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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*)

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Abstract. Oats produce a group of natural products termed avenanthramides. These compounds are produced in both the vegetative tissue and the grain. In leaf tissue they are produced 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 BADH 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 predominate 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 the avenanthramides. Avenanthramides were also detected in the phloem sap, indicating source to sink transport of these metabolites following BTH treatment.

Key Words: Acyltransferase, Phytoalexin, Phytonutrient, Plant Defense Activator.

Introduction

Avenanthramides are a group of phenolic alkaloids produced, among food crops, exclusively by oat. These metabolites are believed to function as phytoalexins in response to crown rust (*Puccinia coronata* Syd & P. Syd) infection (Mayama 1983, Mayama et al. 1995). They also display strong anti-oxidant 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 anti-oxidant 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 (Database 2014). Several studies have also shown strong genotype × environment interaction associated with avenanthramide concentration in the mature grain of oat (Emmons et al. 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 et al. 2013). Avenanthramides are also found in mature oat grain (Collins 1986) and the enzymatic machinery to biosynthesize these metabolites exist in both vegetative tissue (Ishihara et al. 1999, Peterson et al. 2007) and in the grain (Matsukawa et al. 2000, Peterson et al. 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*-hydroxycinnamoyltransferase (HHT), is 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 solution and to uptake radiolabeled avenanthramides from the elicitor solution (Okazaki et al. 2004). Similarly, oat callus tissue in liquid media were 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 reported on the temporal dynamics of avenanthramide biosynthesis in the spikelets and leaves of field grown oat (Peterson et al. 2008). 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

restriction of biosynthesis to the chloroplast. In another study, using crown rust (*Puccinia coronata* f. *sp. avenae*) 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. But, 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, 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 (Ren et al. 2013, Wise 2011, Wise 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 leaflets, 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.

MATERIALS AND METHODS

Chemicals

Methanol and acetonitrile were HPLC grade, purchased from Fisher Scientific as was the HCL and NaHPO₄. MOPS, BisTris, ATP, DTT, EDTA, ethyl acetate, *p*-coumaric acid, and Co-enzyme A came from Sigma, 95% ethanol was from Carolina Biological Supply, formic acid was from Acros Organics, 5-hydroxy-anthranilic acid was purchased from Alfa Aesar, 100% ethanol was from Pharmaco Aaper, and the BTH was kindly provided by Syngenta.

Plant Growth and Treatment

Seeds of cultivar 'Kame' were imbibed in deionized H₂O, with aeration, for three hours at room temperature then planted in 20 cm (D) × 18 cm (H) pots with commercial topsoil and peat moss (50:50) mixed with approximately 5 g of Osmocote. Upon emergence the plants were culled to three per pot and grown under a photon flux of approximately 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (determined with a Li-Cor 250 light meter (Lincoln, NE)) with a 16/8 hr light/dark cycle at 22 °C (light) and 16 °C (dark) and grown to the appropriate Zadok stage (Zadoks 1974). They were then treated with 500 mL of a 1 mM (active ingredient) solution of BTH or with an equal volume of H₂O as 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 hour), 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 processed for RNA or protein extraction. Tissue for avenanthramide extraction were collected in 50 mL Falcon tubes, with small holes bored in 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) and maturing spikelets were harvested at 0 (null), 2, 7, 14, 28 and 56 days (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, then approximately 0.5 g was carefully weighed and the weight recorded. The ground tissue was extracted with 3×10 mL 80% EtOH in 10 mM NaH_2PO_4 buffer, pH=2.0 in a shaker/water bath at 50 °C for 20 min. After each extraction the sample was centrifuged seven 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 re-suspended in 1.0 mL MeOH and filtered through a 0.2 μm nylon membrane. Avenanthramides were analyzed by UHPLC-PDA on a Shimadzu NexEra system (Kyoto, Japan) using a Kinetex 2.1×50 mm, 100Å pore, 1.7 μm diameter, C-18 column (Phenomenex, Torrance, CA) at 40 °C with a Shimadzu SPD-M20A photo diode array detector (PDA). 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 10 to 17% B over 2.1 min, 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 minutes 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 comparison of 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 et al. 2010) and its mass spectral data as determined by LC-MS (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 callous tissue (Wise et al. 2009).

