Distinct Defenses Induced in Wheat Against Powdery Mildew by Acetylated and Nonacetylated Oligogalacturonides

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ABSTRACT

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In wheat, little is known about disease resistance inducers and, more specifically, about the biological activities from those derived from endogenous elicitors, such as oligogalacturonides (OGAs). Therefore, we tested the ability of two fractions of OGAs, with polymerization degrees (DPs) of 2–25, to induce resistance to *Blumeria graminis* f. sp. *tritici* and defense responses in wheat. One fraction was unacetylated (OGAs–Ac) whereas the second one was 30% chemically acetylated (OGAs+Ac). Infection level was reduced to 57 and 58% relative to controls when OGAs–Ac and OGAs+Ac, respectively, were sprayed 48 h before inocu-

lation. Activities of various defense-related enzymes were then assayed in noninoculated wheat leaves infiltrated with OGAs. Oxalate oxidase, peroxidase, and lipoxygenase were responsive to both OGAs–Ac and OGAs+Ac, which suggests involvement of reactive oxygen species and oxilipins in OGAs-mediated responses in wheat. In inoculated leaves, both fractions induced a similar increase in H₂O₂ accumulation at the site of fungal penetration. However, only OGAs+Ac led to an increase in papilla-associated fluorescence and to a reduction of formed fungal haustoria. Our work provides the first evidence for elicitation and protection effects of preventive treatments with OGAs in wheat and for new properties of acetylated OGAs.

Additional keywords: induced resistance, *Triticum aestivum*.

A plant attacked by a pathogen can resist infection by activating its own defense strategies, leading to natural resistance. These defense responses are known to be triggered by a wide range of elicitors which are derived from either the pathogen or the host plant. Among them, some plant cell-wall-derived oligosaccharides, especially oligogalacturonides (OGAs), are considered to be signals that trigger plant resistance. They are released when endopolygalacturonases and endopectate lyases secreted by pathogens degrade pectin homogalacturonans (13). OGAs involved in induced resistance consist of linear chains of α -(1-4)-linked D-galacturonic acid (GalA), with a degree of polymerization (DP) often ranging from 10 to 15 (1,13). In planta, GalA units can be methyl-esterified or acetylated, and their degree of substitution (DS) depends on the source plant (8). It has been shown that the degree of OGA methylation clearly influences plant defense responses. In wild strawberry (*Fragaria vesca*), partial demethylation of OGAs in transgenic fruit enhanced resistance to *Botrytis cinerea* (31). Furthermore, in wheat, Wietholter et al. (51) found a significant difference in methyl esters distribution in OGAs from cultivars susceptible or resistant to stem rust. However, the role of acetylation of OGAs in plant defenses has not been studied thus far.

Different biotic molecules have been extracted from various organisms and applied to plants in order to test their defense-inducing potential. Some of them can mimic a pathogenic interaction and activate physiological and morphological responses in plants. The perception of such elicitors is followed by modifications in membrane permeability, production of reactive oxygen species (ROS) responsible for an oxidative burst, and early defense gene expression. Then, the activation of signal transduction pathways leads to late defense gene expression and to the accumulation of secondary metabolites (52). In dicotyledonous plants, the exogenous application of OGAs was shown to initiate the production of ROS and to induce the accumulation of transcripts and the catalytic activation of phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5), which is the first enzyme in the phenylpropanoid pathway leading to phytoalexins and lignin production (38). More recently, in grapevine, OGAs were shown to initiate a strong hydrogen peroxide (H₂O₂) production and an increase in PAL and expression of various pathogenesis-related (PR) genes (4,5).

Responsible for powdery mildew in wheat, *Blumeria graminis* f. sp. *tritici* is an obligate parasite that develops on leaves and stems and leads to important yield losses. To date, fungicides are used to control fungal development but their negative impacts on the environment and human health justify a decrease of used quantities. Thus, alternative or nonpollutant-associated strategies are being developed, including stimulation of plant defenses. In wheat, a partially induced resistance against *B. graminis* f. sp. *tritici* has been obtained with different elicitors. Some carbohydrates, such as trehalose, a nonreducing disaccharide, or Iodur 40 (Goëmar SA, Saint-Malo, France), a commercial laminarin, induced resistance in wheat against *B. graminis* f. sp. *tritici* (36,37).

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They exhibited common effects, such as an enhancement in H₂O₂ and phenolics accumulation at the penetration site of the fungus. On the other hand, only Iodus 40 exhibited an additional decrease in lipid peroxidation (37). Furthermore, some elicitors can also directly act on the fungus, such as Milsana (Biofa AG, Münsingen, Germany), which is an ethanolic extract from leaves of *Reynoutria sachalinensis* that inhibited the germination of *B. graminis* f. sp. *tritici* conidia (35). These studies indicate partially different modes of action of these elicitors in wheat against powdery mildew.

