Chapter 4 Expression of DsRed under the *cbh1* promoter and the *hex1* promoter with random integration

4.1 Introduction

4.1.1 Comparison of the *cbh1* promoter and the *hex1* promoter

The use of the *cbh1* gene promoter for industrial enzyme production is well established and the expression system continues to be improved for the production of a broad range of proteins (discussed in Chapter 1). However, the use of promoters that are active in the presence of glucose are highly desirable for industrial protein expression in *T. reesei* for the prevention of the simultaneous production of unwanted cellulose hydrolysing enzymes and for the growth of *T. reesei* in simple, inexpensive medium (discussed in Section 1.6). The *hex1* gene promoter has not been investigated previously for the expression of alternative proteins. The current work has established that the expression pattern of the *cbh1* and *hex1* promoters is very distinct and that the *hex1* promoter may offer several unique features that could contribute to making the *T. reesei* system more versatile.

One of the dominant differences between the *cbh1* gene promoter and the *hex1* gene promoter is the effect of growth medium on the level of promoter activity. The *cbh1* promoter is strongly induced by cellulose and is repressed in the presence of glucose (Ilmén *et al.*, 1996a; Ilmén *et al.*, 1997; Margolles-Clark *et al.*, 1997). In contrast, the *hex1* promoter was active in growth medium conducive to *cbh1* promoter induction (cellulose medium), *cbh1* promoter repression (glucose medium) and in neutral conditions in respect to *cbh1* expression (glycerol medium) (Section 3.2.6). The activity

in conditions that repress *cbh1* promoter activity is a beneficial feature of the *hex1* promoter for protein production.

The detection of a peak in *hex1* gene expression seemed to coincide with the active growth phase of the culture, after which the level of expression declined with the reduced level of mycelial growth. Consequently, the active phase of the *hex1* promoter was brief, yet exceeded the level of *cbh1* promoter activity at the early time point (24 h in CLS medium) as the *cbh1* promoter experienced a lag in activity early in the culture period. This lag period was compensated for by the maintenance of strong expression in the mature culture, which greatly exceeded the level of *hex1* expression.

The *cbh1* promoter encodes a protein that is typically secreted from the cell whilst the *hex1* promoter directs the expression of an abundant intracellular protein. The structure of the two promoters is also vastly different. The *cbh1* gene contains a major *tsp* at –83 and –93 from the start codon and strong expression requires as little as 210 bp of the 3' sequence of the *cbh1* promoter even though regulatory elements are located as far as –1492 from the start codon (Ilmén *et al*., 1996a). The *hex1* promoter region appears to be more complicated due to the fact of two alternative *tsp*s at approximately –288 and –1337 from the start codon, which are intervened by a 506 bp intron within the UTR. It has not been determined at this stage, which regions of the *hex1* promoter region are important for the activity of the promoter but the complexity of the promoter structure indicates that regulation may be multilayered.

In light of the differences between the *hex1* and the *cbh1* gene promoters, the *hex1* promoter may offer a unique pattern of activity that may be useful for the production of particular proteins in *T. reesei*. In order to investigate the potential of the *hex1* promoter

for protein production, an expression vector was constructed which contained the gene encoding for the fluorescent reporter protein DsRed under the regulation of the *hex1* promoter. DsRed was also expressed under the control of the *cbh1* promoter to maintain the comparison with the *hex1* promoter. The expression of DsRed under a well characterised promoter such as *cbh1* also provided the opportunity to investigate the option of using DsRed as a fluorescent reporter in fungal studies.

4.2 Results and discussion

4.2.1 Expression vectors

The expression plasmid phexDsR, was constructed to investigate the ability of the *hex1* promoter to drive expression of a heterologous protein (described in Section 2.6.3) (Figure 4-1). The entire 2.4 kb region, 5' to the coding sequence of the *hex1* gene, was used in the phexDsR construct to be sure of inclusion of any *cis* regulatory motifs. The plasmid also contained 1 kb of the *hex1* terminator region at the 3' end of the expression cassette to increase the likelihood of complementary alignment at the *hex1* locus and thereby, homologous integration. The features of the phexDsR plasmid were designed to resemble those of the pHEN54RQ plasmid, which had previously been used successfully to demonstrate expression of a heterologous protein under the *cbh1* promoter (Bergquist *et al*., 2004). The pHEN54RQ plasmid was used to comparatively gauge the level of expression from the *hex1* promoter as well as serve as a positive control for the expression of the novel fluorescent reporter DsRed in *T. reesei*.

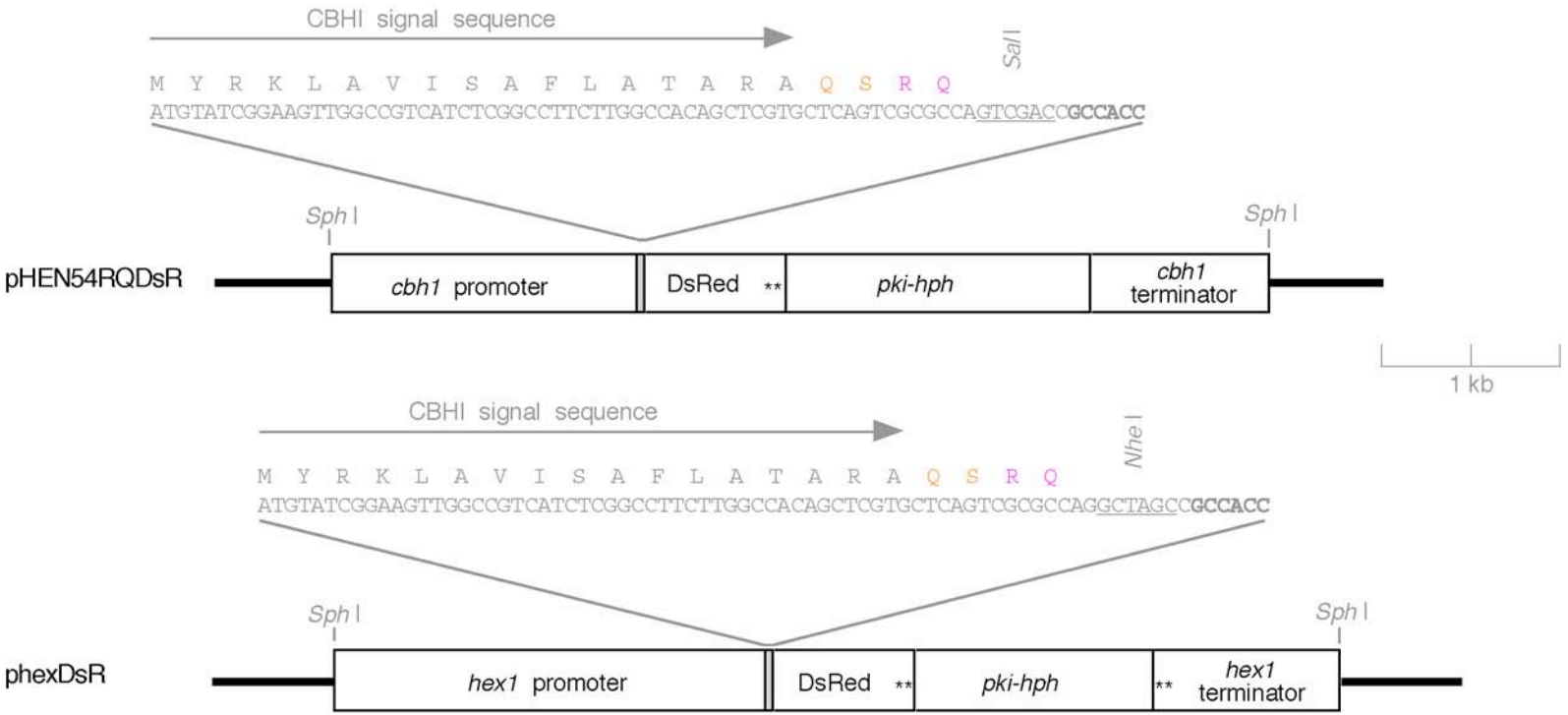

Figure 4-1. DsRed expression vectors pHEN54RQDsR containing the *cbh1* promoter, and phexDsR containing the *hex1* promoter. The leading residues of the *cbh1* core are shown in orange, the Kex2-like cleavage site is shown in pink and the Kozac sequence is in bold. The *hph* gene which imparts resistance to hygromycin B is under control of the *pki1* promoter. The position of stop codons is represented by asterisks.

