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Tissue distribution of avenanthramides and gene expression of hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT) in benzothiadiazole-treated oat (*Avena sativa*)¹

Mitchell L. Wise

Abstract: Oats produce a group of natural products termed avenanthramides. These compounds are produced in both the vegetative tissue and the grain. They are produced in leaf tissue in response to crown rust infection and by chemical plant defense activators and likely other environmental stresses. Grain avenanthramide production tends to be constitutive but concentrations are highly variable and strongly influenced by environmental conditions. In this paper, we report the effect of a plant defense activator [benzothiadiazole (BTH)] on the temporal expression and tissue distribution of avenanthramides in the leaf, stem, root, panicle stem, glumes, lemma/palea, and filling grain in the oat plant. Hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT, a member of the BAHD acyltransferase family and the final enzyme in the biosynthetic pathway to the avenanthramides) activity is also determined in these tissues, as well as the relative expression ratios of HHT mRNA resulting from BTH treatment. Evidence for phloem transport of the avenanthramides is also presented. In summary, following BTH treatment, leaf tissue is the predominant location for avenanthramide biosynthesis. However, significant amounts are also found in the upper and lower stems, roots, panicle stems, and glumes. The lemma/palea and filling grain contained demonstrable but substantially lower amounts of avenanthramides. Avenanthramides were also detected in the phloem sap, indicating a source to sink transport of these metabolites following BTH treatment.

Key words: acyltransferase, phytoalexin, phytonutrient, plant defense activator.

Résumé : L'avoine synthétise un groupe de composés naturels appelés « avénanthramides ». Ces composés sont produits par les tissus végétatifs et germinatifs. Dans les feuilles, ils sont sécrétés à la suite d'une infection par la rouille couronnée et les agents chimiques qui déclenchent les mécanismes de défense de la plante mais aussi, vraisemblablement, par d'autres facteurs de stress environnementaux. Dans le grain, la production d'avénanthramides a tendance à être intrinsèque, mais la concentration de ces composés varie énormément et est très influencée par les conditions ambiantes. Les auteurs examinent les effets d'un activateur des mécanismes de défense végétaux (le benzothiadiazole ou BTH) sur l'expression des avénanthramides dans le temps et sur leur répartition dans le tissu des feuilles, de la tige, des racines, du pédoncule de la panicule, des glumes, du lemme et de la paléa, ainsi que du grain de l'avoine, pendant son remplissage. Ils ont également établi l'activité de l'hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyl transférase (HHT, un membre de la famille des BAHD acyltransférase et enzyme final de la biosynthèse des avénanthramides) dans les mêmes tissus, ainsi que les ratios d'expression relatifs de l'ARNm de la HHT à la suite du traitement avec le BTH. Les auteurs présentent également la preuve que les avénanthramides sont transportés par le phloème. En résumé, après le traitement avec le BTH, la biosynthèse des avénanthramides se concentre essentiellement dans les tissus foliaires. Cependant, on trouve aussi une quantité appréciable de ces composés dans la partie supérieure et inférieure de la tige, le pédoncule des panicules et les glumes. Le lemme et la paléa de même que le grain en train de se remplir en renferment une concentration quantifiable, mais sensiblement moindre. Enfin, on a décelé des

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avénanthramides dans la sève du phloème, signe qu'il y a transport de la source des métabolites à leur puits, après le traitement au BTH. [Traduit par la Rédaction]

Mots-clés : acyltransférase, phytoalexine, éléments nutritifs des plantes, activateur des mécanismes de défense végétaux.

Introduction

Avenanthramides are a group of phenolic alkaloids produced, among food crops, exclusively by oat (*Avena sativa* L.). These metabolites are believed to function as phytoalexins in response to crown rust (*Puccinia coronata* Corda f. sp. *avenae*) infection (Mayama 1983; Mayama et al. 1995). They also display strong antioxidant activity (Dimberg et al. 1993; Emmons et al. 1999) and, based on numerous in vitro and live animal laboratory studies, are thought to possess beneficial nutraceutical properties (Meydani 2009). These include anti-inflammatory activity (Guo et al. 2008; Sur et al. 2008), anti-tumorigenic properties (Guo et al. 2010; Wang et al. 2012), and activation of the antioxidant response element (Fu et al. 2015). Thus, there is growing interest in the oat industry to produce grain with consistently high levels of the avenanthramides. Unfortunately, the level of avenanthramides found in mature grain is highly variable, with one recent study finding concentrations ranging between 1.7 and 330 mg kg⁻¹ (Global Oat Genetics Database 2014). Several studies have also shown strong genotype × environment interaction associated with avenanthramide concentration in the mature grain of oat (Emmons and Peterson 2001; Mannerstedt-Fogelfors 2001; Wise et al. 2008).

Originally discovered in the leaves of rust infected oat (Mayama et al. 1981), biosynthesis of these metabolites is known to be elicited by crown rust infection (Miyagawa et al. 1995) as well as several chemical elicitors (Bordin et al. 1991; Miyagawa et al. 1996; Ren and Wise 2013). Avenanthramides are also found in mature oat grain (Collins 1986) and the enzymatic machinery to biosynthesize these metabolites exist in both the vegetative tissue (Ishihara et al. 1999; Peterson et al. 2007) and grain (Matsukawa et al. 2000; Peterson and Dimberg 2008). The phenylpropanoid and anthranilate precursors originate via the shikimate pathway (Ishihara et al. 1999) and the final enzyme in the biosynthetic pathway: hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT), a member of the BAHD family of acyltransferases (D'Auria 2006). Avenanthramides were also found in root tissue of elicited oat seedlings, although no HHT activity was detected (Wise 2011).

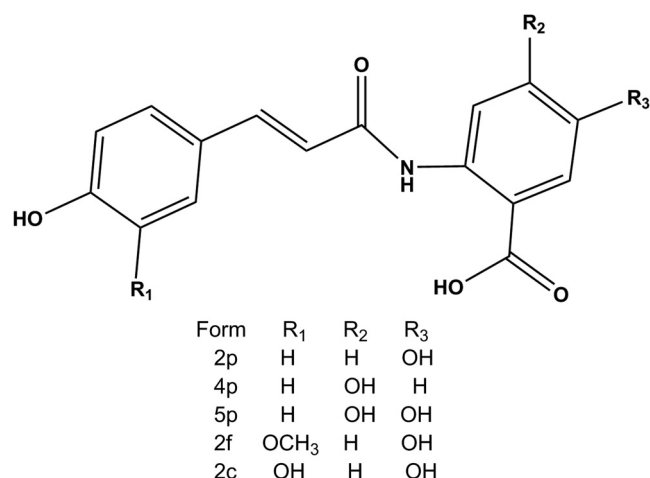
Several lines of evidence demonstrate that avenanthramides are transported across the cell wall. Thus, excised leaves floated in dishes containing elicitor solutions were shown to excrete newly biosynthesized avenanthramides into the solution and to uptake radio-labeled avenanthramides from the elicitor solution

(Okazaki et al. 2004). Similarly, oat callus tissue in liquid media was shown to excrete avenanthramides into the media subsequent to elicitation with crab shell chitin (Wise et al. 2009).