In this study, two distinct biochemical fractions of OGAs were tested for their ability to elicit defenses in wheat and to induce resistance against *B. graminis* f. sp. *tritici*. They consisted of OGAs with DPs of 2 to 25 (OGAs–Ac) and one fraction that was chemically 30% acetylated (OGAs+Ac). We obtained and compared protective efficacies with both acetylated and nonacetylated OGAs. Their effects on conidia germination or haustoria formation were also tested. Moreover, their biological activities and eliciting effect were evaluated; because H₂O₂ is a central molecule in ROS metabolism, we measured enzyme activities which are involved in the production or the metabolism of this molecule, such as oxalate oxidase (OXO) (EC 1.2.3.4), peroxidase (PO) (EC 1.11.1.7), and catalase (CAT) (EC 1.11.1.6). Relationships between these enzyme activities and the accumulation of H₂O₂ or polyphenolic compounds at the penetration site of the fungus were investigated. We also assayed PAL and lipoxygenase (LOX) (EC 1.13.11.12) activities. They are key enzymes of the phenylpropanoid and octadecanoid metabolism pathways, both induced in plant defense. Here, we established several biological activities of these OGAs during the wheat–*B. graminis* f. sp. *tritici* interaction and focused on distinct biological properties obtained by acetylation.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, chemicals were purchased from Sigma (Saint Quentin Fallavier, France). OGAs were produced at the Laboratoire des Polysaccharides Microbiens et Végétaux (Université Jules Verne, Amiens, France). The mixture of OGAs was obtained by thermal degradation of polygalacturonic acid (PGA) from citrus fruit. OGAs with DPs of 2 to 25 (OGAs–Ac) were selected by sequences of purification using acetic acid and isopropanol (33). The proportion of each DP was calculated by microscale colorimetric assay using methoxyhydroxybiphenyl (*m*HBP) (46) after fractionation of the purified oligomers by SEC chromatography on a Bio-gel P6 column (BioRad, France) (15). Dried OGAs–Ac were acetylated using acetic anhydride (S. Rossard, *unpublished data*). After addition of H₂O, the preparation was dialyzed and the acetylated galacturonides were dried by lyophilization. Samples were then dissolved in D₂O 99.96% D; the final concentration was 15 g/liter. Spectra were recorded at 80°C using a Bruker Avance 300 spectrometer (Bruker Biospin SA, France). Spectral width was 3000 Hz, acquisition time was 1.36 s, pulse width was 7 µs, the relaxation time was 1 s, and 256 scans were recorded. The acetylation degree was calculated by integration of signals in the downfield, upfield, and acetyl regions as described in the literature (14).

Plants, treatments, and inoculation with the pathogen. Caryopses of wheat (*Triticum aestivum* L.) cv. Orvantis were provided by Benoît C.C. (Orgerus, France). They were sown in compost and placed in a growth chamber (day temperature, 18°C and night temperature, 12°C) under a 12-h photoperiod (light intensity, 250 µmol m⁻² s⁻¹) and a relative humidity of 70%. Treatments occurred when plantlets were 10 days old. OGAs–Ac and OGAs+Ac powders were dissolved in distilled water. Solutions were freshly prepared before each experiment. Two distinct methods were used to apply treatments. For enzymatic assays or microscopic investigations, OGAs were used at 1 or 5 g/liter.

They were infiltrated inside leaf tissues with a hypodermic syringe without a needle; approximately 50 µl of solution were used to cover an area of 1 cm² in the central part of primary leaves. This area was delineated with a marker pen. Two parts were then considered: an infiltrated area between the infiltration place and the marker line, and a noninfiltrated 1-cm² area above the infiltrated one. Leaves infiltrated with distilled water were used as controls. For microscopic observation, plants were inoculated 2 days after treatment. Leaves were inoculated with 8-day-old *B. graminis* f. sp. *tritici* conidia that were collected by vacuum extraction from fully infected leaves and suspended in Fluorinert FC43 (3M, Cergy-Pontoise, France). This product is compatible with the hydrophobicity of spores, without any effect on the plant or the fungus. The inoculum concentration was adjusted to 5 × 10⁵ spores/ml and conidia were sprayed on leaves. For infection-level assays, plants were sprayed with distilled water (control treatment) or OGA solutions at 5 g/liter, each supplemented with 0.025% of a wetting agent, Citowett (B.A.S.F., Levallois-Perret, France), an alkylaryl polyglycol ether. Only one spray was applied. Two days after OGA treatments, leaves were inoculated with a Fluorinert FC43 suspension containing 2.5 × 10⁵ spores/ml.

Infection level assessment. Eight days after inoculation with *B. graminis* f. sp. *tritici* conidia, 10 plantlets treated in the same conditions were randomly harvested to count the number of sporulating colonies that developed on the adaxial and abaxial surfaces of the first leaf. The percentage of infection obtained with OGA solutions was determined as follows: (average number of colonies on treated leaves/average number of colonies on control leaves) × 100.

Microscopy. For phenolics accumulation assessment, primary leaves were harvested 40 h after inoculation, cleared with a mixture of ethanol:acetic acid (3:1, vol/vol), and stored in lactoglycerol (lactic acid:glycerol:water, 1:1:1, vol/vol/vol). Conidia were stained with trypan blue. A 1.5-cm-long segment was cut from the infiltrated area of leaves and mounted on a glass slide. Samples were used for fluorescence and transmitted light microscopy to visualize autofluorescent phenolic compounds using a Nikon microscope (Champigny-sur-Marne, France) fitted for fluorescence (blue exciter filter, maximum transmittance 400 nm, dichroic mirror, and barrier filter with a transmittance range of 500 to 800 nm). As described by Hückelhoven (22), a yellow fluorescence visualized under UV-light microscopy indicates the presence of polymeric phenolic compounds. When a papilla was observed at the fungal penetration site, it was registered as belonging to one of the seven classes we established: (i) papilla without fluorescence or haustorium, (ii) papilla with a weak fluorescence but no haustorium, (iii) papilla with a weak fluorescence and a haustorium, (iv) papilla with a strong fluorescence but no haustorium, or (v) papilla with a strong fluorescence and a haustorium. In other cases (vi), no papilla was observed but a haustorium was formed. Finally, (vii) some infected epidermal cells showed whole cell fluorescence without haustorium. Each event was expressed as a percentage of the total of penetration sites of the fungus that was recorded.