Both above vectors contain the respective promoter regions followed by the *cbh1* signal sequence, two codons encoding a portion of the CBHI core, and a Kex2-like cleavage site, as a translational fusion to the DsRed-encoding sequence (Figure 4-1). Each expression vector also possessed a Kozac sequence adjacent to the start codon for DsRed to potentially increase translation efficiency and the insertion of the *pki-hph* fragment allowed for the selection of transformants on hygromycin B (Mach *et al*., 1994).

To our knowledge, at the time of this work, this was the first study to use any variant of the fluorescent reporter, DsRed, in filamentous fungi. To predict the functionality of DsRed in *T. reesei*, the codon usage of DsRed1-E5 was determined using the Codonfrequency programme on ANGIS (Section 2.5.2). A difference in codon usage between the endogenous host and the heterologous host can negatively impact upon heterologous protein production and codons are sometimes altered to favour the preferences of the production host (Wantanabe *et al*., 1993; Te'o *et al*., 2000). However, the codons of the DsRed gene sequence and the codon usage of *T. reesei* were found to be compatible.

4.2.1.1 Expression of DsRed in *E. coli*

The DsRed-encoding DNA fragment was cloned under the *LacZ* promoter in pUC18 and expressed in *E. coli* to confirm that the sequence encoded a functional protein (Section 2.6.1). The expression was confirmed by observation using fluorescence microscopy (Figure 4-2). The functional DsRed-encoding fragments were excised and ligated into the fungal expression vectors pHEN54RQDsR and phexDsR as described in section 2.6.

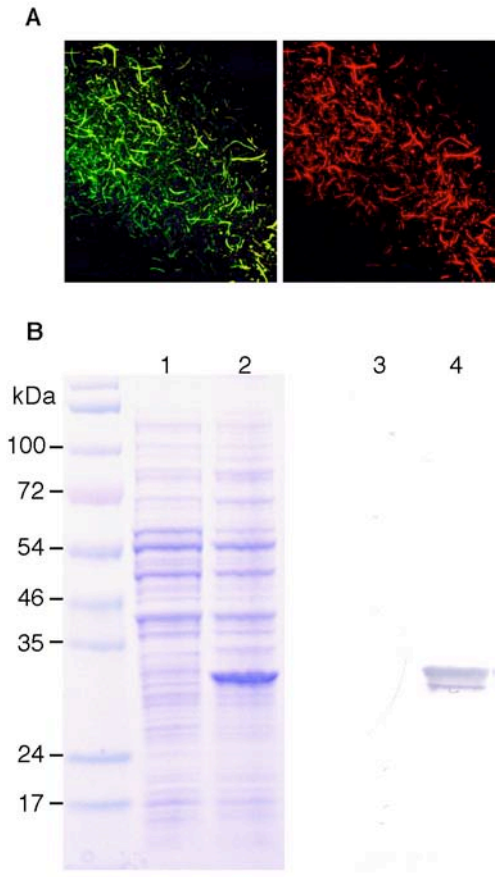

Figure 4-2. Production of DsRed protein in *E. coli*. A. A smear of *E. coli* cells transformed with pUC18-DsRed was exposed to blue and green light causing green and red fluorescence respectively (40x magnification) B. Proteins in the crude cell lysate of *E. coli* transformed with 1. pUC18 plasmid or 2. pUC18-DsRed, were separated by SDS-PAGE and stained with coomassie. The gel was blotted onto nitrocellulose membrane and the result of a Western blot probed with DsRed monoclonal antibody is shown in the right panel. 3. Cell extract from the pUC18 only control 4. Cell extract from the pUC18-DsRed recombinant.

A Western blot of the *E. coli* cellular lysate was carried out. A strong band at approximately 29 kDa labelled positive for DsRed, and was absent in the cellular lysate from *E. coli* transformed with pUC18 plasmid (Figure 4-2). This crude cellular extract from *E. coli,* was used in future experiments as a positive control on Western blots and to assay the relationship between DsRed protein concentration and fluorescence intensity (refer to Section 4.2.6).

4.2.2 Generation and screening of transformants

T. reesei was transformed with the linear expression cassette derived from the plasmids phexDsR or pHEN54RQDsR by biolistic bombardment (Section 2.7.1). Twenty eight transformants carrying the phexDsR expression cassette and 26 transformants carrying the pHEN54RQDsR expression cassette survived two rounds of selection for resistance to hygromycin B. Genomic DNA was extracted and transformants were further screened by PCR and fluorescence microscopy (Table 4-1). Transformants in which the promoter region and DsRed-encoding sequence of the expression cassette had been amplified by PCR, were also screened by PCR for homologous integration into the *hex1* or *cbh1* locus using primers internal to the respective coding regions. None of the transformants of either expression cassette were positive for homologous integration of the transforming DNA into the corresponding *hex1* or *cbh1* locus.

Table 4-1. Summary of the screening procedures of transformants of the expression cassettes phexDsR and pHEN54RQDsR. Intergration of the expression cassette by ectopic or homologous integration was determined by PCR. The expression of DsRed was determined by fluorescence CLSM.

Expression cassette	**Number of transformants**	**Integration of expression cassette into the genome**	**Integration of cassette into the *hex1* locus**	**Presence of intracellular fluorescence**
phexDsR	28	24	0	8
pHEN54RQDsR	26	21	0	13

Transformants were then grown in cellulose medium and observed daily for seven days by fluorescence microscopy. Eight transformants carrying the phexDsR expression cassette and thirteen of the pHEN54RQDsR transformants displayed fluorescence at some point over the seven day period. The presentation of transformants which did not express the heterologous gene is not unusual and is generally attributed to the number

of expression cassettes integrated into the genome and the varied sites of integration (reviewed by Mäntylä *et al.*, 1998).

4.2.3 Fluorescence microscopy observations

4.2.3.1 Optimisation of fluorescence microscopy and sample preparation

Since there was no previous experience with the use of the DsRed variant in filamentous fungi, the growth conditions, sample preparation and microscopy procedures required some investigation to establish protocols.

For the use of fluorescent reporter proteins in filamentous fungi it should be noted that confocal fluorescence microscopy is beneficial, especially due to the relatively large cell size and thickness of hyphae. Initially, fluorescence observations of mycelia were carried out using epi-fluorescence microscopy. Whilst this technique was sufficient to visualise the presence or absence of fluorescence within hyphae, the distribution of fluorescence within the cells appeared different to when observed by the confocal microscope. For example, under the epi-fluorescence microscope the fluorescence within pHEN54RQDsR transformants appeared as uniformly distributed throughout the hyphal cell, but confocal fluorescence microscopy revealed the fluorescence as densely punctate throughout the hyphae with occasional larger fluorescent concentrations.