Aside from grain, leaf, and root tissue, there has been little investigation of the biosynthesis or presence of avenanthramides in other organs of oat plants. Peterson and Dimberg (2008) reported on the temporal dynamics of avenanthramide biosynthesis in the spikelets and leaves of field-grown oat. There have also been studies on the compartmentalization of avenanthramide biosynthesis in the mesophyll cells of excised and chitin-elicited oat seedling leaves (Izumi et al. 2009); these investigators provided evidence for the restriction of biosynthesis to the chloroplast. In another study, using crown rust (*P. coronata*) infected oat seedling leaves and an immunohistological approach, Uchihashi et al. (2011) determined that HHT was exclusively produced in mesophyll cells undergoing a hypersensitive response proximate to hyphal invasion and that avenanthramide A (2p) (Fig. 1) was initially localized to the cells producing HHT. However, in time, 2p could be observed (by immunological staining) in cells distal to those producing HHT. Thus, the avenanthramide appeared to be transported to other parts of the infected leaf.

Recent reports show that treatment of oat with a commercially available plant defense activator, Actigard™ (Syngenta, Wilmington, DE), an *S*-methyl ester derivative of benzothiadiazole (BTH), is highly effective at up-regulating the biosynthesis of avenanthramides in oat leaves and in the mature grain (Wise 2011; Ren and Wise 2013; Wise et al. 2016). The recognition that BTH strongly up-regulates avenanthramide biosynthesis in oat plants (Wise 2011) presents an opportunity to examine the tissue distribution of these metabolites in whole plants and to provide insight into the locations of their biosynthesis as well as organs that might serve as sinks for transported avenanthramides. Here, in an effort to determine the principal tissues in which avenanthramides are produced and to investigate the possibility that they are transported via the phloem, we analyzed the distribution of avenanthramides in individual leaves, upper and lower stems, roots, panicle stems, filling grain, lemma/palea, and glumes subsequent to treatment with BTH by root drench. Also analyzed were HHT gene expression and HHT enzyme activity. Phloem sap was also extracted and analyzed for avenanthramides.

Fig. 1. Avenanthramide structures.



Materials and Methods

Chemicals

Methanol and acetonitrile were high performance liquid chromatography (HPLC) grade, purchased from Fisher Scientific Ltd. (Ottawa, ON), as was the HCl and NaHPO₄. MOPS, BisTris, adenosine triphosphate (ATP), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethyl acetate, *p*-coumaric acid, and co-enzyme A came from MilliporeSigma (St. Louis, MO), 95% ethanol was from Carolina Biological Supply Company (Burlington, NC), formic acid was from Acros Organics (Thermo Fisher Scientific, Springfield, NJ), 5-hydroxy-anthranilic acid was purchased from Alfa Aesar (Ward Hill, MA), 100% ethanol was from Pharmaco Aaper (Greenfield, Toronto, ON), and the BTH was kindly provided by Syngenta.

Plant growth and treatment

Seeds of cultivar 'Kame' were imbibed in deionized H₂O with aeration for 3 h at room temperature and then planted in 20 cm (diameter) × 18 cm (height) pots with commercial topsoil and peat moss (50:50) mixed with approximately 5 g of Osmocote® (The Scotts Company, Marysville, OH). Upon emergence, the plants were culled to three per pot and grown under a photon flux of approximately 300 μmol m⁻² s⁻¹ [determined with a Li-Cor 250 light meter (Li-Cor Biosciences, Lincoln, NE)] with a 16 h light — 8 h dark cycle at 22 °C (light) and 16 °C (dark) and grown to the appropriate Zadok stage (Zadoks et al. 1974). They were then treated with 500 mL of a 1 mmol L⁻¹ (active ingredient) solution of BTH or with an equal volume of H₂O as an untreated control. Three plants from each pot were harvested separately and treated as individual observations; hence, each time point and treatment was analyzed in triplicate. Leaf tissue was harvested immediately prior to treatment (null or *t* = 0 h) and then at intervals as indicated for the individual experiments. Tissue samples were immediately flash frozen in liquid nitrogen (LN₂) and

stored at –80 °C until processing for RNA or protein extraction. Tissues for avenanthramide extraction were collected in 50 mL Falcon tubes with small holes bored into the tops and immediately placed in a –80 °C freezer and subsequently lyophilized before solvent extraction. For filling grain and spikelet tissue, the plants were treated with BTH at the late booting stage (Zadok's Z49 stage) and maturing spikelets were harvested at 0 (null), 2, 7, 14, 28, and 56 d (mature grain) after treatment. The spikelets were dissected to separate the glumes, lemma/palea (analyzed together), and filling seed; the panicle stem was also harvested and analyzed for avenanthramides, HHT gene expression, and HHT activity.

Avenanthramide analysis

Aliquots of lyophilized tissue were ground in a ZM-200 Retsch mill (Haan, Germany) to pass a 0.5 mm sieve and then approximately 0.5 g was carefully weighed and the weight recorded. The ground tissue was extracted with 3 × 10 mL 80% ethanol in 10 mmol L⁻¹ NaH₂PO₄ buffer (pH = 2.0) in a shaker and water bath at 50 °C for 20 min. After each extraction, the sample was centrifuged for 7 min at 1800g. The supernatants were pooled into a 50 mL round-bottom flask and rotary evaporated under vacuum at 50 °C until dry. The residue was resuspended in 1.0 mL methanol and filtered through a 0.2 μm nylon membrane. Avenanthramides were analyzed by ultrahigh performance liquid chromatography photodiode array (UHPLC-PDA) on a Shimadzu NexEra system (Kyoto, Japan) using a Kinetex 2.1 mm × 50 mm, 100 Å pore, 1.7 μm diameter, C-18 column (Phenomenex, Torrance, CA) at 40 °C with a Shimadzu SPD-M20A PDA detector. The mobile phase consisted of buffer A: H₂O with 5% acetonitrile and 0.1% formic acid and buffer B: acetonitrile with 0.1% formic acid. A gradient of 10%–17% B over 2.1 min and then 17%–33% B from 2.3–3.2 min, then to 60% B at 3.4 min, returning to 10% B at 4.0 min at a flow rate of 1.0 mL min⁻¹, was employed. The peaks corresponding to avenanthramide 2c, 2f, and 2p were quantified by their absorbance at 330 nm and comparing the peak area to standard curves developed using the corresponding authentic, synthesized avenanthramides (Wise 2011), 4p and 5p were quantified as 2p equivalents. Avenanthramide 5p was identified by its retention time relative to 2c (Collins and Burrows 2010) and its mass spectral data as determined by liquid chromatography — mass spectrometry (Wise 2011). Avenanthramide 4p was identified by retention time identity and mass spectral data compared to a sample of 4p previously isolated and structurally characterized from oat callus tissue (Wise et al. 2009).