For *in vivo* detection of H₂O₂, we used the 3,3'-diaminobenzidine (DAB) staining method according to Thordal-Christensen et al. (43). At 17 h after inoculation, the primary leaves were excised and the cut end was immersed for 4 h in a solution containing DAB at 1 mg/ml dissolved in water (pH adjusted to 3.8 with HCl). Leaves were then cleared and stored as described above. A 1.5-cm-long segment was also cut in the infiltrated area of leaves and used for microscopy to visualize the H₂O₂ accumulation at the site of fungal penetration. H₂O₂ appeared with different staining intensities in leaves treated either with OGAs or distilled water. A staining intensity scale was applied to identify the degree of H₂O₂ accumulation as no visible DAB staining (the AGT penetrated the epidermal cell but there was no DAB staining), weak staining intensity, and strong staining intensity.

Enzyme activities. LOX activity was measured according to Todd et al. (44) and Avdiushko et al. (3) with some slight modifications. The formation of conjugated dienes from linoleic acid, a substrate of LOX, was assayed spectrophotometrically at 235 nm to estimate LOX activity. Leaf tissues were ground in ice-cold 0.1 M potassium phosphate buffer (pH 7.6) containing 9 mM octylthioglucopyranoside, 2% polyvinylpyrrolidone (PVPP), and 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged at $10,000 \times g$ at 4°C for 15 min and was tested for its LOX activity. Substrate for the LOX spectrophotometric assay was prepared by dissolving 100 μl of Tween 20 (Uniqema Americas, NJ) in 20 ml of 4-morpholinepropanesulfonic acid (MOPS), pH 7.0, adding 20 μl of linoleic acid, mixing thoroughly, and incubating the substrate mixture under N_2 for 10 min at 22°C . The reaction mixture contained 286 μl of substrate and 28 μl of enzyme extract in a final volume of 1 ml of 50 mM MOPS (pH 7.0). Changes in absorbance at 234 nm were followed over a 5-min period. One unit of enzymatic activity is defined as the amount of enzyme that catalyses the conversion of substrate at 1 mol/s ($\epsilon = 23,000 \text{ M}^{-1} \text{ cm}^{-1}$).

OXO activity was assayed according to the procedure of Sugiura et al. (42). Leaf fragments were ground in a mortar at 4°C in 1.5 ml of potassium phosphate buffer 100 mM (pH 7.0). The mixture was centrifuged at $12,000 \times g$ at 4°C for 5 min. Oxalic acid (2 mM) was used as a substrate in 0.1 M succinate buffer, pH 3.5. In all, 900 μl of substrate mixture and 100 μl of plant extract were preincubated at 37°C for 10 min, and then the reaction was stopped by the addition of 200 μl of 0.1 M Tris basic solution and 660 μl of a developing solution, which consisted of 8 mg of 4-aminoantipyrine, 200 μl of peroxidase (1 U/ μl), and 20 μl of N,N-dimethylaniline in 100 ml of 0.1 M potassium phosphate buffer (pH 5.8). The absorbance of the resulting solution was measured at 550 nm. One unit of the enzyme activity is defined as the amount of the enzyme which formed H_2O_2 at 1 $\mu\text{mol}/\text{min}$ in the standard assay system.

CAT activity was measured according to the procedure of Garcia-Limones et al. (20). Foliar segments were crushed at 4°C in a mortar with pestle and 1.5 ml of 0.05 M potassium phosphate buffer (pH 7.0) was added. The mixture was kept at 4°C and

centrifuged at $12,000 \times g$ for 5 min. The substrate consisted of 175 μl of 35% H_2O_2 in 100 ml of 0.05 M potassium phosphate buffer (pH 7.0). Then, 25 μl of the supernatant was placed in quartz cuvettes. The reaction was initiated by adding 975 μl of substrate. The cuvette content was rapidly homogenized using a vortex and placed in the spectrophotometer. The decrease in A240 ($\epsilon = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$) produced by H_2O_2 breakdown was recorded.

For PO activity, foliar fragments (0.1 g) were crushed and homogenized in 1 ml of ice-cold 100 mM potassium phosphate buffer, pH 7.0. Particulate material were removed by centrifugation ($12,000 \times g$, 5 min, 4°C) and the supernatant was kept at 4°C and used as crude enzyme extract. PO was assayed colorimetrically using 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as the electron donor at 20°C , using the method of Mitchell et al. (28) based on the method of Shindler et al. (41). The reaction mixture consisted of 500 μM ABTS, 250 μM H_2O_2 in 25 mM acetate buffer (pH 4.4), and the suitable volume of crude enzyme extract. The formation of the radical cation was rapidly monitored at 412 nm ($\epsilon = 32,400 \text{ M}^{-1} \text{ cm}^{-1}$).

PAL activity was measured as described by Edwards and Kessman (18) with slight modifications. Leaf areas (0.15 g) were homogenized in 1.5 ml of ice-cold extraction buffer (50 mM Tris-HCl buffer, pH 8.5; 1 mM EDTA; 5 mM dithiothreitol; Dowex 1x2 [Dow France SAS, la Plaine Saint-Denis, France] 5% HCl; and 2% PVPP). After centrifugation ($12,000 \times g$ at 4°C for 5 min), the supernatant was used as crude enzyme extract. The assay mixture consisted of suitably diluted enzyme extract in a L-phenylalanine solution (12.1 mM in 50 mM Tris-HCl, pH 8.5). Assay tubes were read against a corresponding control tube in which D-phenylalanine was substituted for L-phenylalanine. The assay was performed at 40°C , the reaction was stopped by adding 100 μl of HCl 5 M, and enzyme activity was monitored by an increase in optical density at 290 nm caused by the formation of cinnamic acid ($\epsilon = 10,000 \text{ M}^{-1} \text{ cm}^{-1}$).

