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Effect of Chemical Systemic Acquired Resistance Elicitors on Avenanthramide Biosynthesis in Oat (*Avena sativa*)

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ABSTRACT: Oats produce a group of phenolic antioxidants termed avenanthramides. These metabolites are, among food crops, unique to oats and have shown, in experimental systems, certain desirable nutritional characteristics such as inhibiting atherosclerotic plaque formation and reducing inflammation. Avenanthramides occur in both the leaves and grain of oat. In the leaves they are expressed as phytoalexins in response to crown rust (*Puccina coronata*) infection. The experiments reported here demonstrate that avenanthramide levels in vegetative tissue can be enhanced by treatment with benzothiadiazole (BTH), an agrochemical formulated to elicit systemic acquired resistance (SAR). The response to BTH was dramatically stronger than those produced with salicylic acid treatment. The roots of BTH treated plants also showed a smaller but distinct increase in avenanthramides. The dynamics of the root avenanthramide increase was substantially slower than that observed in the leaves, suggesting that avenanthramides might be transported from the leaves.

KEYWORDS: benzothiadiazole, phytoalexin, phenylpropanoid, secondary metabolism

INTRODUCTION

Oats produce a group of secondary metabolites termed avenanthramides. These compounds are conjugates of a phenylpropanoid or avenalumic acid linked via an amide bond to an anthranilic acid moiety or a hydroxylated or methoxylated derivative of anthranilic acid.[1,2] There are at least 25 congeners of the avenanthramides,[1] and they appear both in the grain and in vegetative tissue. Avenanthramides 2p, 2f and 2c[3,4] are usually the most highly represented in the grain (Figure 1). The avenanthramides function as phytoalexins in the leaf tissue in response to crown rust infection[5−7] and in cultivars containing the *Pc-2* locus in response to Victoria blight (*Cochiobolus victoriae*).[6,8] A physiological function for the avenanthramides in grain tissue is undetermined.

In addition to their role as phytoalexins there is increasing evidence that avenanthramides provide beneficial health effects in mammals. Studies have shown that avenanthramides are bioavailable in hamsters and provide antioxidant activity.[9] Avenanthramide 2c and its methyl ester were shown to inhibit several benchmarks for atherosclerotic plaque formation in *in vitro* cell culture systems.[10,11] Treatment of endothelial cells and vascular smooth muscle cells with avenanthramide 2c increased NO production in these cell types, potentially enhancing vasodilation and reducing blood pressure in humans.[12] In aortic smooth muscle cells 2c inhibited the hyperphosphorylation of restinoblastosis protein, preventing cell cycle progress. Inhibition of interlukin-1β induced nuclear factor kappa beta (NF-$\kappa\beta$) activation appears to be another mechanism by which avenanthramides reduce plaque formation.[13] A similar mechanism was shown to effectively reduce chemically induced skin inflammation.[14] Avenanthramides were also recently shown to inhibit oleic acid induced steatohepititis (an inflammatory response to fatty acid deposition in the liver) in cultured human hepatocytes.[15]

Although the avenanthramides appear to have beneficial health effects, there are few published reports on efforts to enhance their levels in the oat crop. Problematic is that grain avenanthramide levels are strongly influenced by environmental factors,[16] one of which appears to be crown rust occurrence.[17] Crown rust (*Puccinia coronata*) infection of oat leaves strongly elicits avenanthramide biosynthesis in leaf tissue[7,18,19] as does other molecular mimics of fungal infection.[20,21] Systemic acquired resistance (SAR) is a generalized response in plants to microbial infection that provides long-term resistance in plant tissues distal to the site of infection.[22] There are commercially available chemical stimulants of systemic acquired resistance (SAR). One of the first such compounds was 2,6-dichloroisonicotinic acid (INA), but it proved too detrimental to the plant to be of practical value.[23] More recently a benzothiadiazole derivative, *S*-methylbenzo[1,2,3]thiadiazole-7-carbothiate (acibenzolar-S-methyl, BTH), sold under the trade name Actigard, has found practical application. Neither the benzothiadiazole derivatives nor INA shows any antimicrobial action on its own, but they appear to mimic salicylic acid activation of natural resistance (reviewed in ref 24). Here we describe the effects of BTH treatment on oat with respect to elicitation of avenanthramide production and the upregulation of hydroxycinnamoyl CoA: hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT), the final enzyme in the biosynthetic pathway for avenanthramides, in oat leaf and root tissue.

MATERIALS AND METHODS

Oat cv. 'Belle' was planted in 15.5 cm diameter ×14 cm high pots in a soil formulation of 50:50 peat moss and commercial top soil with 250 mg of Osmocote 14:14:14 (N,P,K) per liter. Plants were grown either in a growth chamber at 21 °C under a 12 h light/dark cycle or in a green house under similar conditions of light and temperature. Unless

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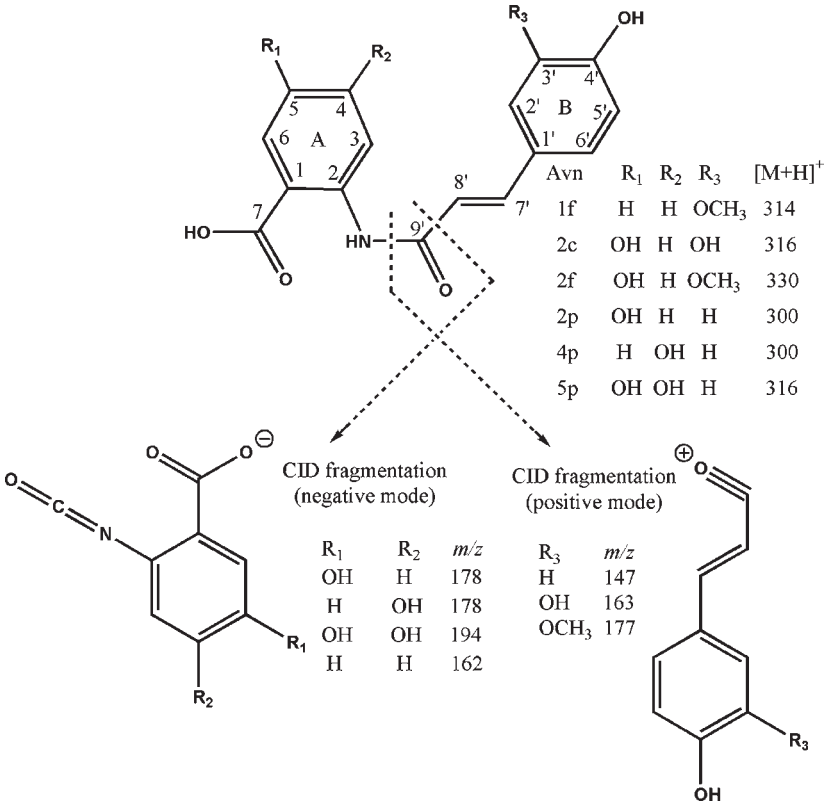

Figure 1. Generalize structure of avenanthramides and the major fragmentation products by LC−MS in an ion-trap mass spectrometer. Collision induced dissociation (CID) in the negative mode results in the isocyanato benzoic acid derivative of the anthranilic acid ring from the parent compound as a major fragment. CID in the positive mode results in the acyl cation of the phenylpropanoid moiety from the parent compound.

otherwise stated, treatments with BTH were administered when the plants reached the Z13 (three leaf) stage.[25]

BTH (Actigard) in a 50:50 w/w formulation with an emulsifying carrier was supplied by Syngenta. Treatment was administered as a root soak in 250 mL of distilled H_2O at various concentrations of active ingredient. Leaves and other tissues were harvested, flash frozen in liquid nitrogen and stored at −70 °C until extracted and analyzed. Unless otherwise stated, three replicate plants were harvested and analyzed separately for each observation. Fresh frozen tissues were extracted by grinding with a mortar and pestle in liquid nitrogen until a fine powder. From 3 to 3.5 g was placed in a tared 50 mL centrifuge tube and the weight recorded. The ground tissue was extracted with 3 × 10 mL of 80% EtOH in 10 mM NaH_2PO_4 buffer, pH = 2 in a shaker/water bath at 50 °C. After each extraction the sample was centrifuged 5 min at 1800*g*. The supernatants were pooled into a 50 mL round-bottom flask and rotary evaporated under vacuum until dry. The residue was resuspended in 1.0 mL of MeOH and filtered through a 0.2 μm nylon membrane (National Scientific, Rockwood, TN).

