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Wheat cells accumulate a syringyl-rich lignin during the hypersensitive resistance response

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Abstract

The stem rust fungus *Puccinia graminis* f.sp. *tritici* is an obligately biotrophic pathogen attacking wheat (*Triticum aestivum*). In compatible host/pathogen-interactions, the fungus participates in the host's metabolism by establishing functional haustoria in the susceptible plant cells. In highly resistant wheat cultivars, fungal attack is stopped by a hypersensitive response of penetrated host cells. This mechanism of programmed cell death of single plant cells is accompanied by the intracellular accumulation of material with UV-fluorescence typical of phenolic compounds. A similar reaction can be induced in healthy wheat leaves by the application of a rust-derived elicitor. We analysed the biochemical composition of this defense-induced phenolic material. Contents of total soluble and cell wall esterified and etherified phenolic acids were determined in rust-inoculated and elicitor-treated leaves of the fully susceptible wheat cultivar Prelude and its highly resistant, near-isogenic line Prelude-Sr5. While no resistance-related changes occured in any of these fractions, the lignin content as determined by the thioglycolic acid and the acetyl bromide methods increased after elicitor treatment. Nitrobenzene oxidation revealed that the entire increase can be explained by an increase in syringyl units only. These biochemical data were confirmed by fluorescence emission spectra analyses which indicated a defense-induced enrichment of syringyl lignin for cell wall samples both from elicitor-treated wheat leaves and single host cells undergoing a hypersensitive response upon fungal penetration.

Keywords: *Triticum aestivum*; Wheat; *Puccinia graminis*; Hypersensitive resistance; Phenolic acids; Syringyl lignin

1. Introduction

Since its detailed description by Stakman (1915), the infection of wheat (*Triticum aestivum*) by its pathogen *Puccinia graminis* f.sp. *tritici*, the causal agent of wheat stem rust, has become one of the most studied systems for analysing the interaction of an obligately biotrophic fungus and its host plant. In susceptible wheat plants, the stem rust fungus invades its host through stomates and establishes functional haustoria within host cells as sophisticated organelles allowing the pathogen to participate in the host's metabolism. The uptake of nutrients is required for further colonisation of the host tissue and the subsequent completion of the fungal life cycle by generating new uredospores (Mendgen and Hahn, 2002). The high reproductive potential can lead to epidemic expansion of plant disease underlining the agricultural significance of this fungus. In highly resistant wheat cultivars, infected plant cells react hypersensitively and die within a few hours after penetration. This mechanism of programmed cell death prevents the formation of a functional haustorium and efficiently stops further infection (Tiburzy et al., 1990). The hypersensitive response is accompanied by the intracellular accumulation of lignin or lignin-like material indicated by the typical yellow autofluorescence under UV-light and by histochemical stains (Beardmore et al., 1983; Tiburzy and Reisener, 1990). Time studies of the formation of fungal haustoria and the occurrence of hypersensitive host cell death (Tiburzy et al., 1990) as well as of the activity of the lignin biosynthetic pathway (Moerschbacher

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et al., 1988), and inhibitor studies affecting different key enzymes in lignin biosynthesis (Moerschbacher et al., 1990; Tiburzy and Reisener, 1990) indicate a direct causal involvement of cellular lignification in hypersensitive cell death and, consequently, resistance (Moerschbacher and Reisener, 1997).

Although more complicated in molecular detail (Boerjan et al., 2003), lignins are, in general, radically polymerised aromatic compounds composed of the differently substituted phenolic monolignols *p*-coumaryl-, coniferyl- and sinapyl-alcohol which are produced via the phenylpropanoid pathway (Barber and Mitchell, 1997; Whetten et al., 1998). Gramineaceous lignins are mainly derived from all three of these monolignols resulting in *p*-hydroxyphenyl, guaiacyl, and syringyl units. In contrast, dicot lignins lack *p*-hydroxyphenyl units, and gymnosperm lignins are substantially built of guaiacyl units only (Higuchi, 2003). In addition, gramineaceous cell walls contain large amounts of covalently bound phenolic acids, mainly *p*-coumaric and ferulic acid (Iiyama et al., 1990).

As both lignin and phenolic acids behave similarly concerning autofluorescence and histochemistry, the question arises to what extent phenolic acids and lignin contribute to the accumulating autofluorescing material in infected resistant plants (Matern and Kneusel, 1988). We have, therefore, analysed the accumulation of cell-wall esterified and etherified phenolic acids and of lignin, as well as the monolignol composition in wheat cells penetrated by a fungal haustorium or treated with a fungal elicitor. While the pools of phenolic acids exhibited no resistance-related changes, a syringyl-rich lignin was found to be deposited in the hypersensitively reacting wheat cells.