In vitro germination of *B. graminis* f. sp. *tritici* conidia. After drying, petri dishes containing agar at 12 g/liter in distilled water were covered with 500 μl of OGAs at 5 g/liter in distilled water or with 500 μl of distilled water (control), each solution containing 0.025% Citowett (vol/vol). After spreading and drying, infected leaves covered by fresh 8-day-old inoculum were shaken above the petri dishes. These were then placed in a growth chamber (18°C , 12-h photoperiod, 70% relative humidity). The length and the number of germ tubes were assessed 24 h later (35).

Statistical analyses. For each experiment, the effect of treatments was statistically assayed. Analysis of variance was carried out with the statistical program Minitab (Minitab Inc., Pennsylvania). The test of Tukey was used to compare means obtained in OGA-treated plantlets with those obtained in water-infiltrated or water-sprayed controls ($P \leq 0.05$). For the enzymatic activities, data were submitted to a logarithmic transformation whereas, for the infection level, DAB staining, and auto-fluorescence recording, data were submitted to an arcsine square root transformation.

RESULTS

Characterization of OGAs obtained from PGA. Unacetylated OGAs with DP ranging from 2 to >25 were obtained by thermal degradation of PGA. After a sequence of purification, OGAs were fractionated by SEC chromatography and the proportion of each OGA was estimated. The preparation used in further experiments contained oligomers with DPs mainly of 3 to 11 (Fig. 1). Acetylated OGAs with a DS of 30% were obtained by chemical substitution of the OGA fraction. Proton nuclear magnetic resonance spectra of acetylated OGAs revealed several signals in the acetyl region (Fig. 2A). They were indicative of the presence of acetyl groups linked either on C2 or on C3 of galac-

Fig. 1. Proportion (%) of each oligogalacturonide (degree of polymerization: 2 to 25) in the purified oligomer preparation obtained from polygalacturonic acid. The proportion of each degree of polymerization was calculated by microscale colorimetric assay after fractionation of the purified oligomers.

turonan residues (Fig. 2B), as described in previous works (6). For all esterification procedures applied, the DS and the repartition of acetyl group on the galacturonan residue were always the same.

Protection efficacy of OGAs–Ac and OGAs+Ac against *B. graminis* f. sp. *tritici*. Plants were inoculated 48 h posttreatment by spraying conidia on leaves, and developed fungal colonies were counted 8 days postinfection. According to the experiment, the mean number of sporulating colonies of the water-control leaves was 55 to 110. A 100% infection rate was attributed to the mean obtained with water-control leaves. Protection against *B. graminis* f. sp. *tritici* infection was obtained for both OGAs–Ac and OGAs+Ac (Fig. 3). Indeed, infection level was significantly reduced to 57 and 58% with OGAs–Ac and OGAs+Ac, respec-

tively. Statistical analysis confirmed a similar efficacy for both treatments. In vitro germination of conidia on agar medium amended with OGAs–Ac and OGAs+Ac was evaluated as described previously (35). Proportion germinated and number and length of germ tubes were not modified on OGA-amended petri dishes when compared with water-control petri dishes (data not shown).

Effects of treatment with OGAs–Ac and OGAs+Ac on activities of defense-related enzymes. All enzyme activities were measured 48 h after treatment in infiltrated areas. Unless otherwise stated, treatments consisted of OGAs–Ac and OGAs+Ac at a 5 g/liter concentration.

OXO activity increased significantly in areas infiltrated with OGA fractions (Fig. 4). The increase reached 153% with OGAs–

Fig. 2. A, ¹H nuclear magnetic resonance spectrum (80°C) of oligogalacturonides (2 < degree of polymerization < 25; 15 g/liter) chemically acetylated; a = downfield region, b = upfield region, and c = acetyl region. H-1, H-2, H-3, H-4, and H-5: signals corresponding to protons in all α-(1-4)-linked D-galacturonic acid residues except α-H-1 and β-H-1 signals corresponding to anomeric protons of residues at the reducing end. **B,** Representation of an oligogalacturonan sequence; * = anomeric protons.

Ac and 111% with OGAs+Ac compared with the water control. Activities obtained with both OGA fractions were not significantly different from each other.

Likewise, significant increases of PO activity were observed in OGA-infiltrated areas when they were compared with water-control areas (Fig. 5). Plantlets infiltrated with OGAs–Ac or OGAs+Ac at 1 g/liter showed a very similar increase in PO activity, which reached 67 and 48%, respectively, when compared with water-control plantlets. However, when used at 5 g/liter, treatment with nonacetylated OGAs led to a higher significant level of PO activity than treatment with acetylated OGAs. The increases reached 132 and 50%, respectively, when compared with water-control plantlets.

LOX activity was enhanced by both OGA fractions but also by water infiltration (Fig. 6). Indeed, infiltrated areas, including water-infiltrated ones, exhibited significantly higher LOX activity compared with the untreated control. Nevertheless, the increase was lower in water-infiltrated areas and significantly different from OGA-treated ones. Treatments with OGAs–Ac and OGAs+Ac resulted in a similar enhancement of the enzyme activity level (83 and 68%, respectively) when compared with water-infiltrated plantlets.

