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BARLEY HORDOTHIONIN ACCUMULATES IN TRANSGENIC OAT SEEDS AND PURIFIED PROTEIN RETAINS ANTI-FUNGAL PROPERTIES *IN VITRO*

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Summary

Transgenic anti-fungal gene expression in heterologous species provides a means to test resistance protein combinations across species barriers. This is the first report of transgenic anti-fungal seed storage protein accumulation in oat seed. An anti-fungal barley (*Hordeum vulgare* L.) hordothionin (*Hth1*) gene was genetically engineered into oat (*Avena sativa* L.) to determine the effect of hordothionin on pathogen resistance. The transgene was expressed in both leaf and seed tissue, with transgenic protein accumulation occurring only in the seed. Transgenic oat line HTH-Av5 expressed *c.* 94 μg HTH/g seed, 19% of native barley seed levels. The anti-fungal activities of HTH fractions from barley cv. Morex and oat (transgenic and control) were tested in an *in vitro* growth assay against an important small grain pathogen, *Fusarium graminearum*. The partially purified HTH fractions from control oat seeds did not inhibit fungal growth, while HPLC-purified HTH positive control, as well as partially purified barley and transgenic oat HTH inhibited growth similarly over a range of concentrations. These results indicate hordothionin can be expressed in a heterologous cereal species and still maintain its anti-fungal properties. Future studies with HTH targeted to additional tissues are planned to test for increased fungal resistance.

Key words: Avena sativa; *Fusarium graminearum*; hordothionin; transgene.

Introduction

Small-grain cereals, such as wheat, barley, and oat, are important crops throughout the world. Over the past 20 yr, they accounted for almost half of the over 700 million hectares of cereal grain harvested annually (http://apps.fao.org/ FAOSTAT agriculture database). These crops are susceptible to a range of bacterial and fungal pathogens, limiting grain production and quality. One of the most prominent of diseases in North America is head blight, mainly caused by *Fusarium graminearum* (Nganje et al., 2001), which infects all of the small grains including oat (Clear et al., 2000).

Pathogen attacks on agricultural crops lead to significant losses annually, and have prompted investigation of various means of genetically enhancing host plant resistance to disease. Field breeding programs have been successful in improving pathogen resistance in many crops; however, additional improvements are needed to meet resistance goals and reduce further losses. Genetic transformation approaches toward characterizing and enhancing resistance are being investigated to complement traditional breeding.

Plants produce anti-fungal, pathogenesis-related (PR) proteins in response to pathogen attack (Antoniw et al., 1980). PR proteins include those that are resident and induced upon infection (van Loon and van Strien, 1999). One recently added category of PR proteins is PR-13, thionin (Epple et al., 1997), a class of small (45–47 amino acids), highly basic, cysteine-rich proteins. Thionins can be broken into four major types based on the origin of plant species or tissue, but all share a conserved cysteine motif (reviewed in Florack and Stiekema, 1994). Type-1 thionins have been found in cereals like wheat (purothionin; Balls and Hale, 1940) and barley [hordothionin (HTH); Redman and Fisher, 1969]. Oat type-1 thionins have also been putatively identified (Békés and Lásztity, 1981); however, the work could not be replicated (R. Lásztity, personal communication). In cereals, type-1 thionin accumulates in the developing endosperm (Bohlmann et al., 1988).

The first report of the anti-bacterial and anti-fungal nature of thionin came shortly after it was first isolated, over 60 yr ago (Stuart and Harris, 1942). The anti-pathogenic properties of HTH have been studied in endogenous, *in vitro*, and transgenic conditions. Ebrahim-Nesbat et al. (1993) showed that incompatibility was directly correlated with concentration of thionin at the powdery mildew (*Blumeria graminis*)/barley leaf interface. An *in vitro* type-1 αHTH treatment of *Neurospora crassa* hyphae resulted in increased calcium ion uptake and permeabilization of the fungal membrane, leading to rupture (Thevissen et al., 1996). High levels of the barley type-1 αHTH protein in tobacco resulted in reduced symptom severity and fewer induced lesions after inoculation with *Pseudomonas syringae* (Carmona et al., 1993).