2. Results and discussion

First we determined the contents of phenolic acids in rust-inoculated and healthy control leaves of the fully susceptible wheat cultivar Prelude and its highly resistant isoline Prelude-Sr5 (Fig. 1). Two and seven days after inoculation, the amounts of free phenolic acids, total soluble phenolic acids (free and esterified), and phenolic acids esterified to the cell walls were determined. All fractions from both cultivars were dominated by ferulic acid and *p*-coumaric acid, and the vast majority was esterified either to soluble compounds (Fig. 1, top) or to the cell wall (Fig. 1, bottom). Since the contents of free phenolic acids were very low (<2 μg (g fr. wt)$^{-1}$, not shown), they hardly contributed to the total soluble fractions. Thus, observed small changes in free phenolic acid contents upon fungal attack are unlikely to be involved in resistance and may be due to the difficult evaluation of chromatograms near

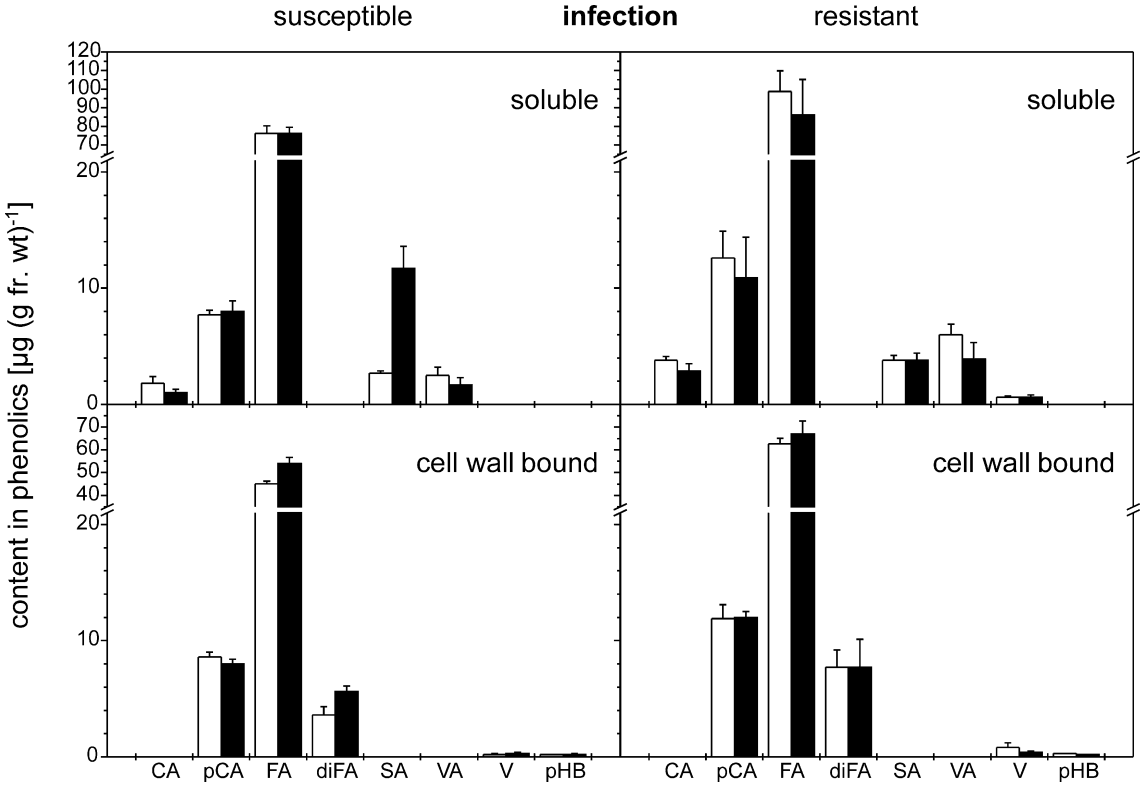

Fig. 1. Content in phenolics of susceptible and resistant wheat primary leaves seven days past inoculation with uredospores of the stem rust fungus. Total soluble phenolics and cell wall esterified phenolics were determined in primary leaves of untreated control plants (white columns) and rust-infected plants (black columns). The phenolics found were caffeic acid (CA), *p*-coumaric acid (pCA), ferulic acid (FA), diferulic acid (diFA), sinapic acid (SA), vanillic acid (VA), vanillin (V), and *p*-hydroxybenzoic acid (pHB). Data are means ± s.d. of three independent experiments; fr. wt: fresh weight.

the limit of detection. However, even in the other fractions, neither successful nor restricted infections caused distinct changes in the amounts of major or minor phenolic acids. Only the content of soluble sinapic acid (SA) esters increased clearly (ca. 4-fold) seven days after inoculation of the susceptible cultivar but not in the resistant one. No infection-related changes were seen in any of the fractions two days after inoculation (data not shown).