Liquid chromatography — mass spectrometry analysis

Liquid chromatography — mass spectrometry was used to confirm the identity of avenanthramides; analysis was performed on an Agilent 1100 liquid chromatography system with a G2445 series ion-trap mass

spectrometer. A 2.1 mm × 30 mm C-18 column (Zorbax SB-C18, Agilent, Santa Clara, CA) was employed. The mobile phase consisted of buffer A: H₂O with 5% acetonitrile and 0.1% formic acid and buffer B: acetonitrile with 0.1% formic acid. A gradient of 13%–30% B over 20 min at a flow rate of 0.2 mL min⁻¹ was employed. 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 (N₂) at 207 kPa (30 psi) and dry gas flow at 8.0 L min⁻¹ 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* 100–1000 (or 100–400 for MS2) at 13 000 *m/z* s⁻¹.

Quantitation of gene expression

Each sample was individually extracted for RNA and each RNA extraction was reverse transcribed, in duplicate, to cDNA. These separate cDNAs were then analyzed, in duplicate, by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Thus, each treatment, at every time point, was performed with triplicate biological repetition (i.e., three plants per treatment per time point) and quadruplicate technical repetition (2 × cDNA × 2 PCR). RNA was extracted using a Qiagen RNeasy plant mini kit (Valencia, CA) with on-column DNase digestion according to manufacturer's instructions. The quality of the RNA extractions was determined using an Agilent 2100 Bioanalyzer. Leaf RNA samples with RNA integrity numbers (RIN) (Schroeder et al. 2006) above 6.5 were deemed suitable for gene expression analysis by RT-qPCR. Duplicate cDNAs were synthesized from 1.0 µg aliquots, determined by UV absorbance on a Nanodrop spectrophotometer (Wilmington, DE) using the iScript cDNA synthesis kit (Bio-Rad Laboratories Ltd., Hercules, CA) and stored at -20 °C until analyzed. RT-qPCR was performed with an Applied Biosystems 7500 Fast System (Thermo Fisher Scientific, Waltham, MA) using TaKaRa SYBR premix Ex Taq (Tli RNase H plus; TaKaRa Bio Inc., Kusatsu, Japan) in a 20 µL reaction volume (Clontech, Mountain View, CA). Primer sequences for the target gene HHT were based on previous work by Uchihashi et al. (2011). The following sequences were used: HHT-F GTGGAGATCGACTGCAACG; HHT-R AAGTCGACGGTGG GGATGA. These primer sequences are 100% conserved in all three of the published full-length oat HHT mRNA sequences (Genebank accessions AB076980, AB076981, and AB076982). Several reference genes were evaluated based on published reports on their validation for use in cereals (Jarošová and Kundu 2010; Ovesná et al. 2012). After comparing their stability with the cDNAs from all time points and treatments using Bestkeeper software (Pfaffl et al. 2004), heat shock protein 70 (HSP-70), HSP-90, and glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) were selected for use as reference genes; the coefficient of correlations to the Bestkeeper index was 0.98 > *r* > 0.90 with a *P* value of 0.001. The following primer sequences were used: HSP-70 F GCTCAACATGGACCTCTTCAGG; HSP-70 R CC GACAAGGACAACATCATGG; HSP-90 F CAAGAAGCTTG TCTCTGCCACC; HSP-90 R ACAGCCCCTCGAACTTCT CCTT; GAPDH F GCCAGTTACTGTCTTTGGCGTC; GAP DH R GGCCTTGTCCTTGTCAGT GAAG. Polymerase chain reaction amplicons for all primer pairs (target and reference genes) using BTH-treated oat ('Kame') leaf cDNA as a template were cloned into TOPO TA (Invitrogen, Carlsbad, CA), sequenced, and the sequence confirmed by a BLASTN search of the NCBI database. Relative gene expression ratios (RER) were determined using the 2^{-ΔΔC_t} method (Livak and Schmittgen 2001; Pfaffl 2006).

HHT assay

Coumaroyl-coenzyme A (CoA) was biosynthesized using a cloned 4-hydroxy-cinnamate:CoA ligase (4-CL, EC6.2.1.12) from tobacco, kindly provided by T. Beuerle (Technical University, Braunschweig, Germany) and prepared following procedures described previously (Beuerle and Pichersky 2002). To a volume of 116 mL of 200 mmol L⁻¹ 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH = 7.5) was added 11.2 mg (68 µmol) *p*-coumaric acid, 28.0 mg (31 µmol) CoA, and 240 mg (0.44 µmol) ATP dissolved in 4 mL 95% ethanol. 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 coumaroyl-CoA was immediately isolated and purified by chromatography on a 2g (12 mL) PrepSep C-18 column (Fisher Scientific, Pittsburgh, PA) pre-equilibrated with 50 mmol L⁻¹ MOPS buffer. The column was washed with six column volumes of MOPS buffer and then one column volume milliQ H₂O. The coumaroyl-CoA was eluted with 100% methanol. The eluate was roto-evaporated to near dryness, resuspended in HCl acidified H₂O (pH = 4.0), and the volume adjusted to provide a final concentration of 2.5 mmol L⁻¹ as determined by the UV absorbance at 333 nm using an extinction coefficient of 21 000 L mol⁻¹ cm⁻¹ (Stöckigt and Zenk 1975).

Depending on availability, a 100–250 mg sample of tissue was ground with a mortar and pestle using LN₂ and the ground tissue was resuspended in 1.0 mL ice-cold 100 mmol L⁻¹ BisTris buffer, pH = 7.2 with 2 mmol L⁻¹ DTT. The vials were immediately placed on ice before centrifuging at 4 °C for 20 min at 14 000g. The supernatant (protein extract) was transferred to a microfuge tube and kept on ice until assayed. HHT assays were conducted as previously described (Wise et al. 2009). Briefly, a 10 µL aliquot of the protein extract was added to 60 µL 100 mmol L⁻¹ BisTris (pH 7.2) with 10 µL of 10 mmol L⁻¹

5-hydroxy anthranilic acid in dimethyl sulfoxide and 20 μL 2.5 mmol L^{-1} coumaroyl-CoA and allowed 20 min reaction at 30 °C. The reaction was quenched by the addition of 20 μL concentrated acetic acid followed by 380 μL methanol (final volume 0.5 mL). An aliquot was analyzed by UHPLC-PDA to determine the amount of 2p formed. All reactions were performed in triplicate and the pkat normalized by the amount of protein added to the reaction [determined by Bradford assay (Bradford 1976) using bovine serum albumin (BSA) as a standard].

