PS3, 1 g of laminarin was dissolved in a mixture of 20 mL of DMF and 2.8 mL of pyridine, and the mixture was heated to 60°C. Sulfation was performed by continuous addition of 5.9 g of SO_3-pyridine complex for 2 h at 60°C. Stirring was continued for another 2 h. After cooling to room temperature, the supernatant was removed by pipetting from the slippery brown precipitate formed during the reaction. The latter was dissolved in a 2.5 M NaOH solution, and the resulting solution was then poured onto 99% EtOH to obtain a final concentration equal to 90% EtOH. After 12 h stirring at 4°C, the white precipitate was isolated by centrifugation and dissolved in distilled water. The solution of PS3 was dialyzed (molecular weight cut off 1000 D) for 7 d at room temperature against flowing deionized water. The solution was then frozen at −70°C and freeze-dried. A similar procedure was used to obtain Lam5S. Lam5 (100 mg) was stirred for 3 h at 60°C with 653 mg of SO_3-pyridine complex in 5 mL of dry DMF containing 350 μL of pyridine. The supernatant was then removed, and the precipitate was dissolved in 2.5 M NaOH solution. Water was then coevaporated with 99% ethanol, and the resulting product was dialyzed 4 d against flowing deionized water with a 500 D cut-off membrane. The curdlan sulfate (CurS) was obtained by activation and sulfation of the bacterial β-1,3 glucan curdlan (Wako Pure Chemical Industries, Osaka, Japan) as described previously (Alban and Franz, 2000).

Sulfate Content Determination

The principle is based on the hydrolysis of the glucan sulfate by trifluoroacetic acid and the quantification of the resulting ions by ion chromatography with conductivity detection as described (Alban et al., 2001).

Molecular Weight Determination

The molecular weight was determined by gel permeation chromatography (Alban et al., 2001) on a Sephadex S75 column using a fast protein liquid chromatography system (Amersham Biosciences Europe, Freiburg, Germany) and a refractive index detector (Waters, Eschborn, Germany). For calibration, neutral pullulans, stachyose, and glucose were used. The samples were dissolved in water to give a final concentration of 1 mg/mL. The eluant was 0.1 M sodium chloride in water.

HPAEC-PAD

HPAEC-PAD was performed using a Dionex chromatograph in the configuration DX500 (Dionex, Sunnyvale, CA), a 50 μL injection loop, a CarboPac PA100 column (4 × 250 mm), and an electrochemical detector with a gold working electrode. Chromatography was performed as described in Lepagnol-Descamps et al. (1998).

Methylation Analysis

The sulfation pattern was evaluated by a modified methylation procedure (Alban and Franz, 1994). The resulting methylated, ethylated, alditol acetates were analyzed by combined gas–liquid chromatography mass spectrometry, providing qualitative and quantitative information about the carbohydrate structure and the distribution of the sulfate groups.

Plant Material and Treatments

Tobacco cell suspensions (*Nicotiana tabacum* cv Xanthi) were grown, maintained, and used for experiments as described (Binet et al., 1998). Tobacco plants, *N. tabacum* cv Samsun H (seeds obtained from Altadis, Institut du Tabac, Bergerac, France), *N. tabacum* cv Xanthi nc, *N. tabacum* cv Samsun NN transgenic for the *ETR* gene and *N. tabacum* cv Xanthi nc transgenic for the *NAHG* bacterial gene, were grown in a greenhouse under controlled conditions. Two- to three-month-old plants were placed 2 to 3 d before treatments in a growth chamber at 22 ± 1°C with a photoperiod of 16 h. *Arabidopsis thaliana* plants were of ecotype Col-0. Seeds of ethylene response mutant *ein2-1* (Guzman and Ecker, 1990) and nonexpresser of PR genes *NPR1* (Cao et al., 1994) were obtained from the Nottingham Arabidopsis Stock Centre Biological Resource Center (accession numbers CS3071 and CS3726). Seeds of *nahg* transgenic Arabidopsis Col-0 plants were a gift from R. Dietrich (Syngenta, Research Triangle Park, NC). Plants were grown with a 12-h light period at 20°C, and 6-week-old plants were used for experiments. Plant treatments were performed by infiltration of aqueous solutions of the elicitors with a syringe into the mesophyll of fully developed leaves. The infiltrated tissue was immediately delineated with a felt-tip marker when necessary. β-Megaspermin was obtained as described (Baillieul et al., 2003). For virus infection, tobacco plants were first infiltrated with water, laminarin, PS3, or curdlan sulfate [CurS(1.7)] at four to six spots per leaf and were challenge inoculated with TMV 3 or 8 d later by rubbing the upper leaf surface with a suspension of purified virus at a 0.1 $\mu g \cdot mL^{-1}$ concentration in the presence of abrasive celite. The number and the size of TMV lesions were then determined 6 d after virus inoculation using an ocular micrometer.

