Phenylalanine Ammonia-lyase, Tyrosine Ammonia-lyase, and Lignin in Wheat Inoculated with Erysiphe graminis f. sp. tritici

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ABSTRACT

The host-parasite response of an *Erysiphe graminis* f. sp. *tritici* race 21 (avirulent to *Pm1, Pm2, Pm3a, Pm3b,* and *Pm4* genotypes)-inoculated isoline of wheat containing the *Pm2* gene was distinguishable from responses of inoculated *pmx, Pm1, Pm3a, Pm3b,* and *Pm4* isolines on the basis of lignin accumulation and level of phenylalanine ammonia-lyase (PAL). Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activity increased significantly in all inoculated genotypes beginning 4 hours after inoculation, reached a peak at 24 hours after inoculation, and then decreased sharply by 48 hours after inoculation. More than twofold increases in PAL were noted at 4 hours after inoculation, which is prior to the time of appressorial development. In the inoculated *Pm2* genotype, the OAL and TAL activity again increased sharply beyond 48 hours, and reached a maximum 96 hours after inoculation. Beyond 48 hours after inoculation, all other inoculated genotypes which were assayed continued to decrease in PAL and TAL activity to levels near that of the noninoculated controls. The isoline containing the *Pm2* gene was the only isoline in which an accumulation of lignin could be detected histochemically 92 hours after inoculation.

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Additional key words: disease resistance, powdery mildew, isolines.

The gene-for-gene hypothesis has been reported to be applicable to the powdery mildew-wheat interaction (17). Ellingboe (6, 7) and Slesinski and Ellingboe (20, 21) have used near-isolines to determine the synchrony of some genes for disease resistance in the powdery mildew-wheat interaction. Their established time sequence for the morphologic expression of resistance in each of several genes for resistance provides reference points for

histochemical and physiological changes attributable to the presence of each of these genes.

Shaw and Samborski (19) reported that radioactivity accumulated at infection sites when phenylalanine-U-^{14}C was fed to mildew-infected leaves. Ellingboe (7) reported that there is a darkening of the collar region in powdery mildew-infected wheat containing the *Pm4* gene for resistance. These reports suggest that there is an increase in synthesis or accumulation of phenolics in the powdery mildew-wheat interaction as proposed by Green (10). Furthermore, the recent reports of lignin accumulation in other host-parasite interactions (1, 8) suggest that phenolics in resistant host-parasite interaction may be accumulating in the form of lignin. This study was therefore initiated to examine two aspects. First, whether lignin accumulates in the resistance reaction of any of the near-isolines. Second, since lignin is believed to be synthesized by phenolic precursors derived primarily from the deamination of L-phenylalanine and possibly also from L-tyrosine (9), the levels of PAL and TAL activities were assayed to estimate the relative role of these two pathways.

MATERIALS AND METHODS.—*Culture of pathogen.—Ersiphe graminis* DC. f. sp. *tritici* Em. Marchal race 21 (22) was maintained on the Red Bobs cultivar of wheat in a growth chamber at approximately 18 ± 2 C. A light regime, beginning at 0800 hours was maintained on a 10-hour light/14-hour dark cycle using Gro-Lux fluorescent tubes with a light intensity of approximately 5,380 lx (500 ft-c). The culture was assayed on the *pmx*, *Pm1*, *Pm2*, *Pm3a*, *Pm3b*, and *Pm4* genotypes. The infection types, as used by Briggle (2), for these lines were 4, 0-1, 2, 1, 3, and 0, respectively.

Near-isolines of wheat were maintained in a growth chamber separate from the mildew culture until they were approximately 15 cm tall. Plants to be inoculated were then moved into the mildew chamber and inoculated by shaking the conidia-bearing plants over the near-isolines. The inoculum density, ascertained by staining with trypan blue and counting the number of conidia on 10 randomly selected leaves, was determined to be 175 ± 25 conidia per cm^2. Enzyme levels are subject to variation within a 24-hour day-night period. Therefore, inoculations were scheduled so that most extractions would occur at 1400 hours each day. On the first day the plants were inoculated at 0600 hours for the 4- and 8-hour period, at 2200 hours for the 12-hour period and at 1400 hours for all other subsequent assay periods.