Phloem analysis

Avenanthramide content in phloem extracts was determined by the EDTA-facilitated method (King and Zeevaart 1974; Tetyuk et al. 2013). Oat plants, three per pot \times six pots, were grown to the Z49 stage; three pots were treated with BTH and three were treated with water as untreated controls. Seven days after treatment, the three plants in each pot were harvested, leaves were removed and the stems cut into segments (approximately 15 cm). One of the plants had the cuts made while submerged in 20 mmol L^{-1} $\text{K}_2\text{-EDTA}$ and the segments then placed and held vertically in 50 mL Falcon tubes containing 15 mL of 20 mmol L^{-1} $\text{K}_2\text{-EDTA}$. These were allowed 6 h to elute before removing the stems and the EDTA solution was then stored at -80 °C until analyzed. The EDTA solution was extracted three times with approximately 15 mL of ethyl acetate; the ethyl acetate was pooled and reduce to dryness using a Rotavapor[®] R-200 (Büchi, New Castle, DE). The dried extract was resuspended in 1.0 mL methanol and analyzed by UHPLC-PDA. The spent stems were stored at -80 °C before being lyophilized and extracted for avenanthramide analysis. A second plant was simply cut into segments and stored at -80 °C until lyophilized and analyzed for avenanthramide to give an estimate of the avenanthramide concentration before exudation. As an additional control, the cut stem segments of the third plant were placed horizontally on the benchtop for 6 h, frozen at -80 °C, and then lyophilized and analyzed to determine if there was significant loss of the avenanthramides without exudation. Thus, for both the BTH treatment and the untreated control, three replicate samples were subject to (i) EDTA-facilitated exudation, (ii) simple extraction of avenanthramide content, or (iii) 6 h on the bench top without exudation followed by simple extraction of avenanthramides.

Statistical analysis

Avenanthramide data were analyzed using MiniTab 16 software (Minitab, Inc., State College, PA). Data were considered statistically significant for P values <0.05 . Avenanthramide levels comparing treated versus non-treated tissue were analyzed by a two-tail t test based

on either equal or unequal variance, using Excel (Microsoft Corporation, Redmond, WA). Equivalence of variance was determined by Levene's test using MiniTab 16 software. Relative (gene) expression response was analyzed for significant increases by a one-tailed t test, assuming non-homogeneity of variance using Excel. Correlation coefficients were calculated on SAS v9.4 (SAS Institute, Cary, NC).

Results

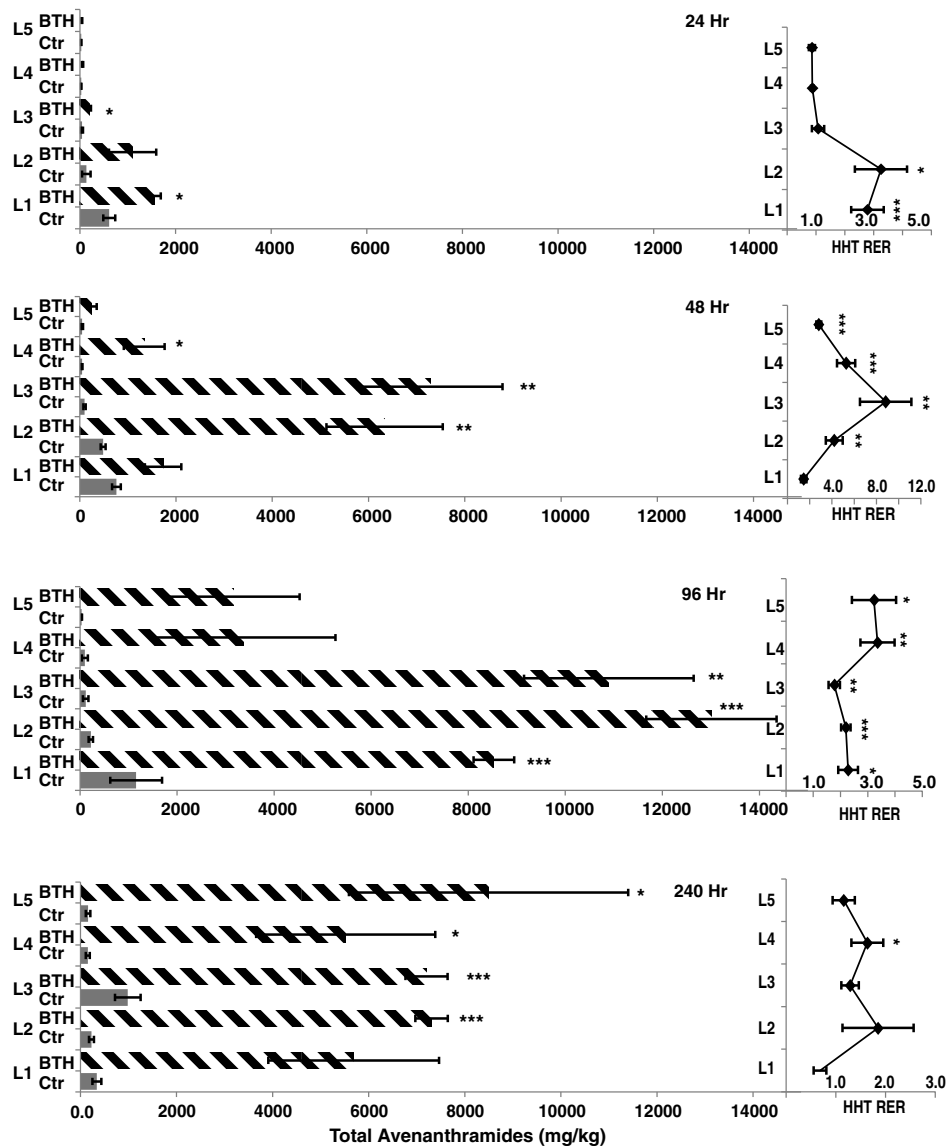
Leaf avenanthramides

Plants at the five-leaf stage (Zadok's Z15 stage), approximately 3 wk old, were treated with BTH and leaves collected at 0 (null, i.e., immediately before treatment), 24, 48, 96, and 240 h after treatment. Figure 2 (and Supplementary Table S1)¹ shows the results from avenanthramide analysis and the RER for the HHT mRNA. Although the lower two leaves had somewhat elevated avenanthramide levels relative to the others prior to treatment (null group), this difference was not significant by analysis of variance; hence, initially there was no difference in total avenanthramide between any of the leaves or treatments. Subsequently, there was a clear temporal lag in the response of individual leaves, with the lower leaves responding first (within 24 h) in both avenanthramide production and HHT gene expression. The highest concentration of avenanthramides occurred at 96 h post treatment with values in the middle leaves (L2, L3) somewhat over 10 000 mg kg^{-1} . The upper leaves (L4, L5) were only about 30% of that at 96 h. By day 10, avenanthramide levels were fairly evenly distributed top to bottom with total avenanthramide concentrations dropping somewhat from the previous sampling and ranging from approximately 5000 to 7700 mg kg^{-1} and, although the HHT RER was above 1.0 in all but the lowest leaf, only the RER in leaf four (second from the top) was statistically elevated relative to the untreated control. Relative gene expression was at its highest ratio at 48 h (RER = 8.8, L3) and dropped off in all leaves subsequent to that time point (Fig. 2). Nevertheless, avenanthramide levels nearly doubled in some of the leaves at 96 h post treatment and enzyme activity remained elevated out to 10 d after treatment. By 10 d after BTH treatment, the plants were headed, hence, panicle stem and immature spikelets were harvested and analyzed for avenanthramide content (see below).

Because of the limited availability of coumaroyl-CoA, leaf three was selected as a representative model for HHT activity, thus only this tissue was analyzed. In leaf three, HHT activity peaked at 96 h with 393 pkat mg^{-1} protein. This was significantly higher ($p < 0.001$) than the untreated control (10 pkat mg^{-1} protein) and, although the HHT RER fell significantly after its peak at

¹Supplementary tables are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/cjps-2017-0108>.