When observing highly fluorescent samples such as the pHEN54RQDsR transformants, a Helium/Neon laser intensity of 20 V was sufficient. However, when viewing samples of low fluorescent intensity such as the phexDsR transformants the Helium/Neon laser intensity was increased up to 66 V. Due to the thickness of the fungal hyphae

(approximately 5 μm) and the tendency of a single hypha to not lie flat on a single focal plane, the images were acquired as a z-stack, typically of 0.2 μm increments.

Sample preparation was important for the visualisation of fluorescence with minimal autofluorescence. Transformants grown on PDA or solid cellulose medium showed a variable level of fluorescence depending on where on the colony (periphery, centre or arial hyphae) the sample was taken. Transformants grown in liquid medium had a more uniform distribution of hyphae representative of the entire mycelium and were found to provide the more suitable material when monitoring changes in expression of fluorescent protein over the course of days.

Transformants grown on PDA containing hygromycin B presented a high level of green autofluorescence over a broad range of wavelengths. In addition, older hyphae (present from day 4 onwards) cultured on any of the media used also displayed green autofluorescence. Under green light excitation there is almost no red autofluorescence in *T. reesei,* which makes the choice of red fluorescent reporter proteins more practical for these applications.

4.2.3.2 Fluorescent microscopy observations of phexDsR transformants

The expression of DsRed in transformants carrying the phexDsR cassette was driven by the *hex1* gene promoter. The fluorescence in phexDsR transformants was of very low intensity and sparsely distributed throughout the mycelium. The transformants were grown in PD broth, CLS and cellulose medium to optimise the level of DsRed fluorescence. However, the growth medium did not appear to have an effect on the intensity of fluorescence, so cellulose medium was adopted as a standard growth

medium in order to be consistent with the growth conditions of the pHEN54RQDsR transformants.

In transformants of the phexDsR cassette, intracellular fluorescence was observed almost solely on day two over the course of a seven day period, in cellulose medium. The fluorescence appeared as small, concentrated spots throughout a hyphal cell (Figure 4-3). The level of green fluorescence was extremely low and often indistinguishable from the autofluorescence of *T. reesei*. Red fluorescent spots were most prevalent at day two where an individual hyphal cell may have contained between 1 to 6 fluorescent spots. There was no correlation in the occurrence of fluorescent spots between apical and subapical cells. In addition, cells containing fluorescence were frequently adjacent to non-fluorescent cells. The distribution of fluorescent hyphae within the mycelium was sparse. Overall, only approximately 20% of a slide preparation contained discernible fluorescent spots at best. Therefore, it appeared that cells showing fluorescent spots were randomly distributed in the mycelium. This may

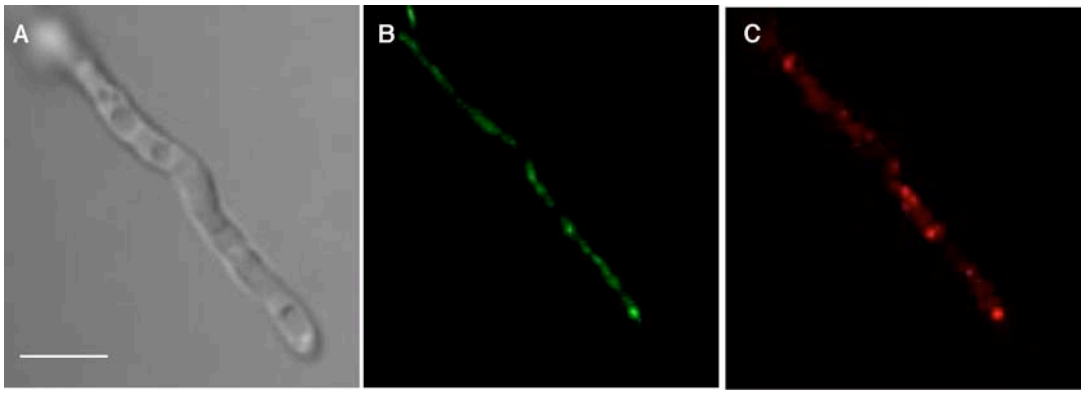

Figure 4-3. Hyphal tip of a phexDsR transformant expressing DsRed at day two grown in cellulase medium. These images show an isolated apical cell which demonstrated the highest concentration of fluorescence observed for phexDsR transformants. The images are DIC (A) or excitation under blue (B) or green (C) light. The scale bar represents 5 μm. The fluorescent images are extended view images compiled from a z-stack. A 3D animation of these images is available on the supplementary DVD.

be an indication that the *hex1* promoter was differentially regulated between cells of the mycelium or that the DsRed protein was transported to these particular cells. The *cbh1* ss was the only discernible targeting sequence fused to DsRed and may have caused the punctate pattern of fluorescence observed by the concentration of DsRed into secretory vesicles. Nykänen *et al*. (2002) has shown that CBHI secretion does not occur solely from the hyphal tips. Therefore, whilst the *cbh1* ss targets protein for the secretory pathway, it seems that it is not solely responsible for targeting the protein to apical cells and this is reflected by the unbias distribution of fluorescence between apical and nonapical cells found in this study. In particular, the lack of a concentration of mature protein (red fluorescence) in the apical cells.

It has been shown that the *hex1* promoter is active from at least 24 h from the time of inoculation (refer to Section 3.2.6). However, fluorescence was not visible until at least day two in any phexDsR transformant. The time difference between the appearance of *hex1* transcripts and the appearance of DsRed expressed under the *hex1* promoter may be attributed to the maturation time of the DsRed fluorophore. The same DsRed variant used in this study took nine to twelve hours from induction in HEK293 mammalian cells and five hours in *C. elegans,* to develop into the red conformation (Terskikh *et al*., 2000). In fungi, other DsRed variants such as DsRed2 took one day to display red fluorescence when expressed under the *gpd* promoter in *Fusarium oxysporum* f. sp. *lycopersici* (Nahalkova and Fatehi, 2003) and the most rapid maturing variant, DsRed-Express also took one day from transformation to display red fluorescence when expressed under the *gpd* promoter in *Penicillium paxilli* (Mikkelsen *et al*., 2003).