Avenanthramides were analyzed by HPLC (Shimadzu SPD-M10A, Kyoto, Japan) on a 4.6 × 50 mm C-18, 5 μm column (Supelco Discovery HS, Bellefonte, PA, USA) using a photo diode array detector (Shimadzu 10A, Kyoto, Japan) with monitoring at 280 and 330 nm. The mobile phase consisted of buffer A, H_2O with 5% acetonitrile and 0.1% formic acid, and buffer B, acetonitrile with 0.1% formic acid. A gradient of 13 to 30% B over 20 min at a flow rate of 1.0 mL/min was employed. All analyses were made with 10 μL injections at room temperature (approximately 22 °C). Avenanthramides 2p and 2f were quantified by comparison of their peak areas at 330 nm with those of a standard curve developed using the corresponding authentic avenanthramide standards. Avenanthramide 4p and 5p were quantified based on the standard curve for 2p.

LC−MS Analysis. LC−MS analysis was performed on an Agilent 1100 liquid chromatography system with a 1946 series ion-trap mass spectrometer. A 2.1 × 30 mm C-18 column (Zorbax SB-C18, Agilent, Santa Clara, CA) was employed with the same solvent system as above at a flow of 0.2 mL/min. The column was operated at 30 °C with 2.0 μL injections. Detection was made by diode array spectrometry monitoring absorbance at 280 and 330 nm and by ion-trap mass spectrometry. Electrospray ionization parameters were as follows: nebulizer gas (N_2) 30 psi, dry gas flow at 6.0 L/min at 350 °C with a capillary voltage set at 3500 V. The ion trap was operated in either the positive mode or the negative mode depending on the analysis made (see Results), scanning from m/z 100 to 1000 (or 100 to 400 for MS2) at 13,000 m/z s^{-1}.

Synthesis of Avenanthramide Standards. Approximately 5 mmol of the appropriate phenylpropanoid (*p*-coumaric, ferulic or caffeic acid) in 2.0 mL of pyridine was acetylated in excess acetic anhydride (10 mL) and allowed approximately 5 h to react. Cold water (50 mL) was added and the solution left at 4 °C for one hour. The precipitant was collected in a Hirsch funnel and washed several times with cold H_2O. The precipitant was dried overnight at 50 °C. The acetylated phenylpropanoid was dissolved in 10 mL of dimethylformamide (DMF) with 1.25 mL of triethylamine. An equimolar amount of benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was dissolved in 5 mL of CH_2Cl_2 and added dropwise to the acetylated phenylpropanoid while stirring on an ice bath. Next a solution of 5-hydroxyanthranilic acid (equimolar to the protected phenylpropanoid) in 20 mL of DMF was added dropwise to the solution. After stirring an additional 30 min on ice the reaction mixture was removed to room temperature and allowed to react an additional two hours. The reaction was stopped by adding 80 mL of 0.5 M HCl and stored overnight at 4 °C. After extraction of the acetoxy avenanthramide into ethyl acetate

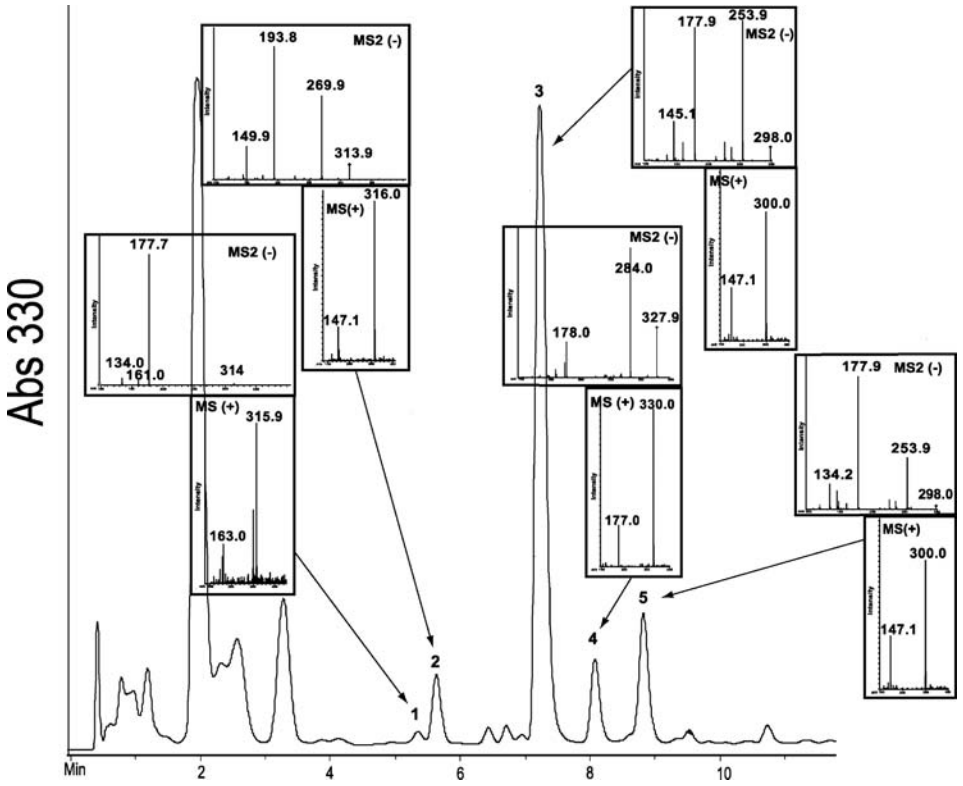

Figure 2. LC−MS chromatogram of a leaf extract treated with 10 mM at 96 h post treatment. Chromatogram shows a UV_{330} trace; insets are the positive ionization MS and negative ionization MS2 spectra. Peaks: 1, avenanthramide 2c; 2, avenanthramide 5p; 3, avenanthramide 2p; 4, avenanthramide 2f; 5, avenanthramide 4p.

and rotary evaporation to dryness, the protecting acetyl groups were removed by addition of 5% pyrollidine in CH_2Cl_2 (10 mL) for approximately 20 min at room temperature. The deprotection reaction was quenched with 20 mL of 1 M HCl and the avenanthramide extracted into ethyl acetate. Purification of the avenanthramide was effected by LH-20 column chromatography as described by Collins.[1] Avenanthramides 2p and 2f were further purified by crystallization in MeOH and H_2O (2c did not crystallize in this manner). The avenanthramides were produced in approximately 40% yield; analysis by LC−MS and ^{1}H NMR was consistent with published data.[1] ^{1}H NMR (400 Mz, CD_3OD, δ): (2c) 8.45 (d, J = 9.0, H-3), 7.50 (d, J = 15.5, H-7′), 7.51 (d, J = 3.0, H-6), 7.07 (d, J = 1.7, H-2′), 7.03 (dd, J = 9.0, 3.0, H-4), 6.97 (dd, J = 8.2, 1.7, H-6′), 6.79 (d, J = 8.2, H-5′), 6.46 (d, J = 15.6, H-8′); (2f) 8.46 (d, J = 9.0, H-3), 7.57 (d, J = 15.6, H-7′), 7.51 (d, J = 3.0, H-6), 7.23 (d, J = 1.5, H-2′), 7.10 (dd, J = 8.2, 1.6, H-6′), 7.03 (dd, J = 9.0, 3.0, H-4), 6.82 (d, J = 8.2, H-5′), 6.57 (d, J = 15.6, H-8′), 3.92 (s, 3H, C3′-OCH_3); (2p) 8.45, (d, J = 9.0, H-3), 7.57 (d, J = 15.6, H-7′), 7.51 (d, J = 3.0, H-6), 7.49 (d, 2H, J = 8.6, H-2′,6′), 7.03 (dd, J = 9.0, 3.0, H-4), 6.82 (d, 2H, J = 8.6, H-3′,5′), 6.53 (d, J = 15.6, H-8′).