Table 1 shows the lignin contents of rust-inoculated and healthy control wheat leaves. No increases were observed even in the resistant cultivar although penetrated plant cells reacted hypersensitively and exhibited yellow autofluorescence under UV light, indicative of cellular lignification (Beardmore et al., 1983; Tiburzy and Reisener, 1990). But since in highly resistant plants, this defence mechanism restricts infection very effectively, further fungal invasion of the plant is prohibited by the hypersensitive reaction of a few cells only. These strongly localised reactions precluded a chemical characterisation of the fluorescent material deposited in the hypersensitive cells.

To overcome the difficulties in detecting weak infection-induced changes in phenolic compounds in front of a strong constitutive background, we injected an elicitor inducing a hypersensitive-like lignification response into the apoplast of intact healthy wheat leaves (Moerschbacher et al., 1986a,b). As no significant differences in the action of the Pgt-elicitor were observed previously in studies comparing the reactivity of different near-isogenic wheat lines (Moerschbacher et al., 1989; Sutherland et al., 1989), only one line (Prelude-Sr5) was used in this study. The phenolic acid contents were similar to those found in the inoculated leaves (Fig. 2). Again, neither two (data not shown) nor seven days after injection, significant changes were observed except for a 4-fold increase of soluble esterified sinapic acid seven days after elicitor-application, comparable to the situation in fully susceptible leaves upon infection (Fig. 1).

The lignin content of elicitor-treated wheat leaves was determined using two independent methods (Table 2). In both cases, an increase in lignin content due to elicitation was evident. However, the thioglycolic acid-based method yielded an increase of 160% compared to the water-treated control leaves whereas the method based on acetyl bromide exhibited a rise of ca. 50% only. In addition to lignin, both methods also record esterified as well as etherified phenolic acids if present in the sample, compromising lignin determinations. Phenolic esters were removed during sample preparation by saponification. The content of phenolic ethers were calculated from the total amount of phenolic acids released under ether-cleaving conditions minus the saponified fraction. Table 3 summarises the results concerning the major phenolic acids demonstrating that neither the esters (as shown above) nor the ethers contribute