4.2.3.3 Fluorescent microscopy observations of pHEN54RQDsR transformants

Over the course of a seven day culture in cellulose medium, the development of fluorescent hyphae by the expression of DsRed under the *cbh1* promoter was as follows. After one day the conidia had germinated and the hyphae had developed branches, however there was no fluorescence distinguishable from that of a non-transformant grown under the same conditions. By day two, faint green fluorescent spots had appeared through some hyphae and by day three, all hyphae contained numerous green and red fluorescent spots of high intensity (Figure 4-4). This pattern remained until day five when the intensity of green fluorescence had decreased in most hyphae and approximately 10% of the mycelium was no longer fluorescent. The number of fluorescent hyphae continued to decrease until day seven when only

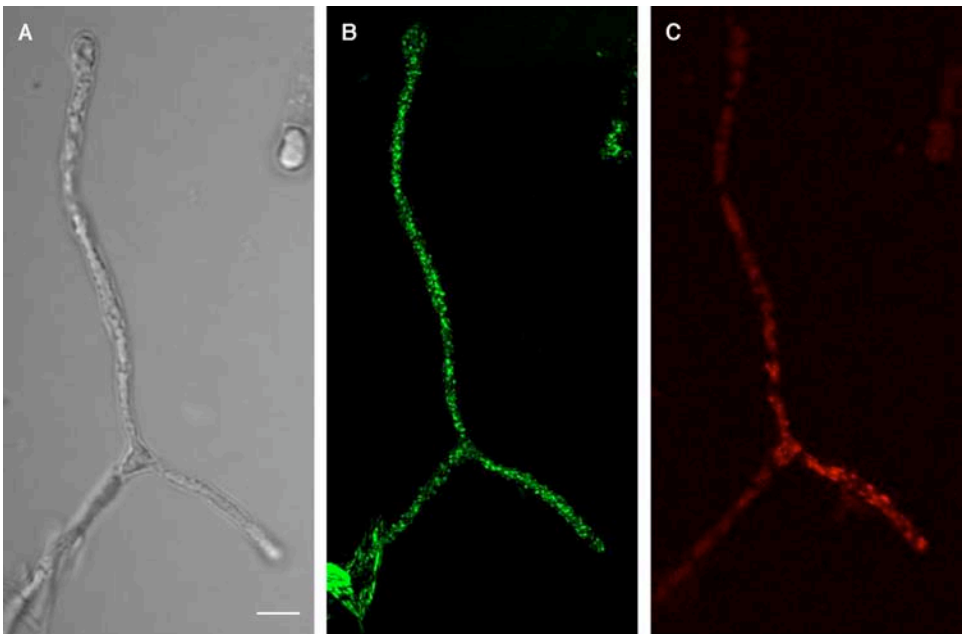

Figure 4-4. Hyphae of a pHEN54RQDsR transformant after three days of culture in cellulose medium. Images were taken using DIC (A) or excitation under blue (B) or green (C) light. The fluorescent images are extended view images compiled from a z-stack. The scale bar represents 5 µm. A 3D animation of these images is available on the supplementary DVD.

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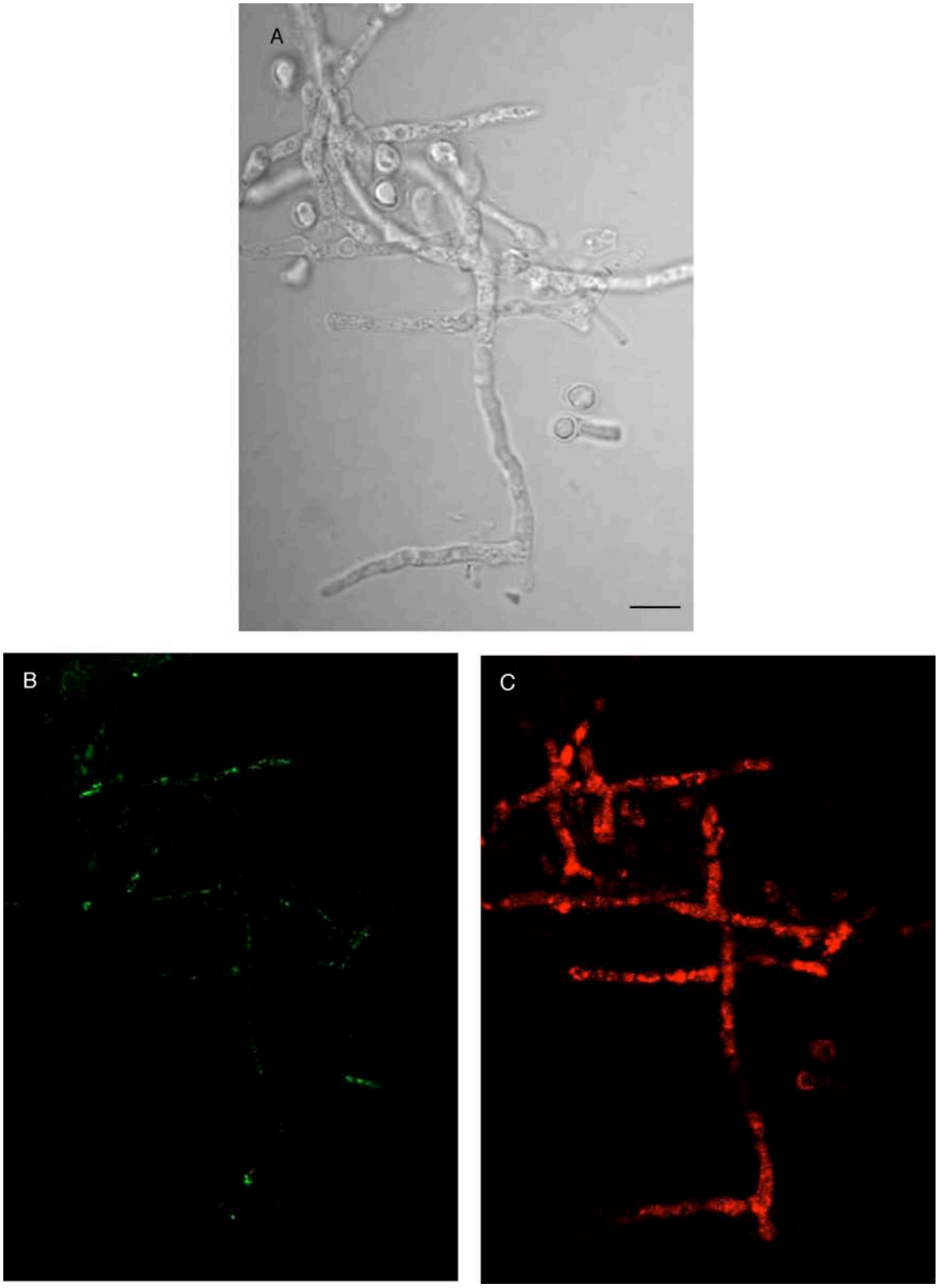


Figure 4-5. Mycelia of pHEN54RQ transformant after four days of culture in cellulose medium. A. DIC image. B. Blue light excitation. C. Green light excitation. Images are a single layer scan. The scale bar represents 10 μm.

approximately 40% of the mycelium maintained red fluorescence. The nonfluorescent hyphae appeared dead as the cellular contents appeared granular and had shrunken away from the cell wall.

The punctate pattern of fluorescence was distributed throughout the entire mycelium and was not concentrated at the hyphal tips (Figure 4-5). There was no discernible difference between the distribution of green and red fluorescent spots. ie. the more recently formed protein co-localised with the more mature protein within the mycelium. The equal distribution of fluorescence between apical cells and non-apical cells suggests that the *cbh1* promoter is active in all cells throughout the mycelium. This distribution of CBHI throughout the mycelium has been observed previously (Nykänen *et al.*, 1997). If the *cbh1* ss influenced translocation of the protein to the hyphal apices, it would have been expected to observe a bias of red fluorescence at the apices and green fluorescence in older cells. The lack of a green/red fluorescence spatial bias provides further evidence that the presence of the *cbh1* ss does not solely target the protein to the hyphal apex.