Two other enzymes, CAT and PAL, were also assayed. Neither OGAs–Ac nor OGAs+Ac infiltrations affected CAT and PAL activities 48 h posttreatment (data not shown).

All these enzyme activities were also measured in upper noninfiltrated areas of OGAs-infiltrated leaves but no significant difference was observed when compared with those of water-infiltrated leaves (data not shown).

Effects of pretreatments with OGAs–Ac and OGAs+Ac on H₂O₂ accumulation in wheat cells at the penetration site of *B. graminis* f. sp. *tritici*. The accumulation of H₂O₂ at the pene-

tration site of the fungal AGT was visualized using DAB staining, and each penetration event was recorded as exhibiting either no staining, a weak staining intensity, or a strong staining intensity (Fig. 7A). In samples treated with OGAs–Ac and those treated with OGAs+Ac, the high staining intensity class represented 31 and 29%, respectively, of the total number of interaction sites. These percentages were significantly different from the 15% recorded in the water-infiltrated control leaves. However, the percentages of interaction sites that exhibited no staining or a weak staining intensity in OGA-infiltrated leaves were not significantly different from those that were observed in water-control leaves (Fig. 7B).

Effects of pretreatments with OGAs–Ac and OGAs+Ac on papilla fluorescence in wheat cells at the penetration site of *B. graminis* f. sp. *tritici* and haustoria formation. Examples of microscopic observations, such as weakly and strongly fluorescent papillae without haustorium, and whole fluorescent cell, are displayed in Figure 8. Leaves infiltrated with OGAs–Ac provided the same distribution of penetration events as water-infiltrated control leaves (Fig. 9A). However, when OGAs+Ac were used, the percentage of strongly fluorescent papillae without haustorium rose from 14.5 to 56%, whereas the percentage of weakly fluorescent papillae without haustoria dropped from 47 to 16%. Moreover, preventive treatment of wheat with OGAs+Ac resulted in a significant decrease of the number of haustoria recorded 40 h after inoculation with *B. graminis* f. sp. *tritici*. The percentage of penetration events showing a haustorium dropped from 22% in water-infiltrated plantlets to 8% in plantlets treated with OGAs+Ac. No effect of OGAs–Ac has been recorded (Fig. 9B).

Fig. 3. Efficiency of protective treatments with oligogalacturonides (OGAs) against *Blumeria graminis* f. sp. *tritici* on wheat plantlets. OGAs were sprayed at 5 g/liter 48 h before inoculation. Data are means of three independent experiments. In each experiment, 10 sprayed leaves from water-control or OGA-treated plantlets were randomly harvested 8 days postinoculation and the number of fungal colonies was counted on both faces. No significant difference was observed between untreated and water-treated control plantlets. A 100% infection level was assigned to the mean number of colonies developed on the water-control, and results obtained for each treatment were compared with this level. Bars with an asterisk are significantly different from the water-control (Tukey's highly significant difference test, $P \leq 0.05$). OGAs–Ac and OGAs+Ac = unacetylated OGAs and 30% chemically acetylated OGAs, respectively.

Fig. 4. Specific oxalate oxidase (OXO) activity in wheat leaves 48 h post-treatments with oligogalacturonides (OGAs). Data represent means of three independent experiments. Bars with an asterisk are different from both untreated and water-control plantlets (Tukey's highly significant difference test, $P \leq 0.05$). OGAs–Ac and OGAs+Ac = unacetylated OGAs and 30% chemically acetylated OGAs, respectively.

DISCUSSION

OGAs are inducers of resistance in wheat to *B. graminis* f. sp. *tritici*. In dicotyledonous plants, OGAs are known as endogenous elicitors, which are released in response to pathogen attack. They are components of cell wall pectins, which are highly complex molecules. Pectin oligosaccharides other than OGAs are reported to be released when an infection occurs. Plant cell wall pectin contents vary depending on plant species and developmental stage at both the quantitative and qualitative levels (48). As we evaluated the protective effect of OGAs in the wheat–powdery mildew pathosystem, we first verified whether the molecules had any fungistatic effect on *B. graminis* f. sp. *tritici*. Indeed, some inducers of plant defenses can also present a direct effect on the fungus. For instance, Milsana, an ethanolic extract of the plant *R. sachalinensis*, was shown to inhibit *B. graminis* f. sp. *tritici* conidia germination (35). We didn't detect any direct effect of acetylated or unacetylated OGAs on conidia germination.

We also observed the effect of pretreatments with OGAs on the pathosystem *T. aestivum*–*B. graminis* f. sp. *tritici*. Two treatment procedures were used to evaluate the protection conferred by the OGAs: infiltration and spraying. When OGAs were sprayed on wheat leaves, protective efficiency was identical for acetylated and nonacetylated molecules, reducing the infection level to 57 to 58% relative to the control. Contrary to dicotyledonous plants, natural endogenous elicitors were not yet known in monocotyledonous plants, and less was known about the elicitor potential of OGAs in these plants. That could be explained by a difference in cell wall composition between monocotyledonous and, more specifically, grasses, and dicotyledonous plants (10). The cell walls of dicotyledonous plants consist of $\approx 35\%$ pectin, whereas gramineous plants usually contain 2 to 10% pectin (48). Therefore, pectins are not considered to be fundamental elicitors in these plants. Furthermore, Morschbacher et al. (29) reported that