In this study, we introduced a type-1 barley hordothionin gene into oat to test its function as an anti-fungal gene in a heterologous cereal species. Using an *in vitro* assay, we challenged an important

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small grain pathogen, *F. graminearum*, with thionin isolated from barley and transgenic oat to show that the protein was produced in biologically significant concentrations and that its anti-fungal properties were maintained.

Materials and Methods

Plant materials and transformation. The oat line, Gaf/Park-1, was used as a donor for transformation explant source (Kaeppler et al., 2000). Mature embryo-derived oat callus was biolistically transformed with two co-bombarded vectors, a *gfp* and a *Hth1* vector, according to Kaeppler et al. (2000). The *Hth1* plasmid vector was pAHC25 (Christensen and Quail, 1996) containing the constitutive maize *ubi*1 promoter, first exon and intron, the *Hth1* gene in place of the *uidA* gene, and a nopaline synthase (NOS) termination sequence (Fig. 1*A*). The barley genomic sequence for the *Hth1* gene was originally cloned over a decade ago (Rodríguez-Palenzuela et al., 1988). We cloned the cDNA encoding the barley seed-specific αHTH from barley cv. Morex endosperm mRNA and used this to transform oat. Transgenic plants and their progeny were grown in a greenhouse with 21°C, 12 h days and 18°C, 12 h nights in pots containing Metro-mix 366 soil (Scotts, Marysville, OH, USA) as in Kaeppler et al. (2000).

Molecular analysis of transgenic status. Nuclear DNA for PCR and blot analysis was isolated using a CTAB protocol (Sambrook and Russell, 2001).

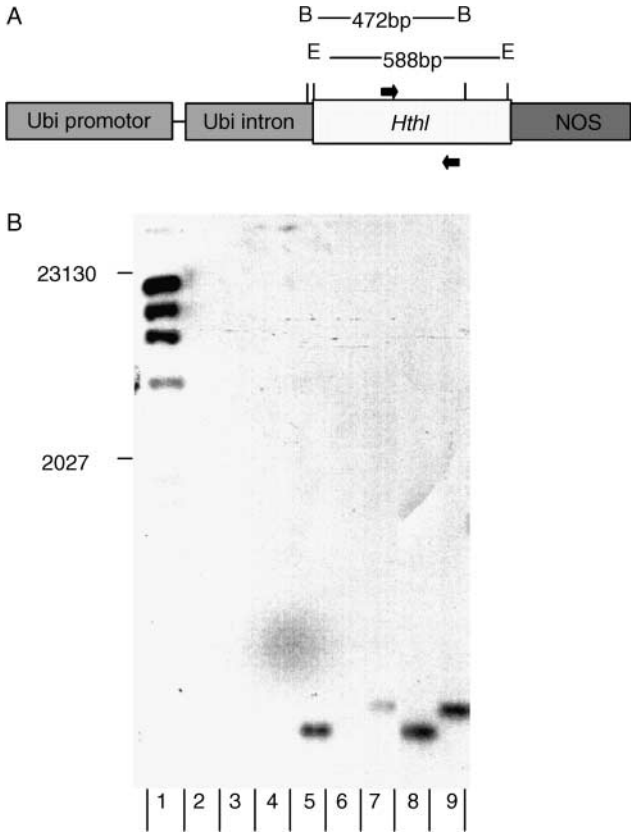

Fig. 1. Evidence of genetic transformation of the *Hth1* gene into oat. *A*, A diagram of enzyme digestion sites (*Bam*HI and *Eco*RI) of the *Hth1* vector and the PCR primer sites (*arrows*) used to make the probe. *B*, A composite of lanes from a DNA gel blot using digested transgenic and control oat DNA along with digested vector and HTH PCR product. *Lane 1* contains Lambda *Hind*III size markers labeled with a Lambda probe. *Lanes 2 and 3* are blank. *Lanes 4 and 6* are Gaf/Park non-transgenic lanes, *5 and 7* are HTH-Av5, and *lanes 8 and 9* are *Hth1* vector DNA. *Lanes 4, 5, and 8* are cut with *Bam*HI while *lanes 6, 7, and 9* are cut with *Eco*RI enzymes.