HTT Assay. Coumaryl-CoA was biosynthesized using a cloned 4-hydroxy-cinnamate:CoA ligase (4-CL, EC6.2.1.12) from tobacco kindly provided by Dr. Till Beuerle (Technical University, Braunschweig) and prepared following procedures described previously.[26] To a volume of 116 mL of 200 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, pH = 7.5, was added 11.2 mg (68 μmol) of *p*-coumaric acid, 28.0 mg (31 μmol) of CoA, and 240 mg (0.44 μmol) of ATP dissolved in 4 mL of 95% EtOH. The reaction was initiated by adding approximately 1.0 mg of the partially purified 4-CL and was monitored by observing the increased absorbance at 333 nm in periodic UV spectra. After 90 min the reaction was stopped by placing the reaction vessel on ice. The coumaryl-CoA was immediately isolated and purified by chromatography on a 2 g (12 mL) PrepSep C-18 column (Fisher Scientific, Pittsburgh, PA) pre-equilibrated with 50 mM MOPS buffer (pH = 7.5). The column was washed with 6 column volumes of MOPS buffer, then 1 column volume of dH_2O. The coumaryl-CoA was eluted with 100% MeOH. The eluate was rotovaped to near dryness and resuspended in HCl acidified H_2O (pH = 4.0), and the volume was adjusted to a final concentration of 3.0 mM as determined by the UV absorbance at 333 nm using an extinction coefficient of 21 mM^{-1}-cm^{-1}.[27]

A 250 mg sample of leaf tissue was placed in a 2.0 mL aluminum bead beater vial with 1.0 mL of ice cold 100 mM Bistris buffer, pH = 7.2 with 2 mM dithiothreitol (DTT). The samples were homogenized by 3 × 30 s shaking on a Biospec Bead Beater (Bartlesville, OK). The vials were immediately placed on ice before centrifuging, at 4 °C, for 20 min at 14000*g*. The supernatant was transferred to a microfuge tube and kept on ice until assayed. HHT assays were conducted as previously described;[28] duplicate 10 μL aliquots were assayed for each observation.

RNA Hybridization (Northern Blot). Total RNA was isolated from approximately 1.0 g of frozen (−70 °C) leaf tissue, using Tripure (Roche, Indianapolis, IN) according to the vendor's instructions. The polyA RNA (mRNA) was extracted from total RNA with a Poly AT tract kit (Promega, Madison, WI). cDNA was then generated using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). A 523 base pair (bp) fragment of HHT corresponding to nucleotides 352 to 874 of AsHHT2 (GenBank accession AB076981[8]) was amplified from cDNA isolated from induced tissue using primers HHTF3, 5′-TGGTGCC-CTTCTACCCGATG-3′ (forward), and HHTR3, 5′-GCGGGA-CAGCTTGAAGATGTC-3′ (reverse). This fragment was cloned into Topo TA, sequenced and termed pHHT1F3R3. Plasmid pHHT1F3R3 was digested with *Eco*RI and the HHT coding insert gel purified on agarose and used as template to generate a ^{32}P labeled probe by random hexamer priming with ^{32}P dCTA using standard methods.[29]

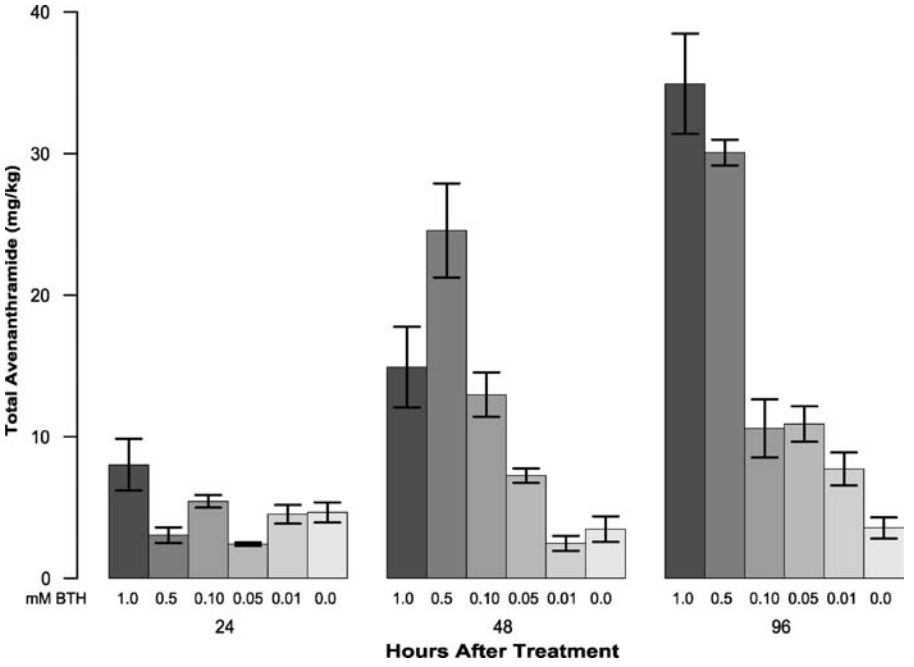

Figure 3. Total avenanthramide levels in oat seedlings treated by root soaking with 1.0, 0.5, 0.10, 0.05, 0.01, and 0.0 mM (active ingredient) BTH. Leaves from the treated plants were harvested at 24, 48, and 96 h after treatment. Bar heights represent leaf tissue mean total avenanthramide level of three plants. Error bars represent ± standard error of mean.

A 454 bp barley PR-1 sequence (GenBank accession Z21494) was previously cloned by PCR and represents most of the transcript sequence.[30] Probe synthesis, RNA blot probing and washing were done according to Sambrook.[29] The hybridization and wash temperature for PR-1 was 55 °C. Blots were initially probed with HHT, stripped, and then reprobed with the PR-1 probe.

RESULTS

The analysis and quantification of the avenanthramides was performed using an HPLC system with a diode array detector. In the early trials BTH treatment resulted primarily in upregulation of avenanthramide 2p with minor amounts of 2c/5p (see below) and 2f as based on similarities in retention times to authentic standards. Selected samples were analyzed on LC−MS to confirm these identifications. In later experimental samples, with more robust responses to BTH treatment, the LC−MS system revealed two peaks at an elution time close to that of authentic avenanthramide 2c (Figure 2). These were not effectively separated on our HPLC. The second peak was identified as avenanthramide 5p (see below). Unfortunately, the crude leaf extracts tended to clog the electrospray needle, requiring frequent cleaning, thus we did not use the LC−MS for routine analysis of all the samples.