Histochemical procedures.—Inoculated leaves were collected at 68, 92, 114, 138, and 186 hours after inoculation. The apical 7-cm of the leaves were excised and immediately immersed in hot 95% ethanol for 2 hours. They were then transferred to cold 95% ethanol for 24 hours with one change of ethanol. After 24 hours, the leaves were removed and placed in a clearing solution (20 g of chloral hydrate in 12 ml of H_2O) for 24 hours. The leaves were then assayed for lignin using the phloroglucinol test (11), the chlorine-sulfite test (12), and the Maule test (4). Two to four replications of 10-15 leaves per replication were used for each near-isoline.

Enzyme extraction and assay.—One and one-half grams of the apical 7-cm of leaf tips were cut and immediately ground in a cold mortar containing 6 ml ascorbate-borate buffer (0.5% sodium ascorbate and 0.05 M sodium borate, at pH 8.8) 1 g glass beads, and 0.1 g Polyclar. This, and subsequent, extractive operations were carried out at 4 C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000 *g* for 10 minutes. The supernatant was assayed immediately for PAL and TAL using slightly revised procedures of Koukol and Conn (14) and of Minamikawa and Uritani (16), respectively. The reaction mixture contained 1.5 ml of enzyme homogenate, 200 μmoles of borate buffer (pH 8.8) and 1 μmole of L-tyrosine with 6.66×10^5 dpm L-tyrosine-U-^{14}C or 2 μmoles of L-phenylalanine with 2.22×10^5 dpm L-phenylalanine-U-^{14}C. The final volume of the mixture was 2.7 ml. The reaction mixture was incubated for 2 hours at 37 C in glass centrifuge tubes, and then terminated with 0.3 ml of 6 N HCl. The *p*-coumaric acid or cinnamic acid was extracted by overlaying the reaction mixture with 10 ml of toluene, stirring vigorously on a Vortex mixer, and centrifuging for 5 minutes at 20,000 *g*. A 5-ml aliquot of the toluene layer was transferred to a toluene-based scintillation counting solution, and the incorporated radioactivity was quantitated using a Nuclear-Chicago liquid scintillation counter.

RESULTS.—The results of all three histochemical tests for lignin were positive for the inoculated near-isolines of wheat containing the *Pm2* gene, but negative for all other inoculated and noninoculated genotypes assayed (Table 1). Leaves of *Pm2* genotypes collected 68 hours after inoculation did not give a lignin reaction, while those checked 92 hours or more after inoculation gave a positive lignin reaction. The phloroglucinol and chlorine-sulfite tests had two observable zones within each reaction site after the lignin test. Beneath the mycelium (stained with trypan blue) there was a brown to reddish-brown reaction several cells in diameter and two or more cells in depth. Within this brown zone, one or two cells developed a deep red color around the cell walls. As the time after inoculation was increased beyond 92 hours, the area of reaction became larger with a darker center. The Maule test showed only a brown reaction without any signs of red.

PAL activity in all inoculated near-isolines increased to levels two to four times greater than that of the noninoculated controls 4 hours after inoculation, four to seven times greater by 24 hours, and then decreased rapidly by 48 hours after inoculation (Table 2). The PAL activity in all inoculated near-isolines, except those containing the *Pm2* locus, continued to decline, reaching a low point 72 hours after inoculation, and thereafter remained relatively low. The PAL activity in the *Pm2* genotype increased between 48 hours and 96 hours after inoculation, resulting in an activity level nearly nine times greater than that of the noninoculated controls. Variations in enzyme activity also occur in noninoculated tissue, as has been noted earlier with other plant species (18). The TAL activity showed increases and decreases similar to that of the PAL activity, but at a much lower level (Table 3). Enzyme activity values of noninoculated control tissues were subject both to variations in host genotypes and to variations associated with differing stages of differentiation which occur with increasing age of the plant.