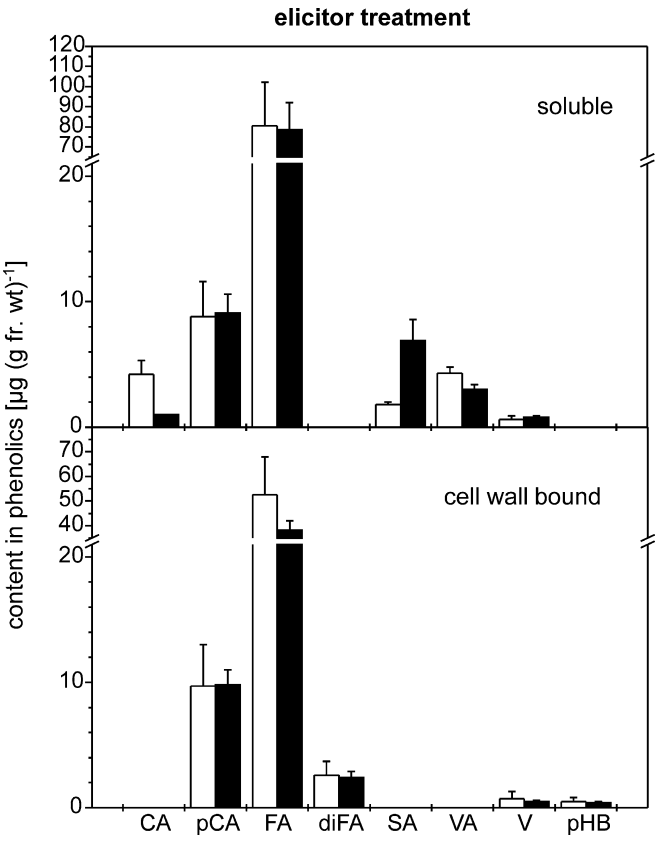

Fig. 2. Content in phenolics of wheat primary leaves seven days past injection of a genuine elicitor of the hypersensitive reaction (Pgt-elicitor, 40 µg glc-equiv ml^{-1}) into the apoplastic space. Total soluble phenolics and cell wall esterified phenolics were determined in primary leaves of untreated control plants (white columns) and rust-infected plants (black columns). The phenolics found were caffeic acid (CA), *p*-coumaric acid (pCA), ferulic acid (FA), diferulic acid (diFA), sinapic acid (SA), vanillic acid (VA), vanillin (V), and *p*-hydroxybenzoic acid (pHB). Data are means ± s.d. of three independent experiments; fr. wt: fresh weight.

Table 1
Lignin contents in stem rust-infected susceptible (Prelude) and highly resistant (Prelude-Sr5) wheat primary leaves seven days past inoculation

Treatment	Prelude [mA (mg cw)$^{-1}$]	Prelude-Sr5 [mA (mg cw)$^{-1}$]
Control	152 ± 8	160 ± 18
Inoculated	170 ± 12	155 ± 5

mA, milliabsorption units; cw, cell walls. Data were determined by the thioglycolic acid method and are means ± s.d. of three independent experiments.

Table 2
Increase of lignin contents in wheat primary leaves 14 days past elicitor-treatment

Treatment	Method	
	Thioglycolic acid [mA (mg cw)$^{-1}$]	Acetyl bromide [% (w/w) cw]
Control	75 ± 11	11.3 ± 1.1
Elicited	193 ± 5	17.0 ± 1.1
Increase (%)	160	50

mA, milliabsorption units; cw, cell walls. Data are means ± s.d. of at least three independent experiments.

Table 3
Contents of the major esterified and/or etherified cell wall bound phenolic acids of wheat primary leaves 14 days past elicitor-treatment

Treatment	*p*-Coumaric acid [% (w/w) cw]			Ferulic acid [% (w/w) cw]		
	$\sum$	Esterified	Etherified	$\sum$	Esterified	Etherified
Control	0.12 ± 0.00	0.08 ± 0.01	0.04 ± 0.01	0.58 ± 0.03	0.22 ± 0.02	0.36 ± 0.05
Elicited	0.12 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.60 ± 0.04	0.18 ± 0.01	0.42 ± 0.05

cw: cell walls; $\sum$: sum of esterified and etherified phenolic acid; contents of etherified phenolic acids were calculated by subtraction. Data are means ± s.d. of triplicate samples.

significantly to the elicitor-induced increase in lignin content.

These results exclude an accumulation of phenolic acids in the hypersensitive resistance of wheat to stem rust. Neither the contents in free phenolic acids or soluble phenolic acid conjugates, nor the contents in cell wall bound, esterified or etherified phenolic acids changed significantly during an infection-induced or elicitor-triggered hypersensitive reaction. The only exception was an increase in the content of a soluble conjugate of sinapic acid in elicitor-treated wheat leaves, small in absolute values but significant compared to untreated control plants. This increase was also seen in susceptible, but not in resistant wheat leaves during rust infection and might be evidence of a detoxification reaction induced by excesses of phenolic acids. We have postulated that the full hypersensitive reaction in infected leaves is regulated by the recognition of a series of molecular triggers, only one of which is the fungal glycoproteogalactan used in this study (El Gueddari and Moerschbacher, 2004; Kogel et al., 1988; Moerschbacher et al., 1986a). As stated previously, this elicitor very strongly induces lignin biosynthetic enzyme activities, but the amount of lignin produced is comparatively low (Moerschbacher et al., 1986a,b). Thus, application of the glycoproteogalactan elicitor alone may lead to an overflow of the sinapic acid produced into a detoxifying conjugation reaction. The same detoxification reaction may be responsible for the generation of the sinapic acid conjugate in rust infected, susceptible plants. We have shown previously that the lignin biosynthetic pathway is initially induced in both resistant and susceptible wheat leaves following rust inoculation (Moerschbacher et al., 1988). However, lignin biosynthesis occurs only in hypersensitively reacting cells in resistant plants (Tiburzy et al., 1990). We have proposed the action of an endogenous suppressor produced by the invading fungus in susceptible host tissue only, preventing execution of the hypersensitive lignification response, thus allowing further fungal development (Moerschbacher et al., 1999; Wiethölter et al., 2003). In the absence of lignin polymerisation, accumulating phenolics may activate the detoxifying overflow valve leading to the conjugation of sinapic acid.