Identification of Avenanthramides. The peaks corresponding to avenanthramide 2p, 2f, and 2c were identified by retention time identity with authentic, synthesized avenanthramide standards, the characteristic ratio of UV absorbance at 330 and 280 nm, and were confirmed by LC−MS. A later eluting peak, avenanthramide 4p, was identified based on retention time identity with the isolated natural product previously characterized.[28] With LC−MS in positive ionization mode this peak (peak 5, Figure 2) yielded an $[M + H]^+$ $m/z = 300$ with an acyl cation fragment resulting from in-source fragmentation $[M_2]^+$ $m/z = 147$, i.e. essentially identical to 2p but with a longer HPLC retention time. An additional metabolite eluting immediately after avenanthramide 2c was also observed and tentatively identified as 5p (peak 2, Figure 2). This identification was based on the $[M + H]^+$ $m/z = 316$, with a substantial fragment $[M_2]^+$ $m/z = 147$ consistent with the acyl cation of *p*-coumaric acid. To further characterize this metabolite we analyzed a high yielding sample by collision induce ionization (MS2) in the negative ionization mode on the selected $[M - H]^-$ $m/z = 314$. In the negative ion mode cleavage of the parent ion between the carbonyl carbon and the double bonded methine results in an isocyanato benzoate derivative of the anthranilate ring. In the case of avenanthramide 5p we observed the expected $m/z = 194$ consistent with a parent molecule having 3,4-dihydroxyanthranilic acid for the A ring. MS2 in the negative mode of avenanthramides 2c, 2f, 2p and 4p all result in major fragments with $m/z = 178$ consistent with the 5-hydroxyanthranilic acid A ring. To further confirm this analysis we subjected synthetic avenanthramide 1p (*N*-(4′-hydroxy)-(*E*)-cinnamoyl anthranilic acid) to the same mass spectral analysis. As expected we found a major MS2 fragment with $m/z = 178$ consistent with isocyanato benzoate. Note also that avenanthramide 5p elutes immediately after avenanthramide 2c and before 2p on C-18 reverse phase chromatography.[31] The UV adsorption spectrum of peak 2 (Figure 2) is nearly identical to authentic avenanthramide 2p (data not shown). With these data the compound in peak 2 is thus identified as avenanthramide 5p. Additional avenanthramides, tentatively identified as 3f (*N*-(4′-hydroxy-3′-methoxy)-(*E*)-cinnamoyl 5-hydroxyanthranilic acid) and 4pd (*N*-(4′-hydroxy)-(*E*,*E*)-avenalumyl-5-hydroxyanthranilic acid) based on mass spectral data and relative retention times, were also observed in some of the treated leaf extracts but only in minor amounts. Because avenanthramides 2c and 5p were not separated on the HPLC system, they are quantified together. However, it should be noted that 5p appears to be the congener

Table 1. Avenanthramide Levels in Leaves of Plants Treated with Low Concentrations of BTH

	BTH treatment (mM)[a]					
Avn	1.0	0.5	0.1	0.05	0.01	0.0
			24 h after Treatment			
5p/2c	1.15 ± 0.39[b]	0.29 ± 0.08	0.97 ± 0.26	0.27 ± 0.03	0.42 ± 0.08	0.71 ± 0.22
2f	0.92 ± 0.22	0.25 ± 0.04	0.58 ± 0.15	0.40 ± 0.26	0.53 ± 0.18	0.53 ± 0.13
2p	4.27 ± 1.11	1.38 ± 0.32	2.46 ± 0.27	0.69 ± 0.14	1.82 ± 0.29	2.22 ± 0.48
4p	1.67 ± 0.14	1.13 ± 0.19	1.42 ± 0.22	1.05 ± 0.13	1.74 ± 0.13	1.19 ± 0.14
total	8.01 ± 1.82	3.05 ± 0.56	5.43 ± 0.45	2.41 ± 0.12	4.51 ± 0.66	4.65 ± 0.74
			48 h after Treatment			
5p/2c	1.39 ± 0.33	2.89 ± 0.51*	2.13 ± 0.49*	0.66 ± 0.49	0.14 ± 0.03	0.31 ± 0.12
2f	1.00 ± 0.15	1.93 ± 0.28*	1.88 ± 0.27*	0.84 ± 0.06	0.21 ± 0.05	0.42 ± 0.17
2p	10.59 ± 2.57*	18.27 ± 3.51*	7.42 ± 0.95	4.20 ± 0.64	1.16 ± 0.24	1.56 ± 0.55
4p	1.93 ± 0.34	1.48 ± 0.09	1.54 ± 0.21	1.56 ± 0.19	0.96 ± 0.28	1.19 ± 0.15
total	14.91 ± 2.85*	24.57 ± 3.32*	12.97 ± 1.57*	7.25 ± 0.51	2.47 ± 0.53	3.47 ± 0.91
			96 h after Treatment			
5p/2c	5.21 ± 0.98*	4.67 ± 0.40*	2.12 ± 0.54	2.28 ± 0.39	0.97 ± 0.41	0.63 ± 0.29
2f	2.95 ± 0.29*	3.16 ± 0.18*	1.16 ± 0.54	1.31 ± 0.15	1.02 ± 0.16	0.40 ± 0.08
2p	25.95 ± 2.62*	21.40 ± 0.99*	6.45 ± 0.54	6.56 ± 0.62	4.77 ± 0.83	1.75 ± 0.30
4p	0.82 ± 0.09	0.85 ± 0.08	0.85 ± 0.46	0.74 ± 0.13	0.96 ± 0.55	0.76 ± 0.11
total	34.93 ± 3.54*	30.07 ± 0.91*	10.59 ± 2.06	10.89 ± 1.25	7.72 ± 1.17	3.56 ± 0.74

[a] Concentration of active ingredient (ai). [b] All values expressed as mg/kg fresh weight ± standard error mean; asterisk (*) indicates significant difference in pairwise comparison to control (Dunnett's test, $\alpha = 0.05$).

most responsive to BTH treatment; hence it likely represents the major portion of these two metabolites in the higher yielding tissues.

Initial efforts to elicit avenanthramide production by spraying the aerial portions of the seedlings did not result in appreciable accumulation of avenanthramides in the leaves. Thus, root soaking with a BTH solution was tried using a range of BTH treatments: 0.01, 0.05, 0.1, 0.5, and 1.0 mM (active ingredient). The response in total avenanthramide production is presented in Figure 3. At 24 h post treatment there is some evidence of a difference in mean total avenanthramide levels (analysis of variance (ANOVA), $F_{5,12} = 4.91$, $P = 0.011$). However, pairwise comparison showed no significant difference in any of the treatments relative to the H_2O control (Dunnett's test, $\alpha = 0.05$). Based on the 95% confidence intervals for the means, the significant differences in the total avenanthramides appear to be between the 1.0 mM treatment and the 0.05 and 0.5 mM treatments. It is clear from Figure 3 that the effect of the BTH treatment is not fully evident until 48 h after treatment. At 48 and 96 h the treatment means were, again, significantly different ($F_{5,12} = 18.10$, $P < 0.001$, and $F_{5,12} = 47.53$, $P < 0.001$ respectively). Pairwise comparison of total avenanthramides showed significant differences in the 0.1, 0.5, and 1.0 mM BTH treatments at 48 h but only the 0.5 and 1.0 mM treatments at 96 h relative to the H_2O treated control (Dunnett's test, $\alpha = 0.05$).