DISCUSSION.—The more than twofold increase in extractable activity of PAL within 4 hours is impressive,

TABLE 1. The lignin response of near-isolines of wheat 92 or more hours after inoculation with *Ersiphe graminis* f. sp. *tritici* race 21

Near-isoline[a]	Host gene designation[b]	Infection type[c]	C.I. Number[d]	Lignin response[e]
Chancellor (Cc)	*pmx*	4	12333	−
Axminstar × Cc^8	*Pm1*	0-1	14114	−
CI13836 × Cc^8	*Pm1*	0-1	14115	−
AsII × Cc^8	*Pm1*	0-1	14116	−
Norka × Cc^8	*Pm1*	0-1	14117	−
Ulka × Cc^8	*Pm2*	2	14118	+
CI12632 × Cc^8	*Pm2*	2	14119	+
Asosan × Cc^8	*Pm3a*	1	14120	−
Chul × Cc^8	*Pm3b*	3	14121	−
Khapli × Cc^8	*Pm4*	0	14123	−
Yuma × Cc^8	*Pm4*	0	14124	−

[a] Cc^8 refers to eight backcrosses to Chancellor.
[b] Gene designation from Slesinski and Ellingboe (20).
[c] Reaction types of Briggle (2).
[d] Seeds acquired from the U.S. Department of Agriculture, Agricultural Research Service, Plant Science Research Division, Plant Introduction Station, Beltsville, Maryland.
[e] The "+" indicates that lignin accumulation was histochemically detected in and around the infection sites using the phloroglucinol, chlorine-sulfite, and Maule tests. The "−" indicates the absence of histochemically detectable lignin accumulation in and around infection sites.

TABLE 2. Phenylalanine ammonia-lyase activity in leaves of wheat inoculated and noninoculated with *Erysiphe graminis* f. sp. *tritici*

			Time after inoculation[b]							
Near-isoline	gene	inoculation[a]	4	8	12	24	48	72	96	144
Chancellor (Cc)	*pmx*	+	47±1	70±3	93±17	419±28	100±4	61±17	150±10	110±5
		−	20±1	23±1	42±1	43±2	72±1	82±3	84±12	20±2
AsII × Cc^8	*Pm1*	+	80±1	141±4	64±11	375±71	192±14	139±18	76±6	73±4
		−	22±1	17±2	26±1	41±4	45±1	49±23	55±16	19±4
12632 × Cc^8	*Pm2*	+	82±1	107±5	121±20	398±47	139±6	490±19	596±13	435±21
		−	26±1	15±1	33±1	37±1	44±5	84±13	68±10	73±28
Asosan × Cc^8	*Pm3a*	+	69±7	81±9	177±1	292±51	185±20	119±8	87±2	56±11
		−	22±7	19±4	30±2	36±8	63±20	102±3	67±10	25±12
Chul × Cc^8	*Pm3b*	+	69±5	96±14	138±27	404±30	196±13	123±5	79±7	54±1
		−	18±2	19±3	48±7	60±5	45±16	107±32	37±6	30±3
Khapli × Cc^8	*Pm4*	+	55±4	98±11	112±21	441±15	132±3	83±8	73±6	35±11
		−	15±3	19±1	18±1	121±8	55±8	46±29	32±6	114±7

[a] + = inoculated; − = noninoculated.
[b] Expressed as nanomoles of cinnamic acid per hour per gram of tissue. Confidence intervals are the average deviation of two replications from the mean.