For further characterisation of the resistance induced lignin, the monolignol composition was analysed using alkaline nitrobenzene oxidation (Fig. 3). Based on lignin determinations via the acetyl bromide method, the total yield of lignin monomers was ca. 10%. As expected for

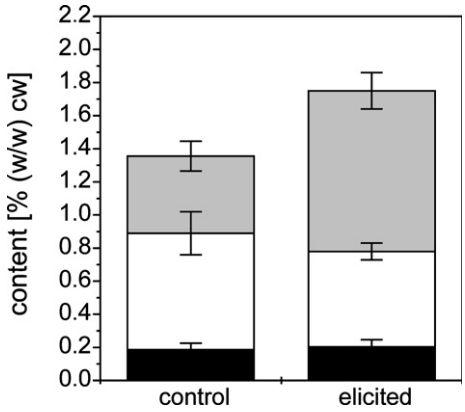

Fig. 3. Lignin composition of wheat primary leaves 14 days past injection of a genuine elicitor of the hypersensitive reaction (Pgt-elicitor, 400 μg glc-equiv ml^{-1}) into the apoplastic space. Control leaves remained untreated. The lignin building units *p*-hydroxyphenyl (black), guaiacyl (white) and sinapyl (grey) were determined by nitrobenzene oxidation of purified cell wall (cw) material. Data of stacked columns are means ± s.d. of three replicate measurements from one experiment; similar results were obtained in a second, independent experiment.

Gramineae, the lignin of the untreated control plants was composed mainly of guaiacyl and syringyl units with small amounts of *p*-hydroxyphenyl units. As demonstrated above, the total lignin content increased upon elicitor treatment. Interestingly, this increase was caused exclusively by an increase in syringyl units. The syringyl to guaiacyl ratio shifted from 0.55 to 1.43. The above described occurrence of a soluble conjugate of sinapic acid only, and not of the more prevalent *p*-coumaric and ferulic acids, is in agreement with the deposition of a syringyl-rich or possibly even pure syringyl lignin during the hypersensitive resistance response. The specific activation of a syringyl lignin biosynthetic pathway in elicitor treated wheat leaves was previously suggested by the induction of a sinapyl alcohol specific isoform of cinnamyl alcohol dehydrogenase (Mitchell et al., 1999). A syringyl-rich lignin was also induced in wheat leaves upon inoculation with non-pathogenic fungi (Ride, 1975), so that this disease resistance mechanism appears to be operating on the level of both non-host resistance and race/cultivar-specific resistance. Differences in the monolignol composition between the lignin formed during the normal development of healthy plants and resistance-related lignin in infected plants have been described in a number of host/pathogen interactions (Vance et al., 1980). In these cases, however, the infection-induced lignin was typically rich in guaiacyl or

p-hydroxyphenyl units. This observation has been explained by the more extensive possibilities of cross-linking these monolignols offer compared to syringyl units. These differing cross-linking properties also explain the apparent differences observed in this study when different methods of lignin quantification were used. Thioglycolic acid cleaves semi-aryl etherbridges only, while acetyl bromide also cleaves C–C-bonds and diarylethers common in lignins rich in guaiacyl or *p*-hydroxyphenyl units. The lack of 5-5′-biphenyl and 4-O-5′-diarylether units in the elicitor-induced syringyl lignin may render it more accessible to the thioglycolic acid method than the constitutive lignin, explaining the higher relative increase detected with this method compared to the more aggressive acetyl bromide method. However, the reaction mechanism of the thioglycolic acid method is not well understood at present, compromising these conclusions (Anterola and Lewis, 2002; Brinkmann et al., 2002). As both chemical methods allow the analysis of only a rather small part of the lignin, typically around 10%, spectroscopic methods need to be used to analyse the entire sample.

Fluorescence emission spectra analysis confirmed the results obtained by chemical analyses (Fig. 4). In reference lignins, the fluorescence maxima of a syringyl-rich reference lignin preparation from *Eucalyptus regnans* is bathochromatically shifted by ca. 15 nm compared to a syringyl-poor reference lignin from *Pinus radiata* (Fig. 4A). A similar shift was exhibited by the emission spectrum of cell wall samples from elicitor-treated wheat leaves compared to those of water-treated control leaves (Fig. 4B). On the microscopic level, the fluorescence maxima of single cells, both after elicitor treatment and after fungal penetration of resistant wheat leaves, show a statistically significant ($P < 0.01$) bathochromatic shift of ca. 10 nm compared to those recorded from xylem vessels (Fig. 4C). No significant difference was observed between the spectra of HR cells triggered by elicitor treatment and fungal infection. These observations indicate that the defense-induced accumulation of syringyl-rich lignin is not restricted to elicitor treatment but also occurs in the authentic incompatible interaction between the stem rust fungus and its host.