4.2.4 The copy number of integrated expression cassettes

Transformants which demonstrated the highest fluorescence intensities were selected for further characterisation. These included two transformants of the phexDsR expression cassette, designated h1 and h24, and three transformants of the pHEN54RQDsR expression cassette designated c20, c23 and c24. The genomic DNA was digested with *Hind*III, which cuts once within the expression cassette but not within the sequence complementary to the probe. A Southern blot was probed for the presence of *dsRed* (Figure 4-6). The appearance of several bands indicated that the expression

plasmids had integrated into the genome of the transformants h1, h24, c23, c24 and c20. No signal was detected for nontransformant *T. reesei* genomic DNA.

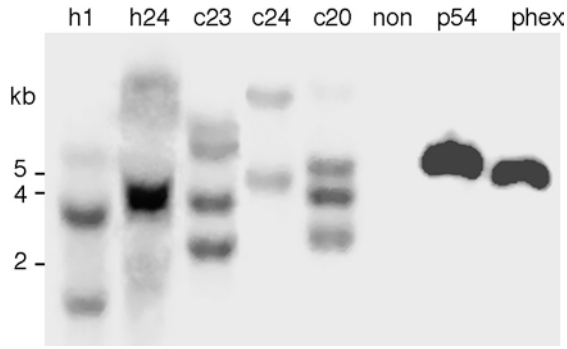


Figure 4-6. Southern blot of phexDsR transformants h1 and h24, pHEN54RQDsR transformants c23, c24 and c20, and a nontransformant (non) probed with *dsRed*. Linearised plasmids pHEN54DsR (p54) and phexDsR (phex) are shown as a positive control for the *dsRed* probe.

There were at least two copies of the DsRed expression cassette integrated into the genome of transformants h1 and c24. It is also likely that greater than one copy of the expression cassette had integrated into the genome of h24 since the single band had a very strong intensity. There were at least three copies of the DsRed expression cassette integrated into the genome for transformants c23 and c20. PCR analysis indicated that none of the expression cassettes had integrated into their respective homologous loci. The difference in fluorescence intensity between pHEN54RQDsR transformants and phexDsR transformants is unlikely to be a direct result of the copy number of expression cassettes integrated into the genomes since both h1 and c24 contained two copies of the expression cassettes. The fluorescence observations demonstrate that the *cbh1* promoter is stronger than the *hex1* promoter. This is consistent with the levels of *cbh1* and *hex1* transcripts when grown in cellulose medium (Figure 3-6).

4.2.5 The expression of DsRed as determined by transcript levels

The level of fluorescence under the *hex1* promoter was sparsely distributed and of low intensity, such that it was necessary to confirm that the fluorescence observed was produced by DsRed. RNA was extracted from cultures grown in CLS (Section 2.9) or glucose medium after 54 h and 5 days for northern blotting analysis. The transformants were grown in CLS medium because it contains the same components as cellulose medium but without the insoluble fraction, which made the extraction of good quality RNA from the mycelium simpler.

The *dsRed* transcript was detectable at 54 h in transformants h1 and h24 grown in CLS and glucose medium (Figure 4-7). Low levels of *dsRed* transcripts were also detectable after five days in CLS medium. The presence of *dsRed* transcripts supports the association between the low fluorescence intensity observed in hyphae of transformants h1 and h24 and the expression of *dsRed* under the *hex1* promoter. No *dsRed* transcripts were detected in the total RNA extracted from the nontransformant *T. reesei*.

However, these results are unusual since the *dsRed* transcript from h1 was approximately 2 kb in size and transformant h24 produced a *dsRed* transcript approximately 1 kb in size (Figure 4-7). The coding sequence for the *cbh1* ss and *dsRed* fusion is 758 bp in length. The transcription termination point is unknown for the expression cassette. However, if it is assumed to be identical in each transformant, this result implies that h1 and h24 used alternative *tsp*s on the expression cassettes. The 1 kb transcript from h24 corresponds to the expected size if transcribed from *tsp* 2 on the *hex1* promoter. The 2 kb *dsRed* transcript from h1 does not correspond to either of the

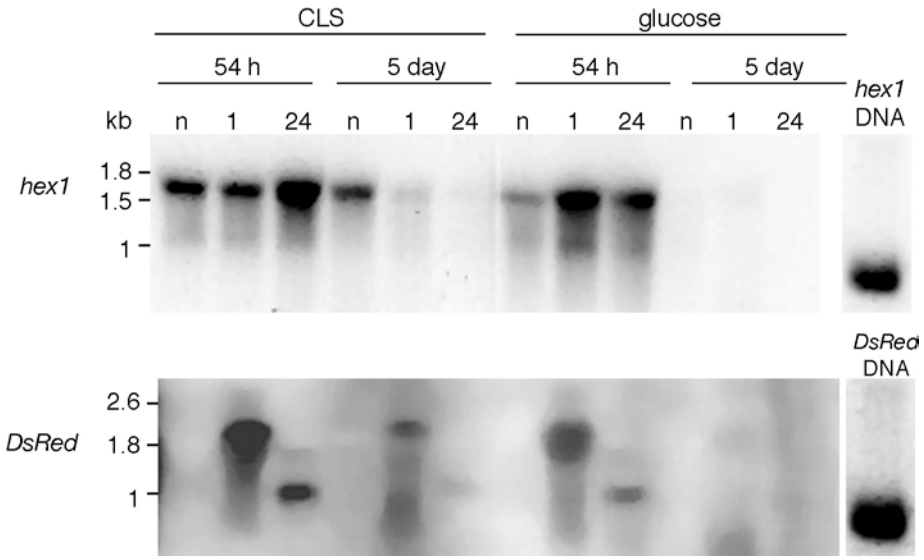


Figure 4-7. Expression of *hex1* and DsRed in phexDsR transformants h1 and h24. Transformants and a nontransformant (n) were grown in CLS or glucose medium and total RNA extracted at 54 hours and five days of culture. Total RNA (20 μg) was probed for *hex1* and *dsRed*. The *dsRed* blot was exposed to film for 30 min to visualise the bands and the *hex1* blot exposed for 5 min.

tsp types previously described (Section 3.2.2). However, a 2 kb transcript would result from transcription from *tsp* 1 and unsuccessful splicing of the intron within the 5' UTR.

Endogenous *hex1* expression appears unchanged in the phexDsR transformants ie. expression is highest early in culture at 54 hours and very low after 5 days and that the dominant *hex1* transcript is 1.6 kb in size (Section 3.2.6), which corresponds to transcription from *tsp* 1 of the endogenous gene promoter. However, h24 produced *dsRed* transcripts from the ectopically integrated expression cassettes indicative of transcription from *tsp* 2. If indeed transcription was from *tsp* 2 on the expression cassette, this result demonstrates that the cell can not only differentiate between endogenous or introduced copies of the *hex1* promoter, but also differentially induce transcription from each *tsp* between the endogenous and introduced copies of the *hex1* promoter simultaneously. If the regulation of the *tsp* is affected by the site of integration

or whether it is a result of an intrinsic factor of the endogenous *hex1* locus, remains unclear.

4.2.6 Secretion of DsRed into the culture supernatants monitored by fluorescence

The use of the fluorescent reporter DsRed in *T. reesei* was explored further by determining if the protein can be targeted for secretion into the culture medium. In addition, it was of interest to determine if a heterologous protein expressed under the *hex1* promoter, N-terminally fused to a secretion signal obtained from the effectively secreted protein CBHI, would be guided into the culture medium.