The polymerase chain reaction (PCR) was used with the oligonucleotides 5′ GCG GAT CCA TGG TGA GCA AG (forward) and 5′ GGG CGG CCG CTT TAC TTG TA (reverse) to amplify a 755 bp *gfp* fragment, and with 5′ GTA GCG GAA AAT GCC CTA CA (forward) and 5′ CAC CTT GCC CTG TGA AAT CT (reverse) to amplify a 266 bp *Hth1* fragment. The 25 μl reactions contained 250 m*M* of each dNTP, 0.5 m*M* of each primer, 1 × *Taq* polymerase buffer (Promega, Madison, WI, USA), 1.5 m*M* MgCl, and 1 U *Taq* DNA polymerase (Promega). The PCR program involved an initial denaturing (94°C/2 min), followed by 35 cycles of melting (94°C/30 s), annealing (60°C/1 min) and extension (72°C/2 min), and finally a 5 min extension at 72°C on a PTC-100 thermocycler (MJ Research, Waltham, MA, USA). Each set of PCR reactions included a positive (vector) and a negative (water) control. DNA blot analysis (Southern, 1975; Sambrook and Russell, 2001) used 10 μg of oat genomic DNA/lane completely digested with 100 U of either *Bam*HI or *Eco*RI. Products were electrophoretically separated in a 0.8% (w/v) agarose gel at 30 V. DNA was transferred to a hybond-N+ positively charged nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). A 266 bp *Hth1* PCR product was purified using low-melting point agarose gel electrophoresis and radiolabeled with ^{32}P dCTP using the Primagene labeling kit (Promega). The membrane was washed once with 2 × SSC/1% SDS for 15 min and three times with 0.2 × SSC/0.1% SDS for 15 min each at 65°C. The hybridized membranes were exposed to Kodak Bio Max MS film between Kodak Biomax MS intensifying screens at −80°C.

Analysis of gene expression. Total proteins were isolated from seeds of barley cv. Morex and oat by grinding 1 g of seed for each sample. Hordothionin was extracted by homogenizing in 5 ml 50 m*M* H_2SO_4 g^{-1} of seed (Carbonero et al., 1980) and partially purified using a modification of Jones and Mak (1977). The homogenate was centrifuged at 8000 rpm for 15 min at 4°C. The supernatant was adjusted to pH 7.5 with NH_4OH and was passed over a small CMC column equilibrated with 0.3 *M* NH_4OAc. The column was washed extensively with 0.4 *M* NH_4OAc, and HTH was eluted with 0.8 *M* NH_4OAc. All NH_4OAc buffers were adjusted to pH 5.2 with NH_4OH. The partially purified HTH was lyophilized and dissolved in 250 μl of dH_2O twice prior to molecular analysis and anti-fungal protein activity assays. Polyclonal antibodies were produced in rabbits following challenge with a 28 amino acid peptide corresponding to a portion of the HTH mature peptide (AnaSpec Inc., San Jose, CA, USA). The protocols for electrophoresis, protein blotting, and immunological detection of HTH were performed as previously reported (Skadsen et al., 2000). HTH from barley cv. Klages was provided by Berne Jones (USDA/ARS, Madison, WI, USA). It was purified according to Jones and Poulle (1990) using CM-cellulose chromatography and judged to be 100% pure by HPLC. The HTH was lyophilized from NH_4OH and quantified by weight.