3. Experimental

3.1. Plants and fungus

Two near-isogenic lines of the wheat cultivar Prelude (*Triticum aestivum* L.), initially provided by Dr. Rohringer (Rohringer et al., 1979) were multiplied at the Max-Planck-Institute for Plant Breeding, Cologne. The parent cultivar Prelude is fully susceptible to race 32 of the wheat stem rust fungus, yielding infection type 4, while the near isogenic line Prelude-Sr5 is highly resistant to that race (infection type 0) (Tiburzy et al., 1990). Plants were raised in prefertilized soil at 20 °C and 70% relative humidity under a 16/8 h photoperiod (15 klx in the visible range) in automatically regulated growth chambers.

Uredospores of race 32 of the stem rust fungus (*Puccinia graminis* Pers. f. sp. *tritici* Erics. & E. Henn.) were cultivated on six-week-old plants of the fully susceptible wheat

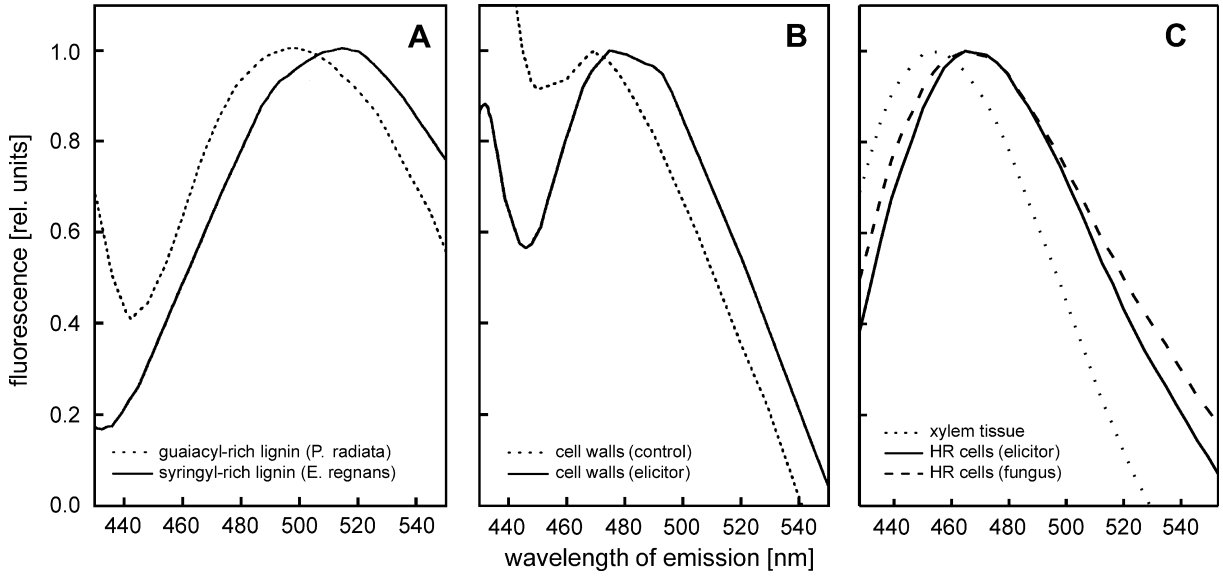

Fig. 4. Fluorescence emission spectra of lignified cell walls. (A) Reference-samples of defined syringyl-poor (*Pinus radiata*, syringyl:guaiacyl-ratio 0.01:1, dotted line) and syringyl-rich (*Eucalyptus regnans*, syringyl:guaiacyl-ratio 3.47:1, solid line) lignin preparations (Lam et al., 1990). (B) Purified cell wall samples of wheat primary leaves 14 days past injection of a genuine elicitor of the hypersensitive reaction (Pgt-elicitor, 400 μg glc-equiv ml^{-1}) into the apoplastic space (solid line); control leaves remained untreated (dotted line). (C) Confocal laser scanning microscopic fluorescence emission spectra of lignified xylem vessels (dotted line) and of hypersensitively reacted mesophyll cells triggered by elicitor treatment (solid line) or fungal infection (dashed line) in destained resistant wheat leaves four days past inoculation with uredospores of the stem rust fungus; spectra shown are means of replicate measurements of at least ten single cells per treatment.

cultivar Little Club (*T. compactum* L.). Inoculation of seven-day-old test plants was performed with spores suspended in 1,1,2-trichlorotrifluoroethane (Moerschbacher et al., 1988). Control plants were sprayed with the solvent only.