small OGAs (dimers and trimers of galacturonic acid) were endogenous suppressors of the disease resistance reaction in wheat against rust. Here, we obtained good levels of protection in wheat against *B. graminis* f. sp. *tritici* with the range of DP of the OGAs we tested. However, when molecules were applied by infiltration, the number of haustoria we observed 40 h postinoculation was significantly reduced following treatment with OGAs+Ac. Among all the penetration events, only 8% displayed a haustorium when plantlets were pretreated with acetylated OGAs, whereas 23% displayed a haustorium when plantlets were pretreated with nonacetylated ones, compared with water-infiltrated control plantlets. Therefore, acetylated OGAs are more efficient than nonacetylated ones when they were infiltrated, although they both provided the same protection level when they were sprayed. It might be more difficult for acetylated molecules to pass through the cuticle and, therefore, to be active because they are more hydrophilic than nonacetylated ones. This could explain the difference in effects when OGAs were sprayed or infiltrated. As mentioned by Shibuya and Minami (40), higher concentrations of OGAs are usually required to show an elicitor activity similar to that of other oligomers, and their penetrability depends more on an optimum size rather than on a specificity of recognition. Acetylation could then counteract the penetration of sprayed OGAs. Therefore, the use of alternative wetting agents has to be considered to enhance the penetration of acetylated OGAs, because they induce a larger extent of defenses compared with nonacetylated ones, provided they are available to the mesophyll cells.

OGAs elicit defense mechanisms in wheat. In order to circumvent difficulties in penetrability, OGAs were infiltrated in wheat leaves when comparing their biological activities. To assess the eliciting activity of both fractions of OGAs, several enzyme activities involved in plant defense reactions were assayed. Most of them are associated with ROS production, which is a key

Fig. 5. Specific peroxidase (PO) activity in wheat leaves 48 h posttreatments with oligogalacturonides (OGAs). Data represent means of three independent experiments. Bars with an asterisk are different from their untreated and water-treated controls; bar with two asterisks is different from both untreated and water-treated controls and other treated plantlets (Tukey's highly significant difference test, $P \leq 0.05$). OGAs-Ac and OGAs+Ac = unacetylated OGAs and 30% chemically acetylated OGAs, respectively.

Fig. 6. Specific lipxygenase (LOX) activity in wheat leaves 48 h posttreatments with oligogalacturonides (OGAs). Data represent means of three independent experiments. Bar with an asterisk is different from untreated control; bars with two asterisks are not significantly different from each other but different from both untreated and water-treated controls (Tukey's highly significant difference test, $P \leq 0.05$). OGAs-Ac = unacetylated OGAs.

element of nonspecific plant defense responses. ROS act as direct antimicrobial molecules or secondary messengers, or are involved in metabolic pathways leading to resistance (21). In different cereal–powdery mildew interactions, nonhost resistance was characterized by the accumulation of H_2O_2 which is linked to penetration resistance and the establishment of hypersensitive response cell death (45). Elicitors often act by mimicking a pathogen attack and lead to the occurrence of an oxidative burst. Therefore, in wheat leaves treated with nonacetylated and acetylated OGAs, we investigated the occurrence of modifications in H_2O_2 biosynthesis. OXO, PO, and CAT activities were assayed 48 h posttreatment in infiltrated leaves of wheat. At the same time, distinct wheat leaves that were pretreated with OGAs for 48 h were inoculated with *B. graminis* f. sp. *tritici*. DAB-staining of H_2O_2 at the penetration site of the fungus was observed microscopically.

We first measured a similar enhancement of OXO activity in plantlets treated with acetylated and nonacetylated molecules. In

wheat and other cereals, OXOs are also called germin-OXOs (G-OXOs), or simply germins, because they were first discovered in germinated embryos (26). This enzyme has been considered to be a marker of pathogen infection in powdery mildew–cereal interactions (9,25). Indeed, OXO generates H_2O_2 and CO_2 from oxalate and O_2 , and its localization inside the apoplast correlates with its involvement in defense mechanisms via the occurrence of an oxidative burst (25). In wheat leaves, germin mRNA levels increased 2 days postinfection with powdery mildew (23). Wheat plantlets that we treated with OGAs exhibited a higher OXO activity 48 h posttreatment. As described by D'Ovidio et al. (17), OGAs elicit oxidative apoplastic defenses but no evidence for an impact of the molecules on OXO activity was previously shown.

Moreover, treatments with OGAs–Ac and OGAs+Ac affected H_2O_2 accumulation at the papillae level in a similar manner. The same increase in high DAB-staining intensity was obtained with both molecules. As described by Thordal-Christensen et al. (43), DAB polymerization requires both the presence of H_2O_2 and PO

Fig. 7. Effect of treatments with oligogalacturonides (OGAs) on H_2O_2 accumulation in wheat epidermal cells at the fungal penetration site, revealed by 3,3'-diaminobenzidine (DAB) staining. Plantlets were inoculated with *Blumeria graminis* f. sp. *tritici* 48 h posttreatment with OGAs, and DAB staining was initiated 17 h postinoculation. **A**, Staining intensity classes. AGT: appressorial germ tube, C: conidia. Bar scale = 20 μ m. **B**, Repartition of penetration events according to staining intensity of H_2O_2 for each treatment with OGAs. Data represent means of three independent experiments. For each treatment, results are expressed as percentage of each DAB staining intensity class observed among 100 penetration events. Results obtained for each intensity class in treated plantlets were compared with those obtained in water-treated controls. No difference was observed between untreated and water-treated plantlets. Bars with an asterisk are significantly different from the water control (Tukey's highly significant difference test, $P \leq 0.05$). OGAs–Ac and OGAs+Ac = unacetylated OGAs and 30% chemically acetylated OGAs, respectively.