HTH in vitro *anti-fungal assay.* *In vitro* assays were used to measure *F. graminearum* spore germination and hyphae growth as a function of HTH concentration and exposure time. The first assay examined two 48-well plates (Fisher Scientific, Pittsburgh, PA, USA); one with 1.5–4.5 μg ml^{-1} HTH inoculated with *F. graminearum* strain NRRL29169 (supplied by K. O'Donnell, USDA, Peoria, IL, USA) and the second with a range of 3.1–12.6 μg ml^{-1} HTH inoculated with a *gfp*/*F. graminearum* strain GZT501 (supplied by T. Hohn, Syngenta, NJ, USA). Each test was replicated twice. The growth of hyphae was documented visually by photographing each well using white light (NRRL29169) and GFP epifluorescence (GZT501). The second assay was performed over time in a 96-well plate (Fisher) at a single concentration of 16 μg ml^{-1} HTH inoculated with strain GZT501. The assay was replicated three times, and measured periodically from 12 to 42 h with a fluorometer (Tecan, Madison, WI, USA). GFP fluorescence in each well was measured using 485 nm excitation and 527 nm emission filters. Both assays were conducted by incubating spores at 30 rpm at 21°C and used 2000 spores ml^{-1}. The medium for growing the spores was an autoclaved organic carrot extract (200 g diced carrots per liter) diluted fivefold with dH_2O.

Results and Discussion

Transgenic status. Five fertile, independent transgenic oat lines expressing HTH were created by biolistic transformation and GFP-based visual selection. The transgenic status of these lines was confirmed with PCR and DNA blot analysis (data not shown). Expression data indicated that one of the lines, HTH-Av5, produced

the highest amount of HTH protein, so it was chosen for transgenic HTH protein isolation and further analysis. A DNA gel blot of T_3 generation HTH-Av5 and control plant DNA, digested with either *Bam*HI or *Eco*RI, showed the expected size bands (472 and 588 bp) in the lanes containing DNA from HTH-Av5 when compared to lanes containing *Bam*HI or *Eco*RI digested vector (Fig. 1*B*).

HTH expression. The line HTH-Av5 was advanced from the T_0 to the T_3 generation via self-pollination. To identify transgenic T_1 plants in the segregating populations, leaves were screened for resistance to 3 mg l^{-1} phosphinothricin (PPT; conferred by the *bar* gene in pAHC25) using a modified leaf assay (Wang and Waterhouse, 1997). T_1 plants from five PPT-resistant lines were selected for RNA expression studies. RNA from the leaves was separated on an RNA gel blot and probed with an *Hth1* probe. All five lines were positive for *Hth1* mRNA expression in the leaf; however, the protein did not accumulate there (data not shown). When the genomic form of *Hth1* was transformed into tobacco, the mature peptide did accumulate in the leaf tissue (Carmona et al., 1993). It is possible that differences between monocotyledonous and dicotyledonous protein targeting systems allowed the seed-specific HTH to accumulate in the leaf of tobacco compared to oat.

T_2 seeds from self-pollinated T_1 plants were tested for accumulation of the seed-specific HTH. The total seed proteins were isolated from single seed preparations and separated on a polyacrylamide gel, protein blotted, and probed with polyclonal HTH antibodies. All five transgenic lines had detectable levels of HTH (data not shown), and the line with the highest level of expression, HTH-Av5, was chosen as the candidate for future transgenic HTH isolation. To standardize the HTH isolation, 1 g each of seed sample from barley cv. Morex, HTH-Av5 and a non-transgenic oat control were ground and the HTH isolated using the H_2SO_4/CMC column thionin enrichment protocol. A second protein gel blot of the enriched samples confirmed the detection of HTH in HTH-Av5, Morex, and the HPLC purified HTH control, but none in the non-transformed oat control (Fig. 2). It is possible that the antibody used did not recognize the endogenous type-1 oat thionin proteins, since their amino acid sequence has only 64% (18/28) conserved homology with barley (in the region used as an epitope to raise antibodies). Given that no band was detected in the non-transformed oat lanes on the DNA blot (Fig. 1*B*), it is likely that the oat thionin sequence is not conserved enough to detect using hordothionin-derived antibodies or probes.