LC-MS analysis

LC-MS 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 2.1×30 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 to 30% B over 20 minutes at a flow rate of 0.2 mL min^{-1} was employed. The column was operated at 30°C with $2.0 \mu\text{l}$ 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) 207 kPa (30 psi), dry gas flow at 8.0 L min^{-1} at 350°C with a capillary voltage set at 3500V. 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 $13\,000 \text{ } m/z \text{ sec}^{-1}$.

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 PCR (RT-qPCR). Thus, each treatment, at every time point, was performed with triplicate biological repetition (i.e., three plants/treatment/time point) and quadruplicate technical repetition ($2 \times \text{cDNA} \times 2 \text{ PCR}$). 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 (Santa Clara, CA). 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, Hercules, CA) and stored at -20 °C until analyzed. RT-qPCR was performed with an Applied Biosystems 7500 Fast System (Thermo-Fisher, Waltham, MA) using TaKaRa SYBR premix Ex Taq (Tli RNase H plus) 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 (Uchihashi et al. 2011). The following sequences were used: HHT-F GTGGAGATCGACTGCAACG; HHT-R AAGTCGACGGTGGGGATGA. These primer sequences are 100% conserved in all three of the published full length oat HHT mRNA sequences (Genebank accessions: AB076980, AB076981, AB076982). Several reference genes were evaluated based on published reports on their validation for use in cereals (Jarošová et al. 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 CCGACAAGGACAACATCATGG; HSP-90 F CAAGAAGCTTGTCTCTGCCACC; HSP-90 R ACAGCCCCTCGAACTTCTCCTT; GAPDH F GCCAGTTACTGTCTTTGGCGTC; GAPDH R GGCCTTGTCTTGTTCAGT GAAG. PCR amplicons for all primer pairs (target and reference genes), using BTH treated oat ('Kame') leaf

cDNA as 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 $2^{-\Delta\Delta C_t}$ method (Livak et al. 2001, Pfaffl 2006).

HTT Assay

Coumaroyl-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 (Beuerle et al. 2002). 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 μ mol) *p*-coumaric acid, 28.0 mg (31 μ mol) CoA, and 240 mg (0.44 μ mol) ATP dissolved in 4 mL 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 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 mM MOPS buffer. The column was washed with 6 column volumes of MOPS buffer, then 1 column volume milliQ H₂O. The coumaroyl-CoA was eluted with 100% MeOH. 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 mM as determined by the UV absorbance at 333 nm using an extinction coefficient of 21 000 L Mol⁻¹·cm⁻¹ (Stöckigt et al. 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 re-suspended in 1.0 mL ice cold 100 mM BisTris buffer, pH = 7.2 with 2 mM dithiothreitol (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 mM BisTris, pH 7.2, with 10 µL of 10 mM 5-hydroxy anthranilic acid in DMSO and 20 µL 2.5 mM coumaroyl-CoA and allowed 20 minutes reaction at 30 °C. The reaction was quenched by addition of 20 µL concentrated acetic acid followed by 380 µL MeOH (final volume 0.5mL). 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 BSA as a standard).

Phloem Analysis

Avenanthramide content in phloem extracts was determined by the EDTA facilitated method (King et al. 1974, Tetyuk et al. 2013). Oat plants, three per pot × six pots, were grown to the Z49 stage; three pots were treated with BTH and three were treated with water as the untreated control. 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 mM K₂-EDTA and the segments then placed and held vertically in 50 mL Falcon tubes containing 15 mL of 20 mM K₂-EDTA. These were allowed 6 hours to elute before removing the stems, 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, reduce to dryness by rotory evaporation, using a Büchi R-200 rotovap (New Castle, DE, USA). The dried extract was resuspended in 1.0 mL MeOH 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. And, as an additional control, the cut stem segments of the third plant was placed horizontally on the benchtop for six hours, frozen at -80°C 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 (1) EDTA facilitated exudation, (2) simple extraction of avenanthramide content or (3) six hours on the bench top without exudation, followed by simple extraction of avenanthramides.