activity; therefore, staining suggested the presence of both H_2O_2 and PO. When testing PO activity 48 h posttreatment, which corresponds to the infection time, we noticed an increase whatever the OGAs used and the concentration tested (1 or 5 g/liter). Considered together, increases we measured in both OXO and PO activities could explain the enhancement of H_2O_2 -DAB-staining, because these activities were enhanced 48 h posttreatment, when infection took place. A synergy between a higher production of H_2O_2 by OXO and an enhanced PO activity could explain an increase in DAB polymerization in papillae. In OGA-treated plantlets, mechanisms involved in resistance were then elicited 48 h posttreatment and enhanced the response to the fungus's attack. Orozco-Cardenas and Ryan (30) observed that wounding induced H_2O_2 accumulation, which could be due to the release of intrinsic OGAs by a wound-induced polygalacturonase. We showed that OGA infiltration in wheat leaves enhanced H_2O_2 production. In soybean cells, an oxidative burst characterized by

H_2O_2 biosynthesis is observed after treatment with a fraction of PGA. The authors suggest a potential use of H_2O_2 for modifications of the cell wall structure but no specific enzymatic activity was measured (27). Here, for the first time, we established relationships between OXO, PO activities, and H_2O_2 production during elicitation by OGAs.

Moreover, concerning CAT activity in treated wheat leaves, no effect was observed, whatever the OGA molecules we used. CAT allows the detoxification of H_2O_2 , and transforms this molecule in H_2O . The nonactivation of CAT in our experiment corroborates the requirement of H_2O_2 production to induce defense. During a plant-pathogen interaction that induces an oxidative burst leading to programmed cell death, ROS detoxifying mechanisms are suppressed (2). Even if OGAs do not lead to a hypersensitive reaction (41), they induce ROS production. In tobacco, CAT activity was inhibited by two inducers of plant defense responses, 2,6-dichloroisonicotinic acid and salicylic acid (11). In wheat,

Fig. 8. Microscopic visualization of polyphenol-associated autofluorescence displayed in wheat epidermal cells in response to fungal penetration on upper face of wheat leaves 40 h postinoculation with *Blumeria graminis* f. sp. *tritici*. Plantlets were inoculated 48 h posttreatments with oligogalacturonides (OGAs). Observations were performed **A**, under transmitted light after trypan blue staining and **B**, under UV light. 1, Weak fluorescent papilla; 2, strong fluorescent papilla; 3, whole fluorescent epidermal cell. AGT: appressorial germ tube, PGT: primary germ tube, C: conidium, P: papilla. Bar scale = 20 μm .

elicitation with OGAs implicated H_2O_2 production but without involvement of the CAT-dependant detoxification mechanisms, because CAT activity was not enhanced. H_2O_2 seemed necessary to trigger induced resistance.

OGAs–Ac and OGAs+Ac signaling may involve the octadecanoid pathway. LOXs are enzymes involved in the first steps of the octadecanoid pathway leading to the synthesis of oxylipins. These lipid-derived molecules and some of their precursors pro-

Fig. 9. Effect of oligogalacturonide (OGA) treatments on fluorescence and haustorium formation at the fungal penetration site. Data are means of observations made on six leaves from two independent experiments. **A**, Each penetration site was observed for the fluorescence displayed under UV microscopy, and the presence or absence of haustoria was also recorded. Each class of event scored was expressed as a percentage of the total number of interaction sites. Bars with an asterisk are significantly different from the corresponding ones in untreated and water-treated controls. **B**, Percentages of haustoria at all the penetration sites considered together. Bar with an asterisk is significantly different from untreated and water-treated controls (Tukey's highly significant difference test, $P \leq 0.05$).

duced through the LOX pathway are potent signaling molecules in plant defense response. LOXs catalyse the addition of molecular oxygen to linoleic and linolenic acids, which are polyunsaturated fatty acids released from the plasma membrane by lipases. LOXs have been classified as 9- and 13-LOX, depending on the location of the oxygenation (9 or 13 position of the linoleic or linolenic acids). Both 9- and 13-LOX are potentially responsive to pathogen attack or treatment with inducers of plant defense (39,50). However, 13-LOX is involved in the biosynthesis of jasmonate, whereas 9-LOX leads to other oxylipin biosynthesis (49).

In our study, LOX activity measured in water-infiltrated control areas was significantly different from that measured in untreated leaves. Therefore, LOX was the only enzyme whose activity we tested that seemed to be affected by the infiltration of water. The increase of LOX activity in wheat in water-infiltrated areas may be related to the physical stress due to infiltration. Indeed, the application of the syringe on the leaf may cause an injury, triggering defense reactions, or the infiltration of a hydrophilic solution may modify the osmotic balance in wheat tissue and disturb LOX activity. For instance, chloroplastic LOX is induced by wounding in *Phaseolus vulgaris* and other plant species (34). However, the level we observed in OGA-treated leaves was clearly higher than that of water-infiltrated leaves and proved a significant effect of these molecules. Bohland et al. (7) showed that three isoforms of LOX were differentially induced in wheat leaves, in response to various eliciting treatments (rust fungus elicitor, chitin oligosaccharides, chitosan, and methyl jasmonate) applied by infiltration. We showed here that both OGAs–Ac and OGAs+Ac enhanced LOX activity. In wheat, the octadecanoid pathway is then responsive to both OGAs–Ac and OGAs+Ac. In tomato, plant-derived OGAs (with DP close to 20) activated plant defensive genes through the octadecanoid pathway, leading to jasmonate production (16). Jasmonate, precursor molecules, or other oxilipins could also be involved in OGA signaling pathways in wheat.