Fig. 2. Mean total avenanthramide levels in individual leaves at each time point. L1–L5 indicate leaves 1 through 5 (leaf one is lowest on the stem). Solid bars, control; diagonal stripe bars, BTH-treated. Line graph on the right side is the relative gene expression ratio (RER). Error bars represent standard error of the mean. Statistically significant differences between individual leafs for BTH-treated vs. control are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as determined by student *t* test, $n = 3$.



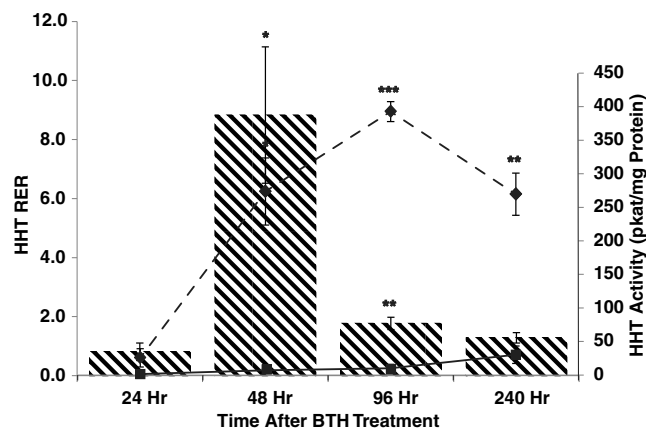
48 h, HHT activity remained fairly high (269 pkat mg^{-1} protein) out to 240 h (Fig. 3). A very strong correlation was observed between HHT activity and avenanthramide content in L3 ($r = 0.96$, $p < 0.0001$).

Upper and lower stems

The stems were divided at the middle to provide upper and lower halves (Fig. 4 and Supplementary Table S2).¹ Although at every time point the BTH-treated plants contained higher concentrations of avenanthramides, only at 240 h post treatment were these levels statistically significant ($\alpha = 0.05$). The lower stem also appeared to have higher avenanthramide levels than the upper stem, although these differences were not statistically

significant. Analysis of the HHT gene expression likewise showed no significant up-regulation. HHT enzyme activity in the lower stem tissue was fairly low, ranging from about 14 to 26 pkat mg^{-1} protein in both the treated and untreated plants with no significant difference between them at any time point. The lower stem also showed poor correlation between HHT activity and total avenanthramide content ($r = 0.42$, $p = 0.47$). In the upper stem, the HHT activity was somewhat lower, approximately 5 pkat mg^{-1} protein for both treatment groups, except in the final time point where the BTH-treated tissue increased to 65 pkat mg^{-1} protein, which was significantly higher ($p = 0.009$) than the control (2.6 pkat mg^{-1}). In contrast to the lower stem, the upper stem did show

Fig. 3. HHT relative expression ratio (RER) and enzyme activity in leaf three over time after BTH treatment. Leaf three was selected as representative of the leaf response in terms of avenanthramide production, HHT gene expression, and HHT activity. Gene expression (bar graph) HHT activity (line graph: diamond, BTH-treated; square, control). Bars represent standard error of the mean, statistically significant differences for BTH-treated vs. control are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, $n = 9$ (RER), $n = 3$ (HHT activity).



some correlation between HHT activity and total avenanthramide content ($r = 0.78$, $p < 0.0001$).

Root tissue

The roots also demonstrated increased levels of avenanthramides in response to BTH treatment (Fig. 5 and Supplementary Table S2).¹ In this case, avenanthramide levels were significantly elevated at 96 and 240 h post treatment. It is also worth noting that the untreated control roots showed no discernable (or statistically significant) increase in total avenanthramide concentration during the course of the experiment. This is in contrast to the leaf data in which the control plants show a small but discernable increase in total avenanthramide concentrations during the course of the experiment. And, as was the case with the stem tissue, HHT gene expression was not demonstrably up-regulated at any time point. Interestingly, however, the C_t values for HHT mRNA were approximately 2–3 cycles lower in the mRNA extracted from the stems and root than that from the leaf tissue (Fig. 6). Furthermore, HHT enzyme activity in the root tissue was relatively high (exceeding 340 pkat mg^{-1} protein at 240 h post treatment) in both the control and BTH-treated plants (Fig. 5), but showed no significant difference between treatment groups. Indeed, the untreated plants were slightly higher at most of the time points analyzed.

Spikelet and panicle stem

By 240 h post treatment, the plants had booted and immature spikelets had emerged. The panicle stems and spikelets were analyzed for avenanthramide content

and gene expression. No effort was made to dissect the spikelet into constituent parts. Both the panicle stem and the spikelet had substantial concentrations of avenanthramide in the treated plants (460 ± 94 and $808 \pm 187 \text{ mg kg}^{-1}$, respectively) while the untreated controls were quite low (4.6 ± 1 and $16 \pm 1 \text{ mg kg}^{-1}$, respectively), these were statistically significant in both tissues ($p = 0.001$ and $p = 0.002$, respectively) (Fig. 7 and Supplementary Table S2).¹ Similarly, the HHT activity in the treated plants was highly up-regulated, particularly in the spikelets, increasing from 4.4 pkat mg^{-1} protein in the controls to 142 pkat mg^{-1} protein in response to BTH treatment. Panicle stem HHT activity increased from 12.7 to $34.5 \text{ pkat mg}^{-1}$ protein with BTH treatment; the response in both tissues was statistically significant ($p < 0.001$ and $p = 0.01$, respectively). No discernable up-regulation of HHT mRNA was observed.

Mature plants

Flag leaf

The flag leaf was clearly the most strongly responsive tissue, with total mean avenanthramide levels reaching nearly 7500 mg kg^{-1} at 7 d post treatment (Fig. 8A). At every time point post treatment, the BTH-treated plants were significantly higher than the untreated plants in total avenanthramide ($\alpha = 0.05$); prior to treatment (null point) there was no difference between treatment groups. There is also a clear downward trend in avenanthramide concentration after 7 d. However, even at 56 d after treatment, when the grain was fully mature and the leaves apparently senescent, substantial levels of avenanthramides (2150 mg kg^{-1}) were still present. The flag leaf also demonstrated a substantial RER for HHT mRNA, peaking at 3.30 at 2 d after treatment then dropping to 2.28 and 2.21 at 7 and 14 d, respectively (Fig. 8F), all of which are significantly elevated ($p < 0.001$) relative to the control treatment. By 28 d, there was no significant RER. By 56 d post treatment, there was insufficient extractable RNA for RER analysis in any of the tissues. Enzyme activity in the flag leaves was also substantial, with activity as high as 240 pkat mg^{-1} protein at 7 d. And, as with the seedling leaf three analysis, there was a strong correlation between avenanthramide concentration and HHT activity ($r = 0.89$, $p < 0.0001$).