Infection types (Prelude: IT 4; Prelude-Sr5: IT 0;) and infection levels (ca. 100 infection sites per cm^2) were verified prior to the chemical analyses.

3.2. Pgt-elicitor

The genuine glycoproteogalactan elicitor (Pgt-elicitor) of the hypersensitive response was isolated from germinated uredospores of the stem rust fungus as described previously (Moerschbacher et al., 1986a). Prior to injection, elicitor solns. were diluted to give 40 or 400 μg carbohydrates (glucose equivalents, glc-equiv) per ml, as indicated. Carbohydrate content was determined using Anthrone reagent (Morris, 1948). Aqueous elicitor solutions were injected into the intercellular spaces of seven-day-old primary wheat leaves using a hypodermic syringe (Kogel et al., 1985; Moerschbacher et al., 1986a). Control leaves were injected with water only.

3.3. Extraction of phenolic compounds

The injected leaf areas extending ca. 3 cm to both sides of the injection site were harvested at the indicated times after injection, and immediately frozen in liquid nitrogen. Phenolic compounds were extracted from these leaf sections as described earlier (Bokern et al., 1987; Strack et al., 1987), with modifications. One gram of frozen leaf-sections were powdered in liquid nitrogen, then ground for 80 s with 10 ml of 50% aq. MeOH in a mortar. The suspension was stirred for 2 h at 20 °C in the dark, soluble compounds were separated from the insoluble residue by filtration, and the residue was washed twice with 50% aq. MeOH. The cell wall containing residue was frozen for further treatment. The clear filtrates were combined, vacuum-dried at 45 °C, and redissolved in 5 ml of hot water (ca. 60 °C). This cell extract contained the total soluble fraction of phenolic compounds.

The fraction of *free phenolic acids* was obtained by acidifying an aliquot of the cell extract with HCl to a pH below 1.5 and extracting the protonated phenolic acids twice with an excess of Et_2O. The combined Et_2O extracts were evaporated to dryness and redissolved in a defined volume of 25% aq. MeOH for HPLC-analysis (see below).

To obtain the *total soluble phenolic acids* (free and esterified), an aliquot of the cell extract was diluted twofold, then saponified with an equal volume of degased 1 N NaOH. The solution was saturated with nitrogen, incubated for 16 h at 20 °C in the dark, before extracting phenolic acids as above.

Cell wall esterified phenolic acids were released from the cell wall containing residue by saponification following successive washings with MeOH:$CHCl_3$ (2:1, v/v), Me_2CO, and water. The purified cell wall fraction was then saponified with 8 ml of degased and nitrogen-saturated 1 N NaOH, incubated for 16 h at 20 °C in the dark, before extracting phenolic acids as above.

For the determination of *cell wall etherified phenolic acids*, 10 g of frozen leaf-sections were powdered in liquid nitrogen, and the soluble components were removed by successive extractions with excess 50% aq. MeOH (×2), MeOH:$CHCl_3$ (2:1, v/v, ×2), Me_2CO (×2), EtOH, and water (×3). The resulting residue, named purified cell wall, was treated according to Iiyama et al. (1990). About 30 mg of freeze-dried purified cell walls was incubated with 5 ml of 4 N NaOH for 2 h in a stainless steel pressure vessel at 170 °C (1 N NaOH and 20 °C for the esterified fraction). After the addition of 50 μl of a solution containing an internal standard (0.5% syringic acid in MeOH), the hydrolysate was centrifuged ($10^4 g$, 10 min), the supernatant was acidified, and the liberated phenolic acids were extracted successively with excess CH_2Cl_2 and Et_2O. The combined organic phases were washed with water, dried with water-free sodium sulfate, then evaporated to dryness. For GC-analysis (see below) the residue was resuspended in a small amount of Et_2O, transfered to an appropriate vial, and dried under a nitrogen stream.