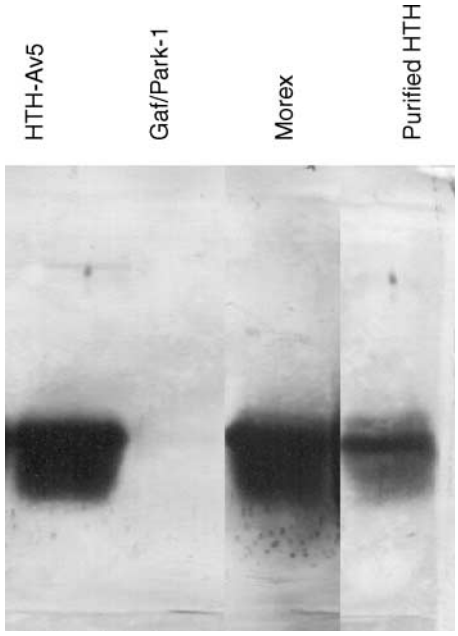

FIG. 2. Protein gel blot of partially purified HTH preparations from Morex, transgenic and non-transgenic oat, and HPLC-purified HTH protein. The oat samples, HTH-Av5 (*lane 1*) and Gaf/Park control (*lane 2*), both had partially purified HTH extract loaded from an equivalent of 5 mg of ground seed. An HTH band is detectable in the transgenic sample and not in the non-transgenic control. The Morex barley sample (*lane 3*) had partially purified HTH extract loaded from an equivalent of 1 mg of ground seed. The positive control, 200 ng of the HPLC-purified HTH, was loaded in *lane 4*. Both barley lanes exhibited the HTH band.

Thionin samples were compared to the HPLC purified standard to estimate amount of HTH produced in the seed of Morex

HTH source	Morex (Barley)				HPLC-HTH		HTH-Av5 (Oat)			
µg of seed	400	200	100	40	Standards		80	800	1600	2400
ng of HTH	200	100	40	20	100	200	n/a	75	150	225

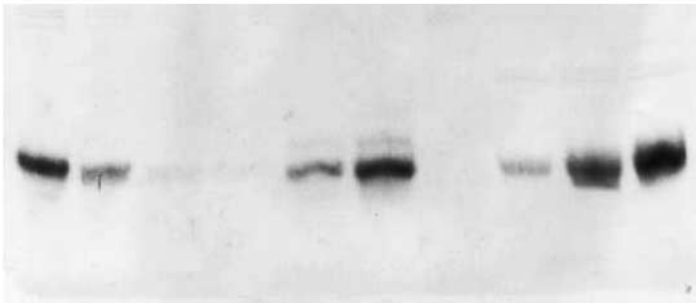

FIG. 3. Protein gel blot with dilution series of HTH from HTH-Av5 and Morex samples to estimate the quantity of thionin produced. Levels of HTH for each lane were visually estimated compared to the known HPLC-purified HTH bands. The equivalent microgram amount of ground seed in each lane is also listed. Using this dilution series, we estimated that barley produces about 500 µg HTH g^{-1} seed and HTH-Av5 produces *c.* 94 µg HTH g^{-1} seed.