Filling grain

The filling grain in the BTH treatment group showed an initial increase in avenanthramide concentrations at 2 and 4 d after treatment followed by decreasing avenanthramide concentrations out to 28 d (Fig. 8B). The mature grain of the treated plants showed substantially higher total avenanthramide concentrations. And, although the BTH-treated plants had grain avenanthramide levels measurably higher than the untreated controls, only at 7 and 56 d after treatment was this difference significantly higher ($p = 0.017$ and 0.002 , respectively). HHT gene expression was only demonstrably higher in the

Fig. 4. Lower and upper stem mean total avenanthramide concentrations at each time point (bar graph: striped, BTH-treated; solid shade, control). Superimposed is the HHT activity in buffer extracted tissue (line graph: diamond, BTH-treated; square, control). Bars represent standard error of the mean. Statistically significant differences for BTH treated vs. control are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as determined by student t test, $n = 3$ (no significant difference was observed for the HHT activity).

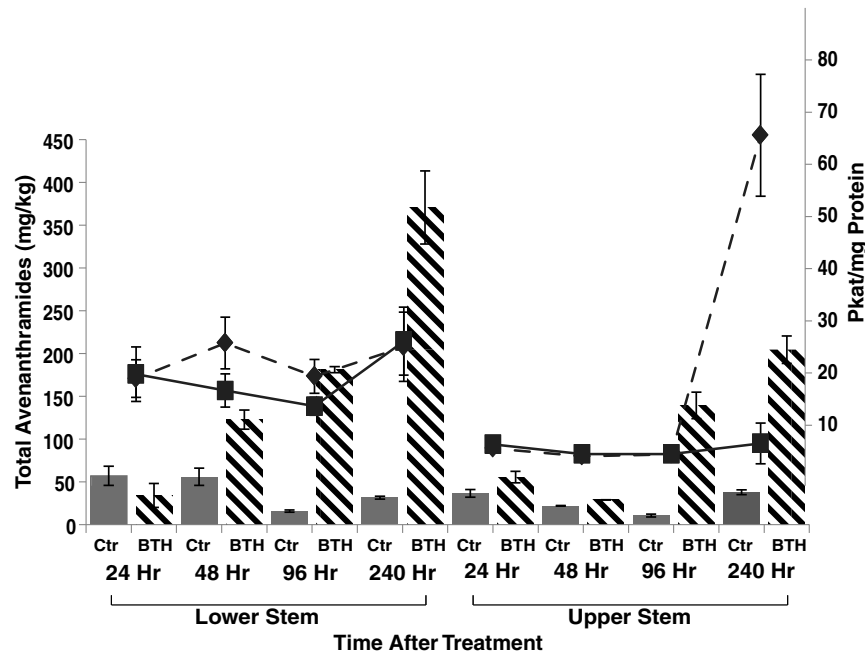
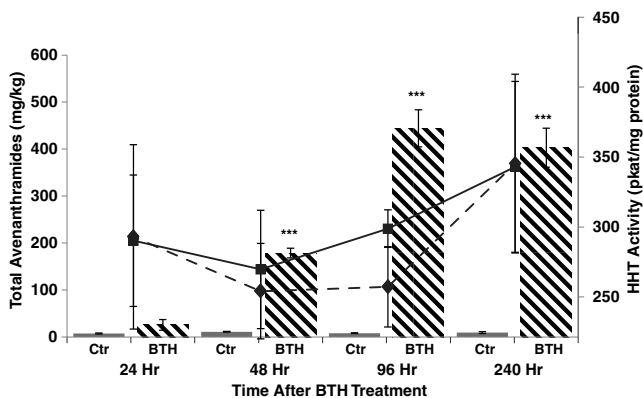


Fig. 5. Mean avenanthramide levels in root tissue at each time point (bar graph). Superimposed is the HHT activity in buffer extracted tissue (line graph: diamond, BTH-treated; square, control). Bars represent standard error of the mean. Statistically significant differences for BTH treated vs. control are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as determined by student t test, $n = 3$ (no significant difference was observed for the HHT activity).



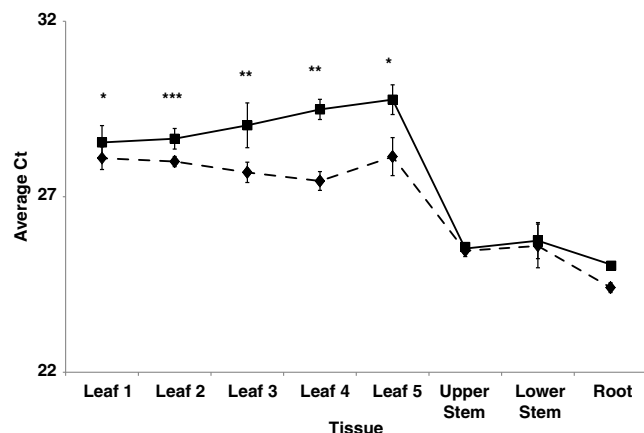
14 and 28 d samples (Fig. 8F) with RER of 1.49 and 1.44, respectively ($p = 0.02$ and 0.006 , respectively). HHT activity was also rather low in the developing seeds, ranging from 2 to 14 pkat mg^{-1} protein for the first 28 d, with no significant difference between treatment groups (there was insufficient tissue to sample for HHT activity prior to the 7 d sample). In the final sample of mature

grain, however, the BTH-treated grain showed 62 vs. 22 pkat mg^{-1} protein in the control. These values were significantly different ($p = 0.008$). HHT activity showed a weak correlation with total avenanthramide concentrations ($r = 0.42$, $p = 0.04$). Also noteworthy are the levels of avenanthramides in the mature grain, these were relatively low, even in the BTH-treated plants (54 mg kg^{-1} in the BTH-treated mature grain and 6.2 mg kg^{-1} in the non-treated mature grain); however, the treatment group was significantly higher ($p = 0.007$) and this data point alone likely accounts for the correlation observed for the developing seed HHT activity and total avenanthramide relationship.

Glumes

The glumes also displayed relatively high levels of avenanthramides, ranging from approximately 20 mg kg^{-1} prior to treatment up to 2200 mg kg^{-1} at 7 d post treatment (Fig. 8C). As was the case with the flag leaves, mean total avenanthramide levels steadily decreased out to 56 d, when the concentration dropped to 379 mg kg^{-1} . The values determined for 7, 14, and 28 d post BTH treatment were all statistically higher than the untreated controls ($\alpha = 0.05$). At 2, 7, and 14 d post treatment, HHT RER (Fig. 8F) was significantly higher in the BTH treatment group ($p < 0.001$) before returning to the level of the untreated plants at 28 d. In contrast to avenanthramide content, the RER for HHT peaked at 14 d post treatment at 2.45. Similar to the flag leaf, HHT activity was highest

Fig. 6. Threshold cycle (C_t) values for quantitative reverse transcription polymerase chain reaction at 96 h post BTH treatment. Solid line (square) represents untreated controls, dashed line (diamond) represents the BTH-treated plants, the associated numbers are the mean C_t values. Note that while the C_t values in the leaves are statistically lower for the BTH-treated plants (indicating higher mRNA levels), those in the stem and root tissue are not. The data from 96 h is taken as representative of the overall response. Statistically significant differences for BTH-treated vs. control are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as determined by student t test, $n = 9$.