For the individual avenanthramides, at 24 h only 2p and 4p showed a significant difference between treatment groups ($F_{5,12} = 5.13$, $P = 0.01$, and $F_{5,12} = 3.29$, $P = 0.042$, respectively). Again comparison of the BTH treatment groups to the H_2O control showed no significant differences. However, in the case of 2p the 95% confidence intervals indicated the 0.05 mM treatment was significantly lower than the 1.0 mM treatment. For 4p the 0.05 mM treatment was significantly lower than the 0.01 mM treatment. At 48 h treatment means were significantly different for three of the avenanthramides: 5p/2c, 2f and 2p ($F_{5,12} = 8.37$, $P = 0.001$, $F_{5,12} = 14.89$, $P < 0.001$, and $F_{5,12} = 12.32$, $P < 0.001$ respectively). Pairwise comparison showed significant effects at the 0.1 and 0.5 mM treatment levels for 5p/2c and 2f and at the 0.5 and 1.0 mM BTH treatment levels for 2p relative to the H_2O control (Dunnett's test, $\alpha = 0.05$). At 96 h there was again a significant difference in means for the same three avenanthramides (5p/2c, $F_{5,12} = 11.99$, $P < 0.001$; 2f, $F_{5,12} = 16.24$, $P < 0.001$; and 2p, $F_{5,12} = 64.19$, $P < 0.001$). Pairwise comparison showed significant differences for all three of these avenanthramides, compared to the H_2O treated control, at the 0.5 and 1.0 mM treatment levels (Table 1).

Salicylic Acid Treatment. Because BTH is reputed to mimic the activity of salicylic acid, we tested this compound on oat seedlings in a manner similar to the BTH treatment. Again, three week old oat seedlings were treated with 0.01, 0.05, 0.10, 0.5, and 1.0 mM salicylic acid and a water treated control. Leaf samples were harvested at 24, 48, and 96 h after treatment. As shown in Figure 4, salicylic acid treatment did not result in avenanthramide levels as high as the BTH treatment. At 24 and 48 h post treatment little evidence for a difference in total avenanthramide means between treatments was observed ($F_{5,12} = 2.44$, $P = 0.095$, and $F_{5,12} = 1.93$, $P = 0.163$ respectively). Even at 96 h there was not a significant difference in total avenanthramide means ($F_{5,12} = 1.21$, $P = 0.363$). Evaluation of the individual avenanthramides, however, showed a difference of means for 5p/2c at 48 h ($F_{5,12} = 7.35$, $P = 0.002$) with the 0.5 and 0.1 mM treatments significantly higher than the control (Dunnett's test, $\alpha = 0.05$). Otherwise the individual avenanthramides were not statistically different from the controls (data not shown). Very small peaks corresponding to avenanthramide 4p appeared in some of the chromatograms but were not quantified.

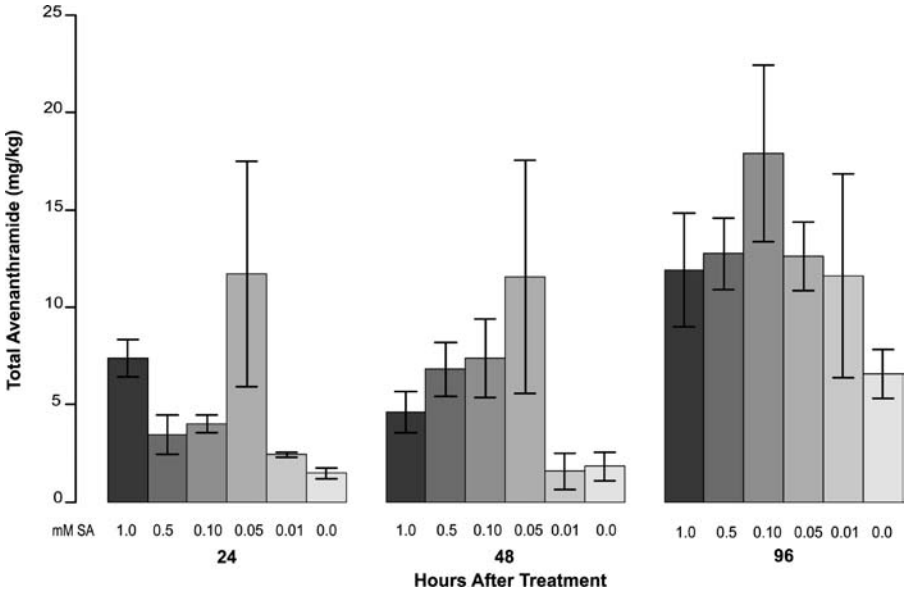

Figure 4. Total avenanthramide levels in oat seedlings treated by root soaking with 1.0, 0.5, 0.10, 0.05, 0.01, or 0.0 mM salicylic acid solution. Leaves from the treated plants were harvested at 24, 48, and 96 h after treatment. Bar heights represent leaf tissue mean total avenanthramide level from three plants. Error bars represent ± standard error of mean.

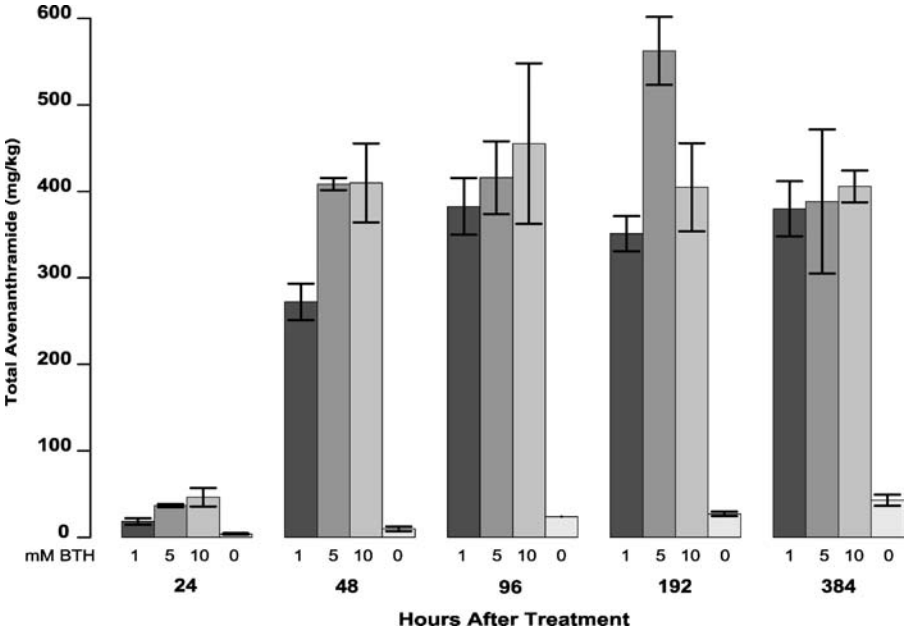

Figure 5. Total avenanthramide levels in oat seedlings treated by root soaking with 1.0, 5.0, 10.0, or 0.0 mM BTH (active ingredient). Leaves from the treated plants were harvested at 24, 48, 96, 192, and 384 h after treatment. Bar heights represent the mean total avenanthramide level from the leaf tissue of three plants. Error bars represent ± standard error of mean.

Higher Level BTH Treatment, Duration of Effect. Our initial experiment to evaluate the effect of BTH appeared to have a dose related response with 1.0 mM showing the maximum effect. Thus, higher BTH concentration treatments were evaluated. Plants were treated with 1.0, 5.0, and 10.0 mM BTH (active ingredient); one group of plants was treated with water as a control. Each pot contained three individual plants, which were harvested and analyzed separately. Leaves were harvested at 24, 48, 96, 192, and 384 h after treatment. At each time point all three plants in each pot were harvested, thus each treatment and time point was replicated in triplicate. Figure 5 shows the total avenanthramide expression in this experiment. The 5 and 10 mM BTH treatments approached maximal avenanthramide production by 48 h after treatment whereas the 1.0 mM treatment did not peak until 96 h after treatment. A significant difference in the means of total avenanthramide content at 24 h post treatment was observed ($F_{3,8}$ = 10.84, P = 0.003), and pairwise comparison shows the 5.0 and 10.0 mM treatment means were different from the H_2O control. Likewise, at 48 h significant differences in mean total avenanthramides were

observed ($F_{3,8}$ = 54.89, P < 0.001) and pairwise comparison showed all three BTH treatments differed from the control. All subsequent time points showed significant differences in mean total avenanthramides ($F_{3,8}$ = 13.85, P < 0.001, $F_{3,8}$ = 44.38, P < 0.001, and $F_{3,8}$ = 14.66, P = 0.001 at 96, 192, and 384 h respectively). Again, pairwise comparison showed all three BTH treatments were significantly higher in total avenanthramides than the control (Dunnett's test, α = 0.05).