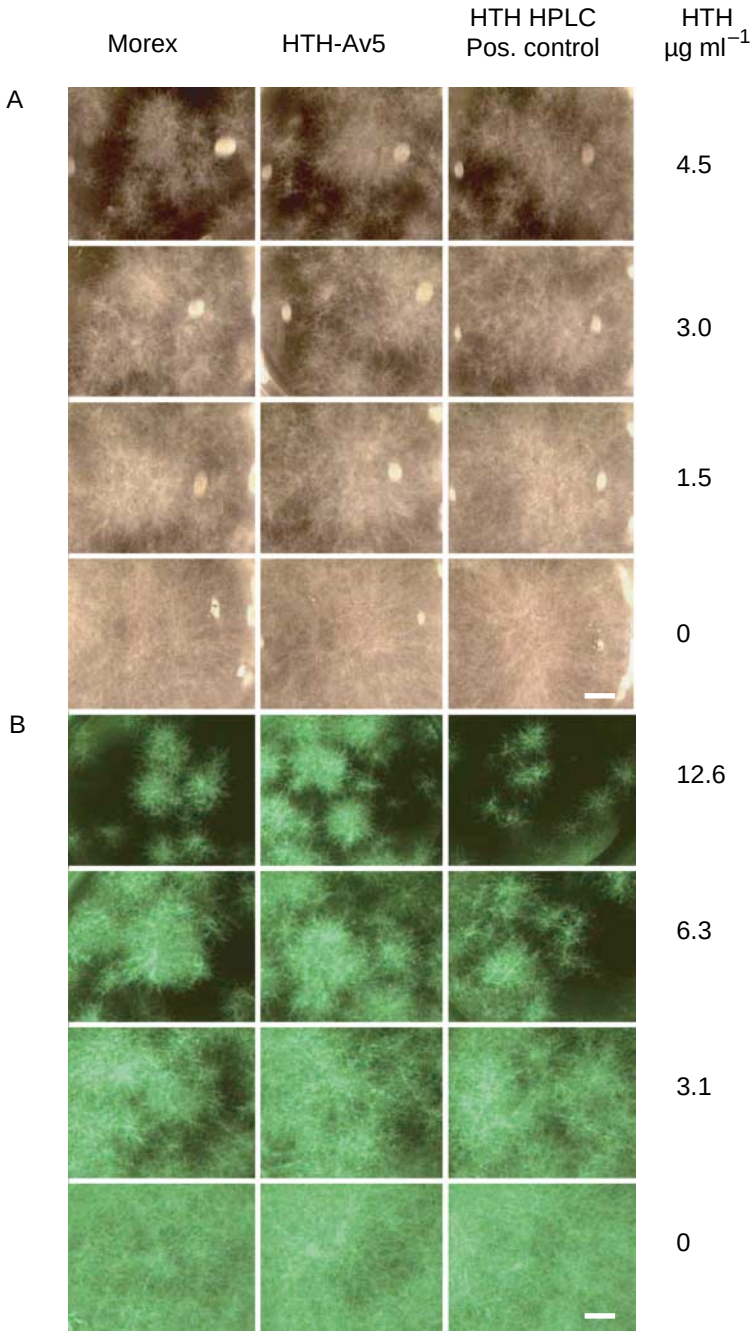

FIG. 4. *In vitro* assay to evaluate the HTH anti-fungal activity against untransformed *F. graminearum* and *gfp*/*F. graminearum*. Growth was observed at 42 h using HTH concentrations ranging from 1.5 to 4.5 μg ml^{-1} (*A*), and from 3.1 to 12.6 μg ml^{-1} (*B*) (using GFP epifluorescent microscopy). The *scale bar* represents 1 mm. The wells are 10 mm in diameter.

and HTH-Av5. Partially purified thionin samples were electrophoresed on a polyacrylamide gel that was either protein blotted and probed with polyclonal HTH antibodies or silver-stained (data not shown) to estimate the concentrations of HTH. The band intensity for the dilution series of the samples was visually compared to the HPLC purified standard, 100 ng μl^{-1} using a protein blot (Fig. 3). After estimating the approximate microgram of HTH production per gram of seed, the volumes of the HTH sample stocks were adjusted to *c.* 100 ng μl^{-1} for the *in vitro* assays of fungal growth. The average amount of HTH produced by transgenic oat line HTH-Av5 was calculated to be 94 μg HTH g^{-1} of oat seed. This was *c.* 19% of that isolated from the barley sample.

In vitro *assay for* F. graminearum *growth*. The oat line, HTH-Av5, could not be tested in an *in planta F. graminearum* assay due to the lack of a developed protocol. Anti-fungal activities of the transgenic oat and endogenous barley HTH proteins were compared

using two *in vitro* assays. Fungal growth was measured at a range of concentrations at one time point and at one high concentration over 42 h to determine if transgenic HTH retained its anti-fungal activity. Both assays were conducted for 42 h at 21°C. HTH inhibited growth of the pathogen but did not kill it completely. The time point of 42 h was chosen because it was the point of confluent fungal growth in the control plate wells.