Defense Response Analyses

The oxidative burst was measured as described (Binet et al., 1998). Staurosporine, $LaCl_3$, and EGTA were purchased from Sigma. For electrolyte leakage measurements, tobacco leaf discs were punched out using a 5-mm-diameter cork borer 3 h after treatments. Fifteen leaf discs from each treated area were placed in 20 mL of water to lower the background signal derived from the wounded cells. Disks were then placed in Petri dishes containing 10 mL of fresh water under continuous light at 24°C. Measurements were taken using a conductivity meter (Conductometer E518; Metrohm, Herisau, Switzerland). Results were expressed as the means and standard deviation calculated from three independent experiments.

Protein extraction was performed from 100 to 200 mg of leaf tissues by grinding in 100 mM Mes buffer (ratio buffer:tissue of 3:1), pH 7.5, containing 15 mM β-mercaptoethanol and charcoal. The crude extract was centrifuged at 13,000 rpm for 20 min, and the supernatant was used to perform acidic and basic PR protein analysis by protein gel blotting as described (Cordelier et al., 2003).

Total scopoletin and SA (free and conjugated forms) were analyzed. Procedures for scopoletin and SA extraction and quantification have been described (Klarzynski et al., 2000; Costet et al., 2002).

RT-PCR Gene Expression Analysis

Total RNA was extracted from leaf tissues using Trizol (Invitrogen, Carlsbad, CA) according to the protocol supplied by the manufacturer.

First-strand cDNA synthesis was made from 2 μg of RNA. The synthesized cDNA was used as a matrix for PCR. PCR was performed with denaturing, annealing, and extension temperatures of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, respectively, for 25 to 30 cycles. Gene-specific primers were as follows: tobacco acidic PR-1 (forward, 5′-GATGCCCATAACACAGCTCG-3′; reverse, 5′-TTTACAGATCCAGTTCTTCAGAGG-3′), tobacco basic PR-3 (forward, 5′-GCCATAGGAGTGGACCTGCTAAAC-3′; reverse, 5′-AAAAGACCTCTGGTTGCCGC-3′), Arabidopsis PR-1 (forward, 5′-CTACGCAGAACAACTAAGAGGCAAC-3′; reverse, 5′-TTGGCACATCCGAGTCTCACTG-3′), Arabidopsis PDF1.2 (forward, 5′-ATGGCTAAGTTTGCTTCC-3′; reverse, 5′-TTAACATGGGACGTAACAGATACAC-3′). As control for equal cDNA amounts in each reaction, PCR was performed with primers for tobacco EF-1α (forward, 5′-TCGCCTTGTGGAAGTTTGAGAC-3′; reverse, 5′-CACCAACAGCAACAGTTTGACG-3′), Arabidopsis EF-1α (forward, 5′-TTGCTCCCACAGGATTGACCACTG -3′; reverse, 5′-TCACTTCGCACCCTTCTTGACG-3′). The gene-specific primers of tobacco acidic PR-1, tobacco basic PR-3, Arabidopsis PR-1, Arabidopsis PDF1.2, tobacco EF-1α, and Arabidopsis EF-1α generate expected product sizes of 535, 336, 222, 243, 1050, and 548 bp, respectively. PCR products were separated on a 1% agarose gel and visualized after ethidium bromide staining. Quantification of the PCR products was made in gel using the Bio-Rad GelDoc apparatus together with the Bio-Rad Quantity One software (Hercules, CA). Control reactions to normalize RT-PCR amplification were run with the EF-1α specific primers and four serial dilutions of each first-strand cDNA. PCR were performed through 25 cycles and resulted in amplification linearly related to RNA amounts.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers CS3071 and CS3726.

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ß-1,3 Glucan Sulfate, but Not ß-1,3 Glucan, Induces the Salicylic Acid Signaling Pathway in Tobacco and Arabidopsis

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