Individual Avenanthramides. At 24 h post treatment ANOVA did not provide evidence for a significant difference in treatments ($F_{3,8}$ =3.78, P = 0.059) for 5p/2c. Avenanthramide 2f was significantly different between treatment groups ($F_{3,8}$ =14.76, P = 0.001); it was elevated by the 5.0 and 10.0 mM BTH treatments as was 2p ($F_{3,8}$ = 15.77, P = 0.001). Avenanthramide 4p was significantly different between treatments ($F_{3,8}$ = 4.85, P = 0.033), with the 10 mM treatment being higher than the control. At 48 and 96 h post treatment 2f, 2p, and 4p were significantly elevated compared to the control at all three treatment levels (48 h, $F_{3,8}$ = 26.46, $F_{3,8}$ = 55.28, $F_{3,8}$ = 69.77, P < 0.001; 96 h, $F_{3,8}$ = 12.73, P = 0.002, $F_{3,8}$ = 13.41, P = 0.002, $F_{3,8}$ = 10.87, P = 0.003 respectively) and 5p/2c was significantly elevated at the 5.0 and 10.0 mM treatments (48 h, $F_{3,8}$ = 33.82; 96 h, $F_{3,8}$ =29.09, P < 0.001). At 192 h after treatment all four avenanthramides were significantly elevated compared to the control at all three BTH treatment levels (all $F_{3,8}$ > 21.89 values with P < 0.001). At the final time point, 384 h (16 days), 2f, 2p, and 4p remained elevated compared to the control at all three treatment levels ($F_{3,8}$ = 6.14, P = 0.018, $F_{3,8}$ = 15.60, P = 0.001, and $F_{3,8}$ = 8.77, P = 0.007 respectively); 5p/2c was significantly higher ($F_{3,8}$ = 7.89, P = 0.009) than the control at the 1.0 mM treatment level but not with the 5.0 and 10 mM treatments (Dunnett's test, α = 0.05). From these data it is clear that avenanthramide 2p was the most strongly elicited of the four avenanthramides analyzed, with a maximum mean concentration of 379 μg/g at 192 h (8 days) after treatment with 5 mM BTH (Table 2).

To evaluate the possibility of volatile signaling from the BTH solution promoting the leaf tissue response, an experiment was performed in which a BTH solution (5 mM (active ingredient), 200 mL total) in two 100 mL plastic beakers was partially buried in the soil, thus preventing contact of the solution with the roots. An H_2O control was treated likewise. Two additional pots were treated with a BTH root soak treatment as in previous experiments. Each pot contained two plants at the Z13 stage. At 48 h after treatment leaf samples from each of the plants were harvested for avenanthramide analysis. The root soak treated plants had a mean total avenanthramide content of 34.0 ± 1.20 (SEM) mg/kg whereas the mock BTH and H_2O control treated plants had mean total avenanthramide levels of 3.05 ± 0.44 and 5.43 ± 1.07 mg/kg respectively. ANOVA showed a significant difference in mean total avenanthramides for the three treatments ($F_{2,9}$ = 320, p-value < 0.001). However, pairwise comparison showed no significant difference in the mean total avenanthramide levels in the mock BTH treatment and the H_2O control (the BTH root soak treatment was significantly different from the H_2O control). Thus, there seems to be no discernible effect from volatile emissions from the BTH solution.

HHT (Hydroxycinnamoyl CoA: Hydroxyanthranilate *N*-Hydroxycinnamoyl Transferase) Activity. Leaf samples from these experiments were also analyzed for HHT activity and results are shown in Figure 6. Under all three BTH treatments HHT activity reached a maximum at 192 h (8 days) after treatment with the 5 mM and 10 mM treatments having 6.3 ± 0.8 and 6.1 ± 0.8 pkat/mg protein (± SEM) respectively, the 1.0 mM treatment was somewhat lower at 3.2 ± 0.3 pkat/mg. By 384 h (16 days) after treatment the HHT activity had dropped to 2.3 ± 0.4, 1.5 ± 0.4, and 0.8 ± 0.3 pkat/mg in the 1.0, 5.0, and 10 mM treatment plants respectively. The H_2O treated control plants had no detectable HHT activity until 384 h after treatment, at which point they showed 0.5 ± 0.2 pkat/mg.

Table 2. Avenanthramide Levels in Leaves of Plants Treated with High Concentrations of BTH

	BTH Treatment (mM)[a]			
Avn	1.0	5.0	10.0	control
		24 h after Treatment		
5p/2c	1.42 ± 0.48[b]	2.55 ± 0.21	2.25 ± 0.84	0.39 ± 0.11
2f	1.06 ± 0.17	1.90 ± 0.21*	2.04 ± 0.14*	0.75 ± 0.13
2p	12.52 ± 2.44	24.23 ± 1.40*	31.63 ± 5.82*	2.40 ± 0.50
4p	3.47 ± 0.69	8.13 ± 0.33	10.57 ± 4.07*	0.42 ± 0.16
total	18.47 ± 3.77	36.81 ± 1.78*	46.49 ± 10.7*	3.96 ± 0.90
		48 h after Treatment		
5p/2c	6.48 ± 1.63	13.04 ± 1.00	24.74 ± 2.95	0.94 ± 0.23
2f	10.98 ± 1.01*	22.35 ± 1.32*	26.38 ± 4.02*	1.67 ± 0.21
2p	188.00 ± 15.6*	298.16 ± 4.27*	293.20 ± 32.9*	6.38 ± 1.67
4p	66.84 ± 3.49*	74.91 ± 3.10*	65.56 ± 6.74*	1.00 ± 0.50
total	272.30 ± 21.1*	408.46 ± 6.99*	409.88 ± 45.6*	9.99 ± 2.60
		96 h after Treatment		
5p/2c	11.58 ± 3.19	28.39 ± 1.92*	34.63 ± 4.00*	2.70 ± 0.35
2f	28.38 ± 2.09*	33.09 ± 3.67*	38.00 ± 7.79*	2.53 ± 0.01
2p	292.50 ± 25.8*	304.70 ± 32.5*	325.70 ± 68.3*	16.29 ± 0.27
4p	50.06 ± 6.32*	49.70 ± 4.80*	56.80 ± 12.9*	2.57 ± 0.18
total	382.52 ± 32.7*	415.88 ± 42.1*	455.13 ± 92.8*	24.09 ± 0.10
		192 h after Treatment		
5p/2c	30.39 ± 4.69*	54.03 ± 1.45*	36.01 ± 4.87*	2.28 ± 0.44
2f	31.08 ± 2.24*	50.99 ± 3.75*	34.23 ± 4.43*	3.62 ± 0.11
2p	245.70 ± 14.9*	379.10 ± 26.2*	279.00 ± 33.3*	17.25 ± 1.72
4p	43.92 ± 2.31*	78.32 ± 9.96*	55.54 ± 8.44*	4.13 ± 0.60
total	351.09 ± 20.2*	562.44 ± 39.2*	404.78 ± 51.0*	27.28 ± 2.62
		384 h after Treatment		
5p/2c	44.70 ± 9.69*	28.37 ± 5.50	27.28 ± 2.08	5.42 ± 1.31
2f	41.30 ± 10.7*	33.21 ± 6.54*	34.31 ± 0.83*	5.81 ± 0.85
2p	221.97 ± 7.82*	272.90 ± 59.1*	285.10 ± 10.7*	26.61 ± 3.70
4p	72.00 ± 14.0*	53.80 ± 12.6*	59.02 ± 5.88*	5.14 ± 0.88
total	379.97 ± 31.7*	388.28 ± 83.2*	405.71 ± 18.3*	42.98 ± 6.34

[a] Concentration of active ingredient. [b] All values expressed as mg/kg fresh weight ± standard error mean; asterisk (*) indicates significant difference in pairwise comparison to control (Dunnett's test, α = 0.05).