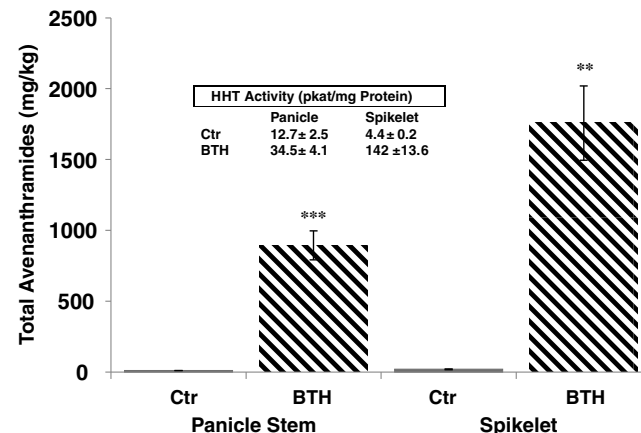


at 7 d, after which it steadily decreased over the next two sampling periods followed by a small, statistically insignificant, increase at 56 d post treatment. Although not as strong as the flag leaf, the glumes also showed a fairly high correlation between mean avenanthramide concentration and HHT activity ($r = 0.85$, $p < 0.0001$).

Panicle stems

The panicle stems of the BTH-treated plants also had substantial levels of avenanthramides. The highest level of avenanthramide was observed at 14 d post treatment with approximately 540 mg kg^{-1} (Fig. 8D). The concentration decreased afterwards and at 56 d was 192 mg kg^{-1} . These dynamics more closely mimicked developing seed avenanthramide production than the flag leaf and glume data, reflecting some delay reaching maximum levels. Interestingly, there is no detectable 2f or 2c in the panicle stems, there is also no detectable 5p or 4p in any of the non-elicited control panicle stems (Supplementary Table S3).¹ Only at 14 d post BTH treatment is the HHT RER (Fig. 8F) significantly elevated in the BTH-treated plants ($p = 0.003$). HHT activity also remained moderately low and although it showed a spike in the BTH-treated plants at day 56, there was no statistically significant difference in HHT activity between treated and untreated plants at any time point. There was also poor correlation between HHT activity and total avenanthramides ($r = 0.22$, $p = 0.20$).

Fig. 7. Panicle stem and spikelet mean total avenanthramide concentrations and HHT activity. By 240 h after BTH treatment the plants headed, samples of the emerging panicle stems and immature spikelets were analyzed. No effort was made to dissect the spikelets into constituent parts. As with the other tissues analyzed at this time point, no significant HHT RER was determined, however, there is substantial HHT activity in the spikelet. Statistically significant differences for BTH-treated vs. control are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as determined by student t test, $n = 9$ (no significant difference observed for the HHT activity).



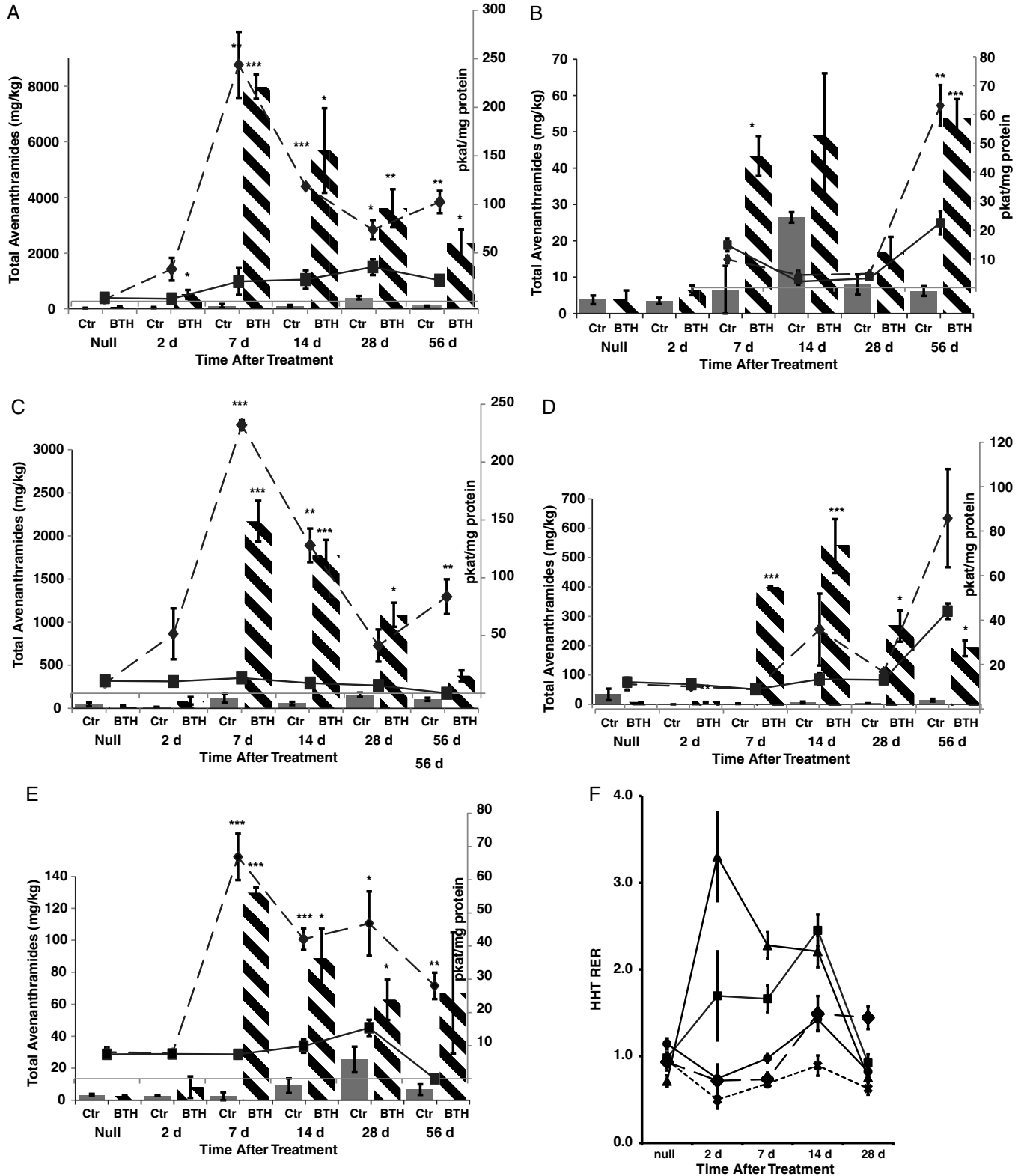
Lemma/palea

The lemma/palea were substantially lower in avenanthramide content than either the flag leaf or the glumes but appeared to mirror the dynamics observed in those tissues (i.e., the peak values; 136 mg kg^{-1} mean total avenanthramides) were observed at 7 d after BTH treatment followed by a steady decline at 14 and 28 d post treatment. Unlike the leaf and glumes, however, the lemma/palea showed a slight increase in mean total avenanthramide concentration at the final time point, although this observation showed a rather large variance (Fig. 8E). RER analysis showed no demonstrable increase in HHT mRNA production resulting from BTH treatment. However, HHT enzyme activity was fairly robust in the BTH-treated plants, with a maximum value of 66 pkat mg^{-1} protein 7 d after treatment, followed by a steady decline to 56 d where it dropped to 28 pkat mg^{-1} protein. These values from 7 to 56 d were all statistically higher than the untreated control plants. The correlation between HHT activity and total avenanthramides was also fairly strong ($r = 0.84$, $p < 0.0001$).