Three transformants, which displayed the highest intensity of intracellular fluorescence expressed under the *cbh1* promoter (c20, c23 and c24) and *hex1* promoter (h1, h5 and h24), were chosen for monitoring the level of DsRed secreted into the supernatant. Transformants were grown in cellulose medium and glucose medium in duplicate in shake flask cultures. The supernatant was sampled daily and the fluorescence ratio was calculated (Section 2.15). In a previous study using the fluorescent reporter GFP in fungi, only fluorescence intensities greater than three times the background level were considered to be a positive indication of secretion (Ma *et al.*, 2001) and this threshold has also been adopted for the current study.

Fluorescence was detectable above background only in the supernatants of transformant c20 in which DsRed was expressed under the *cbh1* promoter (Figure 4-8). Even though fluorescence was observed intracellularly by day two in c20, detectable levels of extracellular red fluorescence were not present until day four of culture. The intensity of

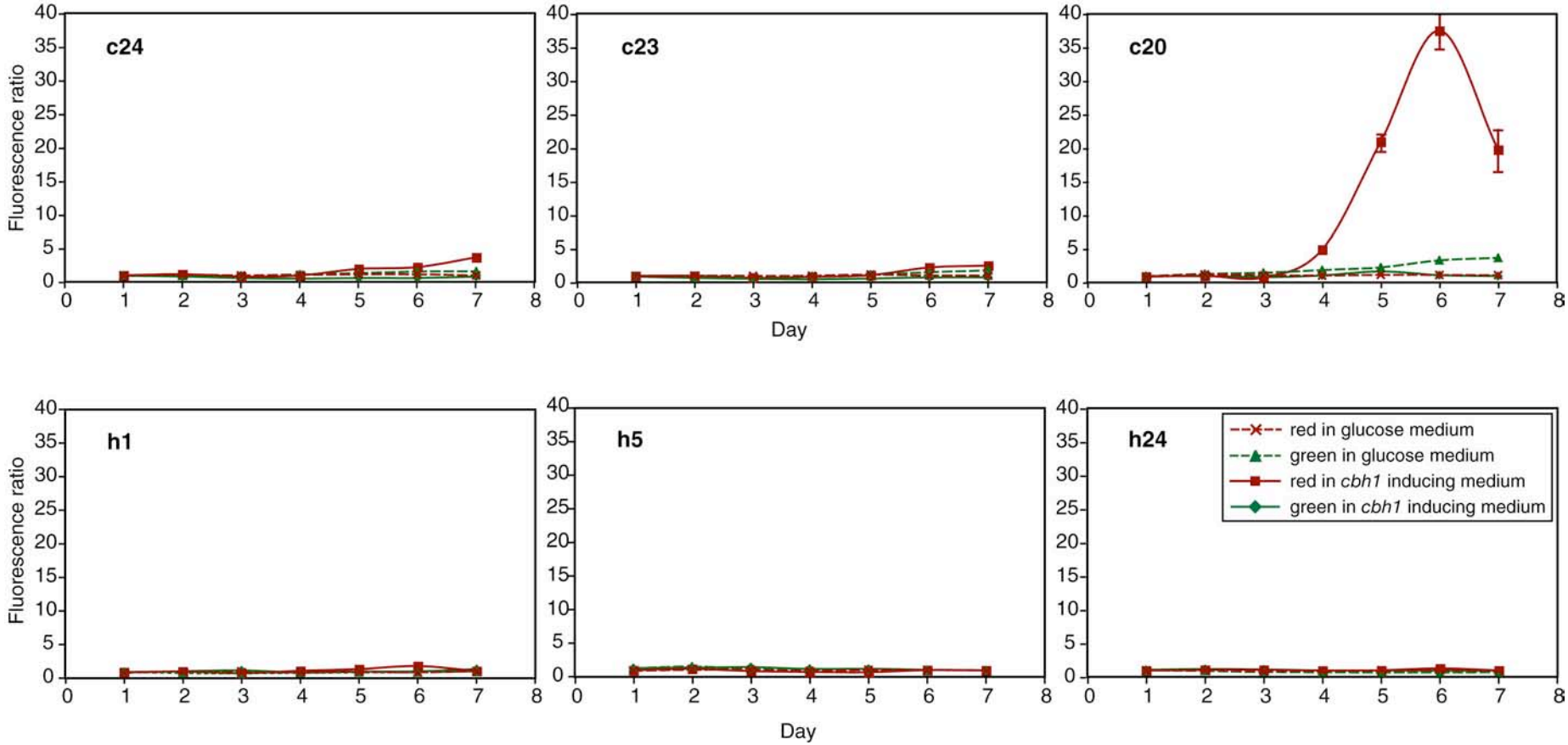


Figure 4-8. Level of DsRed secreted into the supernatant when expressed under the *cbh1* or *hex1* promoters. C24, c23 and c20 are transformants of the pHEN54RQDsR expression cassette and h1, h5 and h24 are transformants of the phexDsR expression cassette.

fluorescence in the supernatant increased sharply to day six and then declined by day seven. The fluorescence was only detectable in supernatants of cultures grown in cellulose medium as expected since the *cbh1* promoter is repressed in the presence of glucose. In addition, the intensity of green fluorescence was not found to be higher than background at any time point, which indicates that the DsRed fluorophore was likely to be in the red conformation by the time of secretion or shortly after secretion such that there was no accumulation of green fluorescence in the supernatant. The time lag between the observation of intracellular fluorescence and detectable levels in the supernatant was slow, considering that CBHI production takes only 11 min from translation to detection of extracellular CBHI in the supernatant (Pakula *et al.*, 2000). The delay in secretion by c20 may be due to a low efficiency of secretion of the heterologous protein or that the accumulation of DsRed in the supernatant was necessary to reach the lower detection threshold.

Transformant c20 demonstrated that the fluorescent reporter DsRed can be expressed and secreted in *T. reesei.* Transformants c23 and c24 had very high levels of intracellular fluorescence, comparable to that of transformant c20 (Figure 4-9), yet these transformants did not have detectable levels of fluorescence in the supernatants. It is unclear why fluorescence was only detectable in the supernatant of transformant c20 whilst the other pHEN54RQDsR transformants did not.

Fluorescence was not detectable in the supernatants of transformants h1, h5 or h24, in which DsRed was expressed under the *hex1* promoter when grown in glucose or cellulose medium. This absence of extracellular fluorescence may be a result of the