RNA Hybridization. In a separate experiment to evaluate the dynamics of HHT production in response to BTH treatment, RNA hybridization analysis (Northern blots) was performed on the leaf tissue treated with a 1.0 mM BHT root soak (Figure 7). Compared to the level of HHT mRNA in the H_2O leaves at 168 h (7 days) post treatment (essentially undetectable), the levels of HHT mRNA appear to increase to detectable levels at 48 h post treatment and reach a maximum at 96 h before diminishing at 168 h post treatment. We also analyzed for pathogenesis related

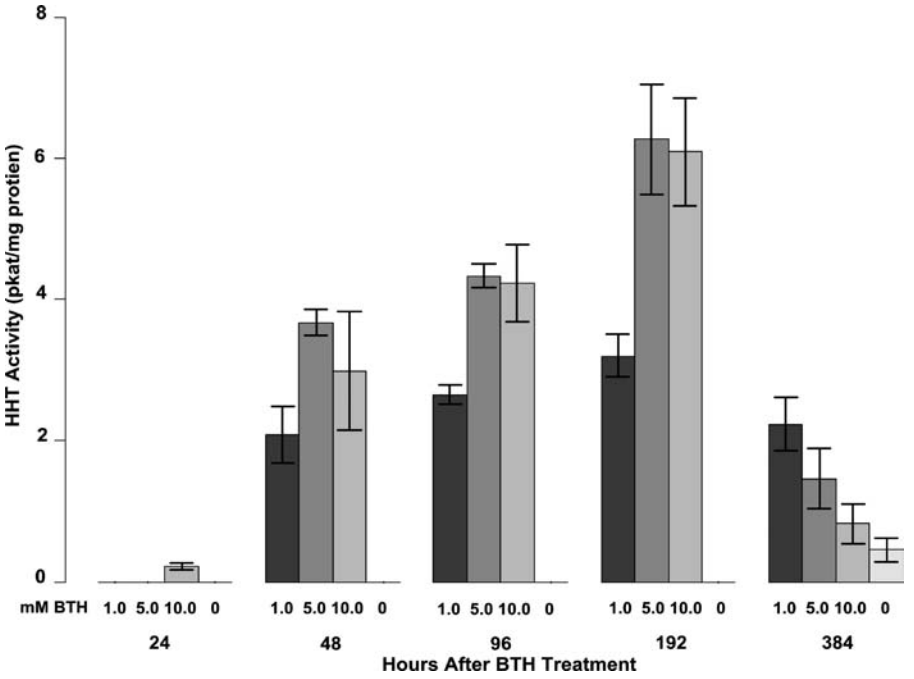

Figure 6. HHT (hydroxycinnamoyl CoA: hydroxyanthranilate *N*-hydroxycinnamoyl transferase) activity in leaf tissue of oat seedlings treated with 1.0, 5.0, 10.0, or 0.0 mM BTH (active ingredient). HHT was extracted from three replicate plants at 24, 48, 96, 192, and 384 h after treatment. Bar heights represents mean HHT activity in pkat/mg protein. Error bars are ± standard error of mean.

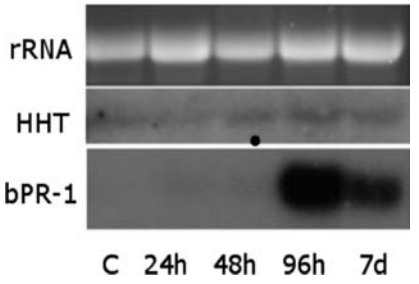

Figure 7. mRNA hybridization (Northern) analysis of oat seedlings treated with a 1.0 mM (active ingredient) solution of BTH at 24, 48, 96, and 168 h after treatment. The top panel (rRNA) is the ethidium bromide stained rRNA loading control, the middle panel (HHT) is the hydroxycinnamoyl CoA: hydroxyanthranilate *N*-hydroxycinnamoyl transferase mRNA hybridization and the bottom panel (bPR-1) is the barley pathogenesis related-1 protein mRNA. C = untreated control mRNA at 168 h after treatment with H_2O.

protein-1 (PR-1), using a barley (*Hordeum vulgare*) PR-1 transcript as a probe. The dynamics of PR-1 expression seem to lag compared to HHT expression, as no PR-1 is evident at 48 h post treatment. Nevertheless, a strong expression of PR-1 is evident at 96 h indicating that between 48 and 96 h post treatment the PR-1 mRNA was elicited. This signal is somewhat diminished, but still clearly evident at 168 h post treatment. The H_2O treated control showed no PR-1 expression at 168 h after treatment (Figure 7).

Root and Leaf Study. In previous experiments in this laboratory, low levels of avenanthramides were observed in the root tissue of field grown oats. To evaluate whether BTH root soak treatment results in avenanthramide production in the roots, oat seedlings were treated at the Z13 stage with 1.0 mM BTH or with H_2O as a control. Duplicate samples were harvested at 2, 4, 7, and 21 days after treatment, and the leaf and root tissue were analyzed for avenanthramide content (Figure 8). As expected we observed an increase in total avenanthramide production in the leaf tissue from a mean of 116.1 mg/kg at day 2 to a maximum mean of 211.4 mg/kg at day 4; then it slowly decreased to a mean of 73.2 mg/kg at day 21. The H_2O treated controls showed a maximum total avenanthramide mean of 4.3 mg/kg at day 14. Again 2p was the principal form of avenanthramide elicited by BTH yielding 83.9 mg/kg on day 2 and 149.3 mg/kg on day 4, accounting for 72% and 71% of the total avenanthramides respectively. This prevalence dropped to 37.5 mg/kg or 51% of the total by day 21. Again, avenanthramide 4p was the next most highly expressed form with 26.4 mg/kg on day 2 and 42.9 mg/day on day 4 (23% and 20% respectively), then dropping to 20.3 mg/kg or 28% on day 21. Avenanthramide 2f ranged from 4.5 mg/kg (4%) on day 2 to a maximum of 13.8 mg/kg (7%) on day 4, then down to 6.7 mg/kg (9%) by day 21. Avenanthramide 5p/2c was the least expressed and showed somewhat different kinetics; it was found at 1.3 mg/kg on day 2 (1%), but the observed maximum did not occur until day 7 with 9.9 mg/kg (5%). It then dropped slightly to 8.72 mg/kg (12%) by day 21. In the H_2O treated controls 5p/2c was below detectable levels until day 21, where it was detected at 0.05 mg/kg.