Phloem sap

To examine whether the avenanthramides are being transported through the phloem we used an EDTA-facilitated exudation method to extract the phloem sap from stems of BTH-treated and untreated plants (see methods). The exudate from the stem segments of three

Fig. 8. Composite of graphs depicting mean total avenanthramide levels in (A) flag leaf, (B) developing seeds, (C) glumes, (D) panicle stems, and (E) lemma/palea. Solid bars, control treatment; diagonal striped bars, BTH-treated. Line graphs represent HHT activity (diamond, BTH-treated; square, control treatment). Note that for the developing seed there was insufficient tissue to do an HHT enzyme activity assay until 14 d post treatment. Error bars represent standard error of the mean. Statistically significant differences between individual leaves for BTH-treated vs. control are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as determined by student *t*-test, $n = 3$. (F) HHT relative expression ratio (RER); triangle, flag leaf; square, glume; circle, panicle stem; diamond (dashed line), developing seed; cross (dotted line), lemma/palea).



plants, analyzed separately, contained 10.8 ± 3.9 (standard deviation) μg of total avenanthramide; no avenanthramide was detected in the phloem exudate from the untreated plants. A separate analysis of whole stems from BTH-treated plants in this experiment showed a mean avenanthramide concentration of $712 \pm 75.3 \text{ mg kg}^{-1}$; the stems following exudation had a concentration of $609 \pm 67.3 \text{ mg kg}^{-1}$, this is significantly lower than the non-exudated sample ($p = 0.04$). This experiment was repeated with similar results, i.e., $7.1 \pm 1.6 \mu\text{g}$ total avenanthramide was extracted from three biological reps of BTH-treated plants, no avenanthramides were detected in the exudate from the untreated plants. Although the mean avenanthramide content in the stems after exudation was considerably lower than the sample measured prior to exudation, in this experiment the difference was not statistically significant (the variance in these data was quite large). BTH-treated oat stems, without EDTA treatment, left horizontally on the bench (to prevent gravity-induced exudation) for the same 6 h period that the exudation was conducted, did not show a significant reduction in total avenanthramide content relative to the stem sample not subject to exudation treatment. The distribution of individual avenanthramides is also interesting. The bulk of the avenanthramide content in the phloem sap is composed of 2p (~50%), 4p (~30%), and 5p (~20%), with only traces of 2c and 2f.

Discussion

The data presented here clearly illustrates that leaves of plants treated by BTH root drench are, by far, the most prodigious producers of avenanthramides. They also show a strong correlation between total avenanthramide production and HHT enzyme activity in leaf tissue as well as the glumes and lemma/palea.

Although avenanthramides were found throughout the plant and in most cases the BTH-treated plants showed significant elevation of avenanthramides relative to the untreated plants, the correlation between avenanthramide content and HHT activity was weak in the lower stem, roots, panicle stems, or the developing seeds. Likewise, HHT mRNA did not seem to be affected by BTH treatment in these tissues. However, it should be noted that HHT mRNA up-regulation is not a particularly sensitive indicator of avenanthramide biosynthesis. Indeed, the eight-fold RER observed in L3 of the seedling experiment (Fig. 2) is the highest RER reported for this enzyme to date, with maximum RER values in the two- to three-fold range being more common (Uchihashi et al. 2011; Wise et al. 2016). In these experiments and in a previous study of avenanthramide production in field treated oat (Wise et al. 2016), HHT RER could only be detected in tissues having total avenanthramide levels exceeding several hundred mg kg^{-1} . HHT also seems to be fairly long lived, as the activity is retained for extended periods after gene expression has subsided.

The aggregate of data from these experiments can be interpreted to suggest that much of the observed avenanthramide content is being transported via the phloem. Analysis of the phloem sap clearly indicates that some avenanthramide content is to be found there. The root data is somewhat enigmatic in that both HHT activity and mRNA levels are relatively high in both the treated and the untreated plants, but total avenanthramide levels are dramatically higher in the BTH-treated plants. There are several possible explanations including (i) the bulk of the avenanthramides is being transported to the root, (ii) BTH treatment up-regulates another necessary part of the biosynthetic pathway, or (iii) avenanthramides are normally exported from the root tissue (possibly to the surrounding soil) and BTH treatment inhibits this transport.

The dynamics of avenanthramide accumulation in flag leaves of the adult plants appeared to follow the same early pattern as in the seedling experiment, i.e., a detectable increase at 2 d after treatment and peaking by day 7 (or between days 4 and 10 in the seedlings), followed by a steady decrease out to the end of the experiment. The glumes essentially followed the same pattern and in both tissues HHT activity paralleled avenanthramide content, except for a (statistically insignificant) spike at the mature stage. This is more likely due to the low total protein content in the senescent tissue than an increase in HHT levels. It is interesting to note, however, that HHT remains active in these tissues in spite of a near total lack of mRNA.

In contrast, the panicle stems did not peak in avenanthramide content until 14 d, with concentrations approximately 10-fold lower than the flag leaf and four-fold lower than the glumes. HHT activity is also quite low and there is no significant difference between HHT activity in the treated vs. the untreated plants. And, similar to the phloem sap in the previous experiment, the avenanthramide composition is exclusively 2p, 4p, and 5p.

The developing seeds showed a similar dynamic to the panicles, with avenanthramides peaking at 14 d and then decreasing at 28 d in the BTH-treated plants. However, mean total avenanthramides were quite low throughout the experiment but spiked again in the mature grain where they were significantly higher in the BTH-treated plants relative to the controls ($p = 0.007$). This phenomenon was observed previously in field-treated oats (Wise et al. 2016). Also interesting was that the HHT activity in the BTH-treated mature grain was significantly elevated relative to the control. It is also worth noting that avenanthramide 2c became the predominant form of avenanthramide in the mature grain (54%, Supplementary Table S3).¹ This dynamic has been observed previously in field-grown oat (Peterson and Dimberg 2008; Dimberg and Peterson 2009) without BTH treatment. The biochemical mechanism for this transition in avenanthramide composition remains to be determined. The mature grain did not yield sufficient RNA to

accurately assess the effect of BTH treatment on gene expression.

Thus, it appears that avenanthramides are distributed throughout the oat plant but that the major locations for their biosynthesis are the leaves and the glumes. Observing avenanthramides in the phloem sap provides compelling evidence that these metabolites are transported from source to sink. The root data, in concert with the moderately high levels of avenanthramide and low HHT activity in the stems, and despite the relatively high HHT activity, strongly suggest import of avenanthramides via the phloem.

Author Contribution Statement

M.L. Wise conceived, designed, and carried out or directed the carrying out of all experiments, analysed the data, and prepared the manuscript.

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