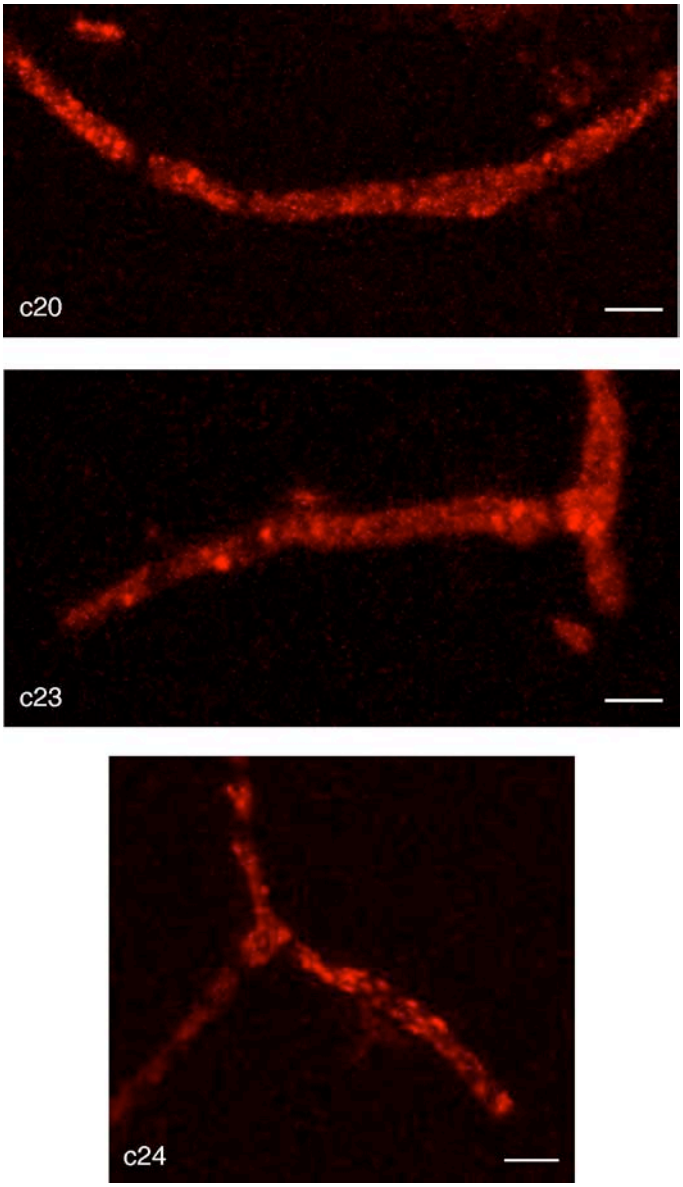


Figure 4-9. DsRed expression in pHEN54DsR transformants c24, c23 and c20. Hyphae are five days old grown in cellulose medium. Images are an extended view compilation of a z-stack. Scale bars represent 5 μm.

very low level of expression which was demonstrated by a low level of intracellular DsRed, compared to that of DsRed expressed under the *cbh1* promoter, and a low level of DsRed transcripts (Figure 4-7). This low level of expression can be attributed to the short duration of *hex1* promoter activity which prevented the accumulation of DsRed protein both intra or extracellularly.

4.2.6.1 Possible explanations for the low rate of DsRed secretion

The absence of extracellular fluorescence may be due to either inefficient secretion of the protein or instability of the fluorophore in the supernatant. Proteolysis of secreted heterologous proteins can be a problem for protein production in *T. reesei* (Section 1.4) due to the activity of endogenous proteases and has presented problems for monitoring fluorescent reporter protein in fungal supernatants previously (Gordon *et al.*, 2000a). Transformant c20 contained at least three copies of the expression cassette ectopically integrated throughout the genome. The integration of the expression cassette at any of these sites may have deleted or interrupted loci important to the expression, secretion or activity of endogenous proteases, which would have enabled the accumulation of secreted DsRed in the supernatant.

T. reesei culture medium becomes acidified over the culture duration. In this study, the pH of the culture supernatants was found to decrease to as low as pH 4. The DsRed fluorophore is unstable in acidic or alkaline conditions, which causes a shifts in the red emission spectra from 583 nm to 386 nm at pH 4.5 (Terskikh *et al.*, 2000). It is possible that secreted DsRed was denatured under the acidic conditions of the culture medium.

The stability of DsRed in *T. reesei* supernatants is explored further in Chapter 5. Unfortunately, the ability of transformant c20 to secrete a high level of DsRed will not be explored further as the transformant did not survive storage at –20 °C.

4.2.6.2 Determination of the DsRed protein concentration based on the fluorescence ratio

The level of DsRed in the culture supernatants was expressed as the ratio of fluorescence intensity of the sample against a negative control and provided the factor

by which the fluorescence intensity of the sample was above background intensity. If the detection of fluorescence in the supernatant was limited by a DsRed concentration below the detectable threshold, it was important to establish an indication of the relationship between the fluorescence ratio and the concentration of DsRed protein. Therefore, a standard curve of the fluorescence ratio and protein concentration was calculated (Figure 4-10). Firstly, the concentration of DsRed protein within the *E. coli* lysate from cells that expressed DsRed (Figure 4-2), was determined. The lysate was run alongside a concentration scale of BSA protein on SDS-PAGE. The concentration of the DsRed band (running at approximately 30 kDa) within the *E. coli* lysate was calculated to be 0.28 μg/μL based on densitometry analysis (Section 2.15.1.2).

The *E. coli* lysate, containing a known concentration of DsRed protein, was diluted in the soluble fraction of cellulose medium to measure the red fluorescence emission intensity. The fluorescence intensity of *E. coli* lysate that did not contain DsRed was also determined and used to calculate the fluorescence ratio of the diluted lysate containing DsRed. The fluorescence ratio was linearly correlated to DsRed concentrations below 2.8 μg in 200 μL (Figure 4-10). Above this concentration, the fluorescence intensity began to plateau with increasing DsRed concentration as the fluorescence reached a point of saturation. The fluorescence ratio at day four and five was 4.9 and 20 respectively for transformant c20 (Figure 4-8). These ratios correlate to concentrations of 3.4 μg/mL and 10.5 μg/mL of DsRed in the supernatants. A fluorescence ratio of 37 for transformant c20 at day six of culture was beyond the linear relationship but indicates that there was at least 34 μg/mL DsRed protein in the supernatant. These concentrations seem low if compared to the fact that the total protein

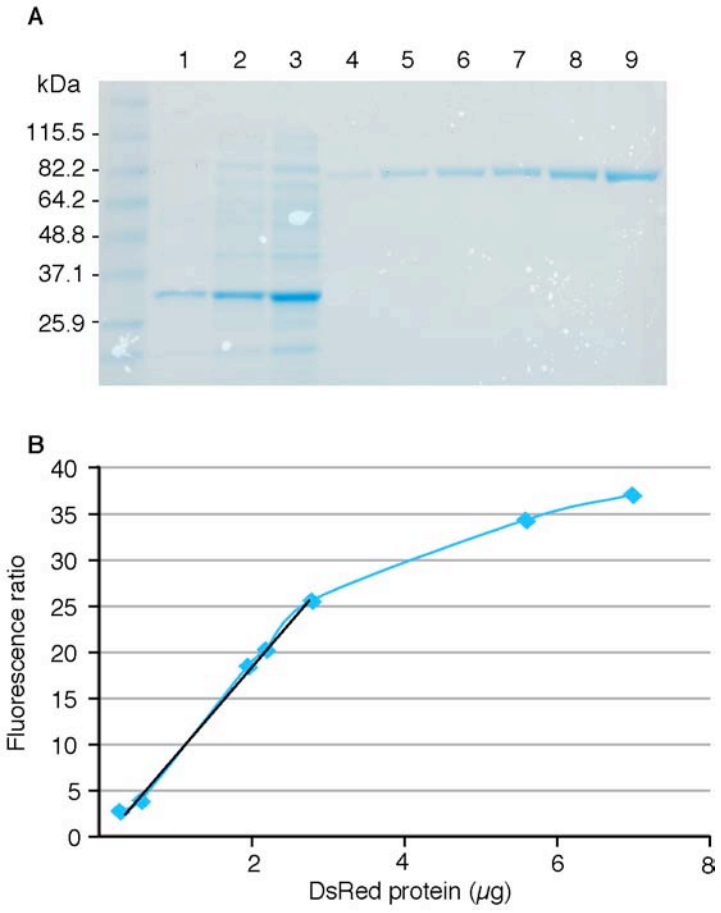


Figure 4-10. The relationship between DsRed protein concentration and the fluorescence ratio. A. Crude extract from *E. coli* cells expressing DsRed was separated by SDS-PAGE along with BSA concentration standards.1. 2 µL *E. coli* lysate 2. 5 µL *E. coli* lysate 3. 10 µL *E. coli* lysate 4-9. 0.25 µg, 0.5 µg, 0.75 µg, 1 µg, 1.5 µg and 2µg of BSA. B. A dilution series of crude *E. coli* lysate from cells transformed with pUC18 only or pUC18-DsRed, was added to fresh cellulose medium and the level of red fluorescence was determined. The fluorescence ratio was calculated in duplicate and the mean was plotted against the concentration of DsRed in the medium.