3.4. Determination of lignin

The determination of lignin contents via the *thioglycolic acid based method* was performed as described by Moerschbacher et al. (1989), with minor modifications. Briefly, 30 mg of purified cell walls were saponified (see above) prior to lignin determination to eliminate any phenolic acids. To a defined aliquot of these pretreated cell walls, 5 ml of 2 N HCl and 0.2 ml of thioglycolic acid were added. After incubation for 4 h at 100 °C, the sample was cooled down and centrifuged ($2 \times 10^3 g$, 10 min). The pellet was washed twice with water and then incubated with 5 ml of 0.5 N NaOH for 16 h at 20 °C. Insoluble material was removed by centrifugation ($2 \times 10^3 g$, 10 min), and the pellet was resuspended twice in 2.5 ml of distilled water. The combined extracts were adjusted to pH 3 using 6 N HCl, then incubated at 4 °C for 16 h to facilitate crystallisation of lignothioglycolic acid complexes. The pellet of the subsequent centrifugation ($9 \times 10^3 g$, 15 min, 4 °C) was washed with 1 ml of 0.1 N HCl, then redissolved in 1 ml of 0.5 N NaOH. After removing insoluble material by centrifugation ($10^4 g$, 3 min), the absorbance of the supernatant was recorded at 280 nm.

The *acetyl bromide based method* for lignin determination was carried out according to Iiyama and Wallis (1988). Briefly, 10 mg of vacuum-dried purified cell walls were suspended in HOAc (5 ml) containing 25% (v/v) acetyl bromide and 0.1 ml 70% perchloric acid. The sealed vessel was shaken gently and incubated for 30 min at 70 °C. After cooling down, the reaction mixture was transfered to a 50 ml volumetric flask containing 10 ml of 2 N NaOH and 12 ml of HOAc. The flask was adjusted to 50 ml with

HOAc, shaken, and after sedimentation of insoluble material, the absorption of the clear solution. was recorded at 280 nm. Lignin content was calculated using the extinction coefficient (20.0 l g^{-1} cm^{-1}) for gramineaceous lignin (Iiyama and Wallis, 1988).

The determination of monolignol contents was performed using the *alkaline nitrobenzene oxidation* optimised for wheat lignin, exactly as described previously (Iiyama and Lam, 1990).

3.5. Chromatography

Phenolic acids were separated by high-performance liquid chromatography (HPLC) using an LKB-system and a reversed-phase column (Waters, Nova Pak C18, 150 mm × 3.5 mm i.d.) operated with a set of linear elution profiles (eluent A: MeOH:acetonitrile (1:1, v/v), eluent B: 10 mM ammonium formiate, pH 3.0; gradients: 0 min: 10% A, 20 min: 14% A, 30 min: 20% A, 60 min: 30% A). Prior to injection, cinnamic acid was added to the samples as an internal standard. The compounds were identified and quantified by comparison with authentic reference substances (relative retention times, spectra, calibration curves) using a diode array-detector (Beckmann).

Etherified phenolic acids and products released after alkaline nitrobenzene oxidation were analysed using gas liquid chromatography (GC) on a HP-system (series II 5890, FID) with a BP-1 bonded-phase (0.25 μm) vitreous silica capillary column (25 m × 0.25 mm i.d.). The temps. were 280 °C for the injector and 290 °C for the flame ionisation detector (FID). The ramping-profiles depended on the samples analysed (phenolic acids: (a) 10 min at 230 °C, (b) 5 °C min^{-1} to 290 °C, (c) 10 min at 290 °C; products of alkaline nitrobenzene oxidation: (a) 5 min at 160 °C, (b) 3 °C min^{-1} to 290 °C, (c) 12 min at 290 °C). Prior to injection, the dried samples were trimethylsilylated using *N*,*O*-bis(trimethylsilyl) acetamide at 105 °C for 10 min. Identification and quantification of compounds was done by comparison (calibration factors, co-migration) with authentic reference substances.

3.6. Fluorescence emission spectra analysis

For spectral analysis of purified cell walls and lignins, appropriate samples were milled and suspended in glycerol. Fluorescence emission spectra were recorded from 420 nm to 620 nm at an excitation wavelength of 410 nm using a fluorescence spectrophotometer (Hitachi 650-10s). The reference lignins isolated from syringyl-rich (*Eucalyptus regnans*, syringyl:guaiacyl-ratio 3.47:1) and syringyl-poor (*Pinus radiata*, syringyl:guaiacyl-ratio 0.01:1) wood species, respectively, were generous gifts from Dr. Lam (Lam et al., 1990).

Emission spectra of xylem tissue and of single epidermal and mesophyll cells which had undergone hypersensitive cell death upon fungal penetration were recorded from leaf tissues destained with 70% ethanol using a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Heidelberg). Spectra were recorded from 430 nm to 550 nm at an excitation wavelength of 364 nm from at least ten cells per treatment, and data given represent the mean to the fluorescence emission maxima.

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