Statistical Analysis

Avenanthramide data were analyzed using MiniTab 16 software. 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 un-equal variance, using Excel. Equivalence of variance was determined by Levene's test using Mini-Tab 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.

Results

Leaf Avenanthramides

Plants at the five leaf stage (Zadok Z15), approximately three weeks old, were treated with BTH and leafs collected at 0 (null, i.e. immediately before treatment), 24, 48, 96 and 240 hours after treatment. Figure 2 (and Supplementary Table S1) shows the results from avenanthramide analysis and the relative expression ratio (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 ANOVA, hence, initially there was no difference in total avenanthramide between any of the leaves or treatments. Subsequently, there was a clear temporal lag in response of the individual leaves, with the lower leaves responding first (within 24 hours) in both avenanthramide production and HHT gene expression. The highest concentration of avenanthramides occurred at 96 hours post treatment with values in the middle leaves (L2, L3) somewhat over $10\,000\text{ mg kg}^{-1}$. The upper leaves (L4, L5) were only about 30% of that at 96 hours. 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 $5\,000$ to $7\,700\text{ mg kg}^{-1}$, 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 hours (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 hours post treatment and enzyme activity remained elevated out to 10 days after treatment. By 10 days 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 hours with 393 pkat mg⁻¹protein. 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 48 hours, HHT activity remained fairly high (269 pkat mg⁻¹protein) out to 240 hours (Fig 3). A very strong correlation was observed between HHT activity and avenanthramide content in L3 ($r = 0.96$, ($p < 0.0001$)).

Upper/Lower Stems

The stems were divided at the middle to provide upper and lower halves (Fig 4 and Supplementary Table S2). Although at every time point the BTH treated plants contained higher concentrations of avenanthramides, only at 240 hours 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 upregulation. HHT enzyme activity in the lower stem tissue was fairly low, ranging from about 14-26 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 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). In this case, avenanthramide levels were significantly elevated at 96 and 240 hours post treatment. It's 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 Ct 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 hours post treatment) in both 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 hours 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), 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 $= 0.002$, respectively) (Fig 7 and Supplementary Table S2). 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 pkat mg⁻¹ protein with BTH treatment; the response in both tissues was statistically significant ($p < 0.001$ and $= 0.01$, 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 7 500 mg kg⁻¹ at 7 days 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 days. However, even at 56 days after treatment, when the grain was fully mature and the leaves apparently senescent, substantial levels of avenanthramides (2 150 mg kg⁻¹) were still present. The flag leaf also demonstrated a substantial RER for HHT mRNA, peaking at 3.30 at 2 days after treatment then dropping to 2.28 and 2.21 at 7 days and 14 days, respectively (Fig 8F), all of which are significantly elevated ($p < 0.001$) relative to the control treatment. By 28 days there was no significant RER. By 56 days 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 days. And, as with the seedling leaf 3 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 days following treatment followed by decreasing avenanthramide concentrations out to 28 days (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 days after treatment was this difference significantly higher ($p = 0.017$ and 0.002 , respectively). HHT gene expression was only demonstrably higher in the 14 and 28 day 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 days, with no significant difference between treatment groups (there was insufficient tissue to sample for HHT activity prior to the 7 days sample). In the final sample of mature grain, however, the BTH treated grain showed 62 pkat mg^{-1} protein 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, 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 what correlation is 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 2 200 mg kg^{-1} at 7 days post treatment (Fig 8C). As was the case with the flag leaves, mean total avenanthramide levels steadily decreased

out to 56 days, when the concentration dropped to 379 mg kg^{-1} . The values determined for 7, 14 and 28 days post BTH treatment were all statistically higher than the non-treated controls ($\alpha = 0.05$). At 2, 7, and 14 days post treatment HHT RER (Fig 8F) was significantly higher in the BTH treatment group ($p < 0.001$) before returning to the level of untreated plants at 28 days. In contrast to avenanthramide content, the RER for HHT peaked at 14 days post treatment at 2.45. Similar to the flag leaf, HHT activity was highest at 7 days, after which it steadily decreased over the next two sampling periods followed by a small, statistically insignificant, increase at 56 days 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 days post treatment with approximately 540 mg kg^{-1} (Fig 8D). The concentration decreased afterward and at 56 days 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). Only at 14 days 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 ($r = 0.22$, ($p = 0.20$)).