TABLE 3. Tyrosine ammonia-lyase (TAL) activity in leaves of near-isolines of wheat inoculated and noninoculated with *Erysiphe graminis* f. sp. *tritici*

			Time after inoculation[b] (hours)							
Near-isoline	gene	inoculation[a]	4	8	12	24	48	72	96	144
Chancellor (Cc)	*pmx*	+	0.7±0.1	0.7±0.1	1.4±0.3	4.9±0.9	2.0±0.1	1.3±0.5	3.7±0.4	3.5±0.1
		−	0.5±0.0	0.5±0.0	0.1±0.0	0.4±0.0	1.2±0.0	1.6±0.1	1.2±0.3	0.1±0.1
AsII × Cc^8	*Pm1*	+	0.6±0.1	1.0±0.2	0.6±0.1	3.1±1.5	4.0±0.1	2.7±0.3	1.2±0.1	1.2±0.1
		−	0.6±0.0	0.2±0.1	0.0±0.0	0.0±0.0	0.8±0.0	0.9±0.4	0.8±0.2	0.2±0.1
12632 × Cc^8	*Pm2*	+	0.7±0.1	0.7±0.1	2.1±0.7	4.3±0.6	1.9±0.1	10.7±1.2	14.4±1.9	11.6±0.1
		−	0.5±0.0	0.4±0.1	0.1±0.0	0.4±0.1	0.6±0.1	1.7±0.2	0.8±0.0	1.4±0.3
Asosan × Cc^8	*Pm3a*	+	0.8±0.1	1.5±0.1	2.7±0.3	5.4±0.8	4.8±0.2	2.2±0.2	1.2±0.1	0.7±0.0
		−	0.6±0.1	0.3±0.1	0.2±0.1	0.4±0.2	1.3±0.4	2.3±0.0	1.3±0.0	0.4±0.1
Chul × Cc^8	*Pm3b*	+	0.8±0.1	0.7±0.0	2.4±0.5	4.9±0.9	4.0±0.4	2.9±0.1	1.4±0.2	0.6±0.0
		−	0.5±0.0	0.2±0.0	0.2±0.0	0.4±0.1	0.9±0.4	2.5±0.7	0.6±0.1	0.5±0.3
Khapli × Cc^8	*Pm4*	+	0.6±0.1	0.7±0.0	1.8±0.5	3.8±0.1	2.7±0.0	1.8±0.2	1.2±0.2	0.6±0.1
		−	0.4±0.0	0.4±0.1	0.0±0.0	2.5±0.3	0.8±0.2	0.9±0.7	0.4±0.0	3.0±0.1

[a] + = inoculated; − = noninoculated.
[b] Expressed as nanomoles of *p*-coumaric acid per hour per gram of tissue. Confidence intervals are the average deviation of two replications from the mean.

considering the low percentage of plant cells which are in contact with the fungus at this time. This early increase suggests that the host-parasite interaction is induced prior to appressorium development (6) and papilla (collar) formation (5, 15), and that it required only the presence of a germinating conidium on the leaf surface to induce the initial host response. The similarities of the PAL activity for all six inoculated genotypes through the first 48 hours after inoculation also indicate that increased PAL activity is a common reaction for this host-parasite interaction and initially does not appear to be a specific response of a given resistance reaction. It is only between 48 hours and 72 hours after inoculation that the *Pm2* locus can be distinguished from the other genotypes on the basis of increased PAL activity. Approximately 90 hours after inoculation the *Pm2* locus is distinguished on the basis of histochemically-detectable lignin. Although the *Pm2* reaction results in lignin accumulation in and around the infected host cell walls, a substantial part of the lignin accumulates at a time subsequent to the expression of resistance (7). The rapid increase in PAL activity between 48 and 72 hours after inoculation suggests that a portion of this latter response overlaps the period in which the morphological expression of this interaction occurs (7). Lignin accumulation may be only a portion of the total *Pm2* resistance response. Hypersensitive necrosis, as seen in the *Pm2* resistance reaction, has recently been viewed as a consequence rather than a cause of disease resistance (3, 13).

TAL activity in terms of μmoles of product deaminated per gram of tissue extracted was considerably lower than the PAL activity. These data suggest that the TAL pathway may have only a secondary role in synthesizing shikimic acid-derived lignin precursors in powdery mildew-infected wheat leaves. Also, these results are consistent with the interpretation that a given host-parasite interaction influences the levels of multiple enzymes or proteins (23), even though the ultimate control of resistance is localized within a given locus.

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