In the root tissue total avenanthramides were detected at approximately 1.0 mg/kg in both the treated and untreated roots on days 2 and 4 after treatment. At day 7 mean total avenanthramides had increased to 5.7 mg/kg in the BTH treated plants compared to 0.2 mg/kg in the controls. Mean total avenanthramide increased to 11.8 mg/kg on day 14 and up to 53.4 mg/kg on day 21. Control mean total avenanthramide remained at 0.2 and 0.1 mg/kg on days 14 and 21 respectively. Again 2p represented the bulk of the avenanthramide found in the root, increasing from 0.8 mg/kg on day 1 to 25.7 mg/kg on day 21. Avenanthramide 4p ranged from less than 0.1 mg/kg on day 1 up to a maximum of 19.1 on day 21. Avenanthramide 5p/2c was not

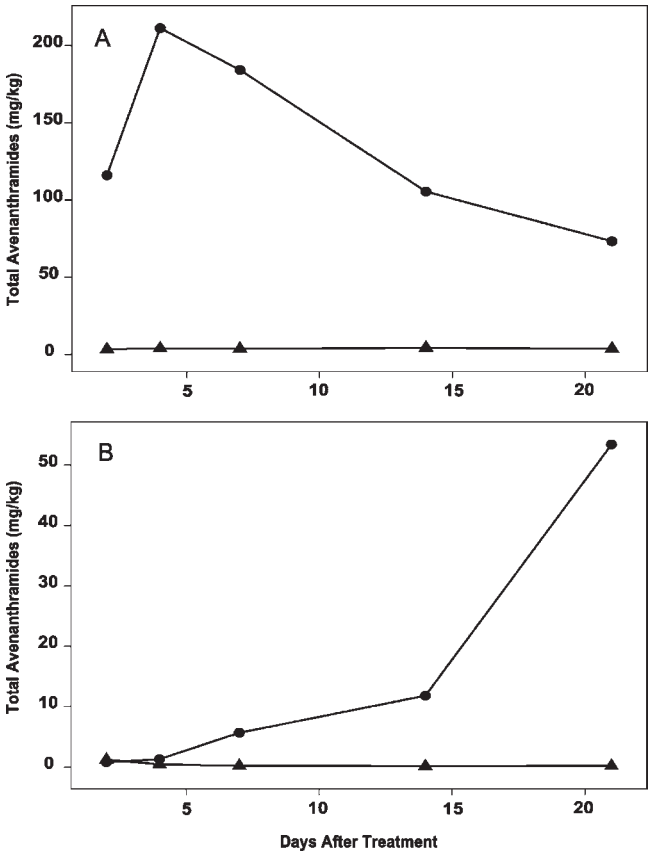

Figure 8. Total avenanthramide concentrations of leaf (A) and root (B) tissue at 2, 4, 7, 14, and 21 days after treatment with 1.0 mM (active ingredient) BTH (●) or H_2O (▲). Each point represents the average of duplicate samples.

detected in any of the untreated root tissue at any of the time points and only at mean levels of 0.13 and 1.3 mg/kg at days 14 and 21 post treatment in the BTH treated plants respectively. The root tissue was analyzed for HHT activity; no detectable activity could be detected. Avenanthramide 4p was not quantified.

DISCUSSION

Oat plants respond to crown rust by producing phytoalexins termed avenanthramides. These metabolites are produced largely in the mesophyll of the affected leaves. Furthermore, there is evidence that avenanthramide biosynthesis is localized within the chloroplast of these tissues.[32] To date, upregulation of avenanthramide biosynthesis has been demonstrated to result from crown rust infection of live plants,[5,19] in elicitor treated excised leaves[18,20,33] or in chitin elicited suspension cultures of oat callus.[28] The results of the experiments presented here clearly show that avenanthramide biosynthesis in oat leaves can be elicited by soaking the roots with a solution of BTH and represents the first demonstration of this effect in whole plants through an abiotic interaction.

The ability of BTH to elicit a SAR response is well established.[23,34] The observation of PR-1 upregulation following BTH stimulation described here is suggestive of a SAR response. This fact, coupled with previous observations of a role as phytoalexins[5−7,19] and the fact that the observed avenanthramide production is distal to the site of BTH application, also suggests that avenanthramide biosynthesis and accumulation is an aspect of the SAR response in oat.

Previous reports on avenanthramide production in unelicited seedlings are somewhat contradictory. Early investigations on avenanthramide biosynthesis in oat leaves reported that none were detectable in unelicited plants.[18,35] However, a more recent study found two oat cultivars, 'Gem' and 'Vista', grown in a controlled environment (ostensibly free of crown rust) did produce detectable levels of avenanthramides.[36] Indeed, maximum expression of total avenanthramide (2c, 2f and 2p) was seen in 'Gem' in 14 and 21 day old seedlings at approximately 29 μg/g (dry weight). In the experiments reported here similar levels of total avenanthramides were observed in some of the untreated controls (Table 1), however, these were measured on a wet weight basis, indicating higher actual concentrations than those reported in the Peterson study cited above.

In a separate experiment (in the same greenhouse used for all the experiments reported here) plants were grown in the absence of BTH treated plants and analyzed for avenanthramide at the three leaf stage. These plants showed a total avenanthramide level of 1.55 ± 0.13 mg/kg (SEM, n = 12) and no detectable 5p/2c was observed. There is ample evidence for plant to plant volatile signal communication in response to herbivore or microbial pathogen attack.[37,38] Indeed, BTH was recently shown to produce interplant volatile signaling in lima bean (*Phaseolus lunatus*) resulting in priming of defense against a bacterial pathogen.[39] To reduce the possibility of plant-to-plant signaling in the experiments reported here all plants were separated from the untreated plants by 1−2 m but were contained in the same greenhouse in an effort to equalize other environmental factors. Thus, a complete randomized experimental design was not used in these experiments. Whether the H_2O treated control plants experienced some degree of elicitation from airborne signals cannot be clearly discerned. They did seem to show higher avenanthramide levels after prolonged exposure to BTH elicited plants than is normally observed. Nevertheless, it is clear that the untreated plants did not produce avenanthramides at the level of the BTH treated plants. The results of the mock BTH treatment experiment showed that volatiles from the BTH solution did not significantly affect avenanthramide production.

Root Tissue Response. The dynamics of the avenanthramide levels in the root tissue following BTH treatment is intriguing. In previous experiments low levels of avenanthramides were observed in field grown oats. Thus, a determination if BTH treatment would result in increased levels of avenanthramides in the root tissue was of interest. This proved to be the case, however, the increase was only observed after a substantial delay (between 4 and 7 days after treatment). There are several possible explanations. (1) The biosynthesis of avenanthramides is simply delayed in the root tissue. (2) Avenanthramides may be synthesized in the roots and immediately either exported into the surrounding soil or transported to other parts of the plant and only after time built up in the stimulated tissue. (3) The avenanthramides may be synthesized in the leaf tissue and transported to the roots. Although not conclusive from the data presented here, this last explanation seems the most plausible. No HHT activity was observed in the root tissue of either the BTH treated or untreated plants. It also seems unlikely that the response to BTH treatment in the tissue most immediately contacted would be delayed by more than 48 h relative to the leaf tissue. Failure to detect HHT activity does not preclude the

possibility that it is simply too low to detect. Certainly the relatively low levels of avenanthramides observed in the roots would be consistent with this, but even in the 21 day post treatment roots where substantial avenanthramide levels were observed no HHT was detected. That avenanthramides are readily transported out of the cells and tissues in which they are biosynthesized is well documented.[28,40] Thus, the possibility that avenanthramides are produced in the leaf and transported to the root seems likely.

These experiments provide clear evidence that signaling from the oat root system results in upregulation of avenanthramide biosynthesis in leaf tissue. Whether this can cause an increase in the avenanthramide content in the grain is unclear. Additional experiments to determine the relationship between BTH treatment and production of avenanthramides in tissues other than the leaves, to include filling grain, are currently underway.

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Notes

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