In addition to the octadecanoid pathway leading to oxylipin synthesis, we also investigated the phenylpropanoid pathway, leading to salicylic acid and polyphenolic compounds synthesis. PAL is the starting enzyme of the phenylpropanoid pathway, involved in many physiological processes, such as synthesis of salicylate and other phenolic compounds. In our study, PAL activity was not responsive to OGAs in infiltrated leaves 48 h posttreatment. Morschbacher et al. (29) showed that very small oligomers, such as dimer and trimer of PGA, reduced PAL activity in infiltrated wheat leaves, whereas pentamers and hexamers were inactive. In this study, we tested OGAs with a large range of DP of 2 to 25. According to biochemical assessments, the majority consisted of OGAs with DPs of 3 to 11. PAL activity was not affected by this oligomer mixture 48 h posttreatment, whereas PO, OXO, and LOX activities were enhanced after treatment. After infection, no modification in polyphenolic compound accumulation at the penetration site of the fungus was observed with OGAs–Ac, whereas we noticed a significant increase after treatment with OGAs+Ac. Because PAL activity leads to polyphenolic compound synthesis and its increase is observed in infectious conditions only, one could suggest that PAL could be potentiated with acetylated OGAs and only activated when the fungus attacks the leaf, therefore suggesting a priming effect of these molecules on the phenylpropanoid pathway.

No defense response was elicited by OGAs in upper non-infiltrated leaf areas. In our study, all enzymes we assayed in infiltrated areas from noninoculated leaves were also tested in their upper noninfiltrated areas. No significant difference was noticed between OGA-treated and control plantlets. OGAs generated in planta consecutively to a pathogen attack are relatively immobile and are considered to be localized inducers (16). They are supposed to set off signals via the octadecanoid pathway,

leading to the expression of defensive genes. Their receptors are not yet known but some oligouronides interact with tomato and potato plasma membrane and cause the phosphorylation of specific plasma membrane proteins and ion fluxes (19). In our study, no enzymatic activity was affected in noninfiltrated upper areas. We conclude that no eliciting effect appeared in upper areas; nevertheless, potentialization may have occurred and systemic induction of defense remains to be investigated (12).

The range of biological activities of OGAs is modified by a 30% acetylation. OGAs–Ac and OGAs+Ac displayed partially different effects on PO activity in wheat leaves, on autofluorescence, and on haustoria frequency detected at the penetration site of the fungus.

Plant POs lead to the generation of ROS via their hydroxylic cycle and to the synthesis of physical barriers against the pathogen's intrusion via their peroxidative cycle. They catalyze the reduction of H_2O_2 and allow the oxidation of various compounds, including the oxidative coupling of phenolics or lignin precursors (32). When treated with both OGA fractions, wheat leaves exhibited a higher PO activity 48 h posttreatment. However, when 5-g/liter solutions were used, the enhancement was significantly higher with nonacetylated than with acetylated molecules. At 21 h postinoculation, the response of leaves to the fungus intrusion was observed under fluorescence microscopy. This allowed the observation of natural fluorescence of polyphenolic compounds, ascribed to lignins (21). Fluorescence was observed in the papillae formed at the site of penetration of the fungus. Surprisingly, only OGAs+Ac-treated plantlets showed a higher rate of strongly fluorescent papillae than the control plants; nonacetylated molecules did not affect the intensity of fluorescence of the papillae. In contrast, PO activity was clearly enhanced with both molecules and, particularly, with nonacetylated ones. This shows that the increase assayed in PO activity with nonacetylated molecules is not correlated with the occurrence of polymerized phenolic compounds. H_2O_2 and POs could contribute to cross-linking of other cell wall components, such as extensin, a proline-rich protein (24).

The significant decrease in haustoria formation observed only with acetylated molecules could be the result of the enhancements measured in OXO and PO activities, leading to the production of H_2O_2 and polyphenolic compounds, respectively. On the other hand, in wheat leaves treated with nonacetylated OGAs, the occurrence of haustoria was not affected when compared with the controls. However, OXO and PO activities were enhanced and H_2O_2 accumulated in papillae, whereas no effect was noticed on papillae autofluorescence. Therefore, a partially different mode of action exists for nonacetylated and acetylated OGAs. In plant cell walls, the degree of acetylation of native OGAs differs from plant to plant (8). The role of OGA acetylation in plant defenses had not yet been investigated. Vander et al. (47) showed that the degree of acetylation of chitosan modifies its mode of action, and that partially acetylated chitosans resulted in a maximum elicitation in wheat leaves. However, little is known about the link between acetylation of molecules and resulting enhanced induced resistance.

In conclusion, OGAs–Ac and OGAs+Ac are inducers of defense in wheat leaves, leading to protection. As described in our experiments, a partially different mode of action exists between both fractions (OGAs–Ac and OGAs+Ac) and the 30% chemical acetylation of OGAs confers new properties to these molecules. It leads to a significant decrease in haustoria formation and to an increase in accumulation of autofluorescent compounds in papillae of infiltrated wheat leaves. As a perspective to our study, we could suggest that different naturally substituted pectin oligomers could be tested for their resistance-inducing potential in wheat. As alternative methods to fight pathogen attack, oligosaccharides may offer a large choice of molecules able to induce different defense reactions.

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