The first assay tested a range of HTH at low concentrations (1.5–4.5 $\mu g\,ml^{-1}$) inoculated with *F. graminearum* strain NRRL29169 (Fig. 4*A*) and a range of HTH at medium concentrations (3.1–12.6 $\mu g\,ml^{-1}$) inoculated with a *gfp*/*F. graminearum* strain GZT501 (Fig. 4*B*). The inhibitory effect of HTH on fungal growth could possibly be observed at a concentration of 1.5 $\mu g\,ml^{-1}$. Increased fungal inhibition with increased HTH supplementation was observed at all levels (Fig. 4*B*).

The second assay tested a single, high concentration of HTH (16 $\mu g\,ml^{-1}$) inoculated with a *gfp*/*F. graminearum* strain GZT501, and measured GFP relative fluorescent units (RFU) from 12 to 42 h post-inoculation (Fig. 5). There was no detectable difference in growth across all of the treatments and controls at 12 h post inoculation. By 24 h, the uninhibited fungi (non-transformed oat and water controls) entered the log phase of growth, while all HTH-treated samples maintained a stationary level of GFP fluorescence that was not different from the initial measurement, indicating no detectable hyphae growth.

Thevissen et al. (1996) studied the inhibitory effect of HTH concentration on *N. crassa* hyphae using non-metabolite, α-aminoisobutyric acid uptake as an indicator of membrane permeability. They found that 1–20 $\mu g\,ml^{-1}$ of HTH increased membrane permeability to α-aminoisobutyric acid and inhibition of fungal growth. Our studies found a similar range of growth inhibition (1.5–16 μg HTH ml^{-1}) against *F. graminearum* (Figs. 4, 5). Overall, fungal growth was inhibited equally among the partially purified Morex and transgenic oat HTH fractions and HPLC-purified HTH.

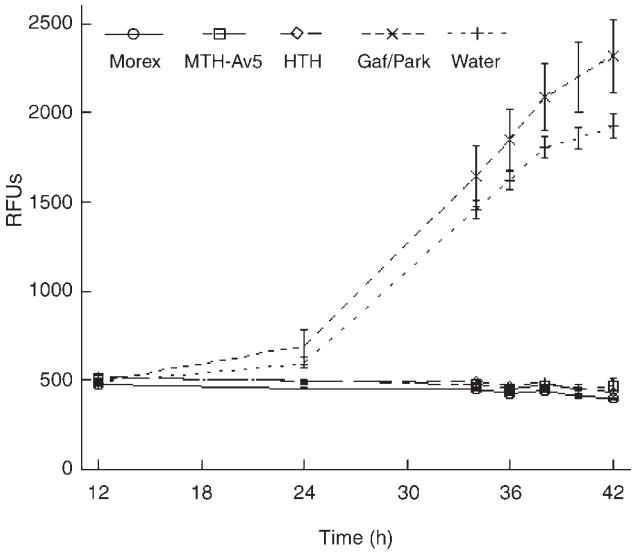

FIG. 5. Effect of 16 $\mu g\,ml^{-1}$ HTH on growth rate of *gfp*/*F. graminearum*. Each HTH treatment and control was grown in three replicates for 42 h. The mean of the RFU growth value is plotted with the standard error of the means. Gaf/Park and the water control yield the same lag/log growth curve, but Gaf/Park outgrew the water sample throughout the assay. None of the HTH samples grew over the 42 h period.

Future considerations and conclusions. This research has established that hordothionin, expressed as a transgene, retains its ability to act as an anti-fungal protein that reduces growth of *F. graminearum in vitro*. This transgenic approach could be applied not only to oat, but to other grain crops as well, to manipulate their levels of accumulated thionin protein and potentially increase their resistance to fungal pathogens. If the protein could be retained in the leaf it may also serve as a transgenic form of resistance to leaf-based pathogens. Future tests are planned to examine if and how expression of HTH, a seed storage protein as well as an anti-fungal agent, has changed the nutritional qualities of the oat seed.

In conclusion, this is the first report of the anti-fungal seed storage hordothionin accumulating in oat. In addition to expression and protein accumulation, maintenance of the protein's anti-fungal property was confirmed. Transgenic hordothionin anti-fungal activity was comparable to the native barley HTH, based on a *F. graminearum in vitro* assay.

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