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 days following BTH treatment followed by a steady decline at 14 and 28 days 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 days after treatment, followed by a steady decline out to 56 days where it dropped to 28 pkat mg^{-1} protein. These values from 7 to 56 days 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 plants, analyzed separately, contained 10.8 ± 3.9 (SD) 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 ± 1.6 μg 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 six hour 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 2016). In these experiments and in a previous study of avenanthramide production in field treated oat (Wise 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: (1) The bulk of the avenanthramides are being transported to the root or (2) BTH treatment up-regulates another necessary part of the biosynthetic pathway or (3) 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 days after treatment and peaking by day 7 (or between day 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 days, with concentrations approximately 10 fold lower than the flag leaf and 4 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 days, and then decreasing at 28 days in the BTH treated plants. However, mean total avenanthramides were quite low throughout the experiment, but they 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 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 predominate form of avenanthramide in the mature grain (54%, Supplementary Table S3). This dynamic has been observed previously in field grown oat (Dimberg et al. 2009, Peterson et al. 2008) 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 the avenanthramides, via the phloem.

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Figure Captions

Fig. 1 Avenanthramide Structures

Fig. 2 Mean total avenanthramide levels in individual leaves at each time point. L1-L5 indicate leaf 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: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) as determined by student t-test, $n = 3$

Fig. 3 HHT relative expression ratio (RER) and enzyme activity in leaf 3 over time after BTH treatment. Leaf 3 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: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), $n = 9$ (RER), $n = 3$ (HHT activity)

Fig. 4 Lower and upper stem mean total avenanthramide concentrations at each time point (bar graph, stripped = 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: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) 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: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) as determined by student t-test, $n = 3$ (no significant difference was observed for the HHT activity)

Fig. 6 Threshold cycle (Ct) values for RT-qPCR at 96 hours post BTH treatment. Solid line (square) represent untreated controls, dashed line (diamond) represent the BTH treated plants, the associated numbers are the mean Ct values. Note that while the Ct 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 hours is taken as representative of the overall response. Statistically significant differences for BTH treated vs control are indicated by: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) as determined by student t-test, $n = 9$

Fig. 7 Panicle stem and spikelet mean total avenanthramide concentrations and HHT activity.

By 240 hours 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: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) as determined by student t-test, $n = 9$ (no significant difference observed for the HHT activity)

Fig. 8 Composite of graphs depicting mean total avenanthramide levels in: Flag Leaf (A), Developing Seeds (B), Glumes (C), Panicle Stems (D), and Lemma/Palea (E). Solid bars = control, diagonal stripe 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 14d post treatment. Error bars represent standard error of the mean. Statistically significant differences between individual leafs for BTH treated vs control are indicated by: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) as determined by student t-test, $n = 3$. Panel (F) is the HHT relative expression ratio (RER), flag leaf = triangle, glume = square, panicle stem = circle, developing seed = diamond (dash line), lemma/palea = cross (dotted line)

Form	R ₁	R ₂	R ₃
2p	H	H	OH
4p	H	OH	H
5p	H	OH	OH
2f	OCH ₃	H	OH
2c	OH	H	OH

112x97mm (300 x 300 DPI)

279x384mm (300 x 300 DPI)

203x147mm (300 x 300 DPI)

203x147mm (300 x 300 DPI)

203x147mm (300 x 300 DPI)

203x147mm (300 x 300 DPI)

203x147mm (300 x 300 DPI)

279x384mm (300 x 300 DPI)