secreted from *T. reesei* in cellulose medium under similar conditions is typically around 3 mg/mL at day 4 (discussed later in Chapter 6). However, 30 µg/mL is comparable to the yields obtained for other heterologous proteins when expressed under the *cbh1* promoter in similar conditions (Section 1.5.1, Table 1-3). For example, calf chymosin was produced to a concentration of 20 µg/mL (Harkki *et al.*, 1989) and human Fab antibody fragments were produced to a concentration of 40 µg/mL (Nyyssönen *et al.*, 1993).

4.3 List of main findings

- A red fluorescent reporter protein is useful for fluorescent microscopy in *T. reesei* due to the low level of autofluorescence in this range of wavelengths.

- DsRed can be a useful tool to study protein expression and secretion in *T. reesei.* However, disadvantages include a long maturation time of the fluorophore and the possible instability of the fluorophore in the culture supernatants due to proteolysis or acidic conditions.

- The low intensity, sparse distribution and temporally brief *hex1* promoter activity prevented the accumulation of DsRed such that intracellular fluorescence was barely detectable. The mycelium displayed the highest level of fluorescence at day two which is in accordance with the pattern of *hex1* expression determined by northern blotting.

- The low level of DsRed protein and sparse distribution of fluorescence suggests that *hex1* expression is regulated spatially and temporally through the mycelium. Alternatively, the weak fluorescence produced under the *hex1* promoter may be a result of the exclusion of important gene regulatory elements in the design of the expression cassette.

- An equal distribution of green and red fluorescence throughout the mycelium of transformants expressing DsRed under the *cbh1* promoter suggests that the *cbh1* promoter is active equally throughout the mycelium and that the protein was not particularly translocated to the apical cells.

- The level of DsRed expression under the *hex1* promoter was not increased by increased copy number of the expression cassette.

- Transformant h1 and h24 may have utilised different *tsp*s of the *hex1* promoter within the expression cassette to transcribe DsRed, whilst the endogenous transcription of *hex1* maintained transcription from the major *tsp*.

- The regulation of transcription of the *hex1* promoter seems to be complicated and may be dependent on intrinsic factors of the *hex1* locus.

Chapter 5 Modified expression vectors containing a fusion to a portion of *hex1* gene sequence

5.1 Introduction

In Chapter 4, it was shown that the expression cassette designed to express the heterologous fluorescent protein DsRed under the *hex1* promoter, resulted in very low levels of intracellular fluorescence and the absence of extracellular fluorescence. The level of expression of DsRed was weaker than the expression of the endogenous HEX1 as indicated by the level of *dsRed* and *hex1* transcripts. Also, transcription from the phexDsR expression cassette in two individual transformants seemed to have been from alternative *tsp*s with inefficient splicing of the intron from the 5' UTR. Intracellular DsRed fluorescence was most visible on the second day of culture, however, the fluorescence was faint and the cells displaying fluorescence were sparse throughout the mycelium. In addition, fluorescence was not detectable in the supernatants of these cultures. It is possible that a *cis*–regulatory component intrinsic to the *hex1* locus was missing from the expression cassette designed or that a feature of the expression cassette was detrimental to efficient expression of DsRed. The design of the phexDsR cassette was reviewed and a series of new expression cassettes were designed based on the following considerations.

5.1.1 Considerations for improvement of the phexDsR expression cassette

5.1.1.1 Kozac sequence

The expression of DsRed from the phexDsR plasmid seems to have been limited by the low amount of DsRed transcript. However, inefficient translation of the transcript may also have been involved. The *hex1* gene has a putative Kozac sequence (GCCAAGATGG) adjacent to and encompassing the start codon of the *hex1* coding sequence. However, in phexDsR the insertion of the *cbh1* ss disrupted the continuation of the Kozac sequence between the non-coding and coding regions (refer to Figure 4-1). Even though an alternative conserved Kozac sequence (CGCCACCATGG) (derived from the pTimer plasmid) was included further downstream from the ss, the disruption of the *hex1* Kozac sequence by the *cbh1* ss may have effected the efficient translation of the *dsRed* transcripts. The expression cassettes designed in this Chapter contain several codons of exon 2 downstream from the promoter sequence, thereby including the full *hex1* Kozac sequence (Figure 5-1 and Figure 5-2).

5.1.1.2 Introns and gene expression

The *hex1* gene of *T. reesei* contains two introns, one within the 5' UTR and another of a variable length within the coding sequence. The intron within the 5' UTR contains several putative promoter regulatory motifs such as CCAAT boxes and a GATA-factor binding site, as described in Section 3.2.5. This intron also contains a region highly complementary to a pyrimidine-rich region of the intron within the coding sequence, which may suggest some direct interaction between the two sites and implication of the second intron in gene expression.

Introns flanked by gene coding sequence can be vital for expression of proteins by several mechanisms. Introns can influence the level of mRNA accumulation (Xu *et al.*, 2003) by affecting mRNA stability (Mascarenhas *et al.*, 1990; Lugones *et al.*, 1999) or by influencing the initiation of transcription through regulatory motifs within their sequences (Melkonyan *et al.*, 1998). The expression of the *Pisolithus* hydrophobin gene (*hydPt-1*) in the basidiomycete *Hebeloma cylindrosporum* occurred only when the fungus was transformed with the genomic copy of the *hydPt-1* gene containing three introns, but not when transformed with the *hydPt-1* cDNA (Tagu *et al.*, 2002). In addition, the inclusion of an intron from the SC6 hydrophobin gene onto the 3' end of the GFP coding sequence increased the level of GFP transcripts and the intensity of fluorescence in *S. commune* (Lugones *et al.*, 1999). In the basidiomycete *Phanerochaete chrysosporium,* the fluorescent marker GFP was placed under the manganese peroxidase promoter or the glyceraldehyde-*p*-dehydrogenase promoter. However, no transformants showed fluorescence. When GFP was expressed as a fusion to the *gpd* exon 1 (6 bp), intron 1 (55 bp) and the first 9 bp of exon 2, the level of GFP RNA and fluorescence increased. Likewise, when the same *gpd* exon and intron sequences were included between the *mnp1* promoter and GFP-encoding sequence, GFP was successfully expressed (Ma *et al.*, 2001). In a similar case, the expression of the *Agaricus bisporus* hydrophobin gene *ABH1* in *Schizophyllum commune* under the native hydrophobin promoter, required the inclusion of the native introns (Lugones *et al.*, 1999). Lugones *et al.* (1999) also determined that the level of transcription was not affected by the presence or absence of introns and that the events affecting expression of the protein occurred at a post-transcriptional level. The insertion of an artificial intron, possessing conserved 5' and 3' splice sites and branch sites of the lariat, was also able to enhance the accumulation of transcript and the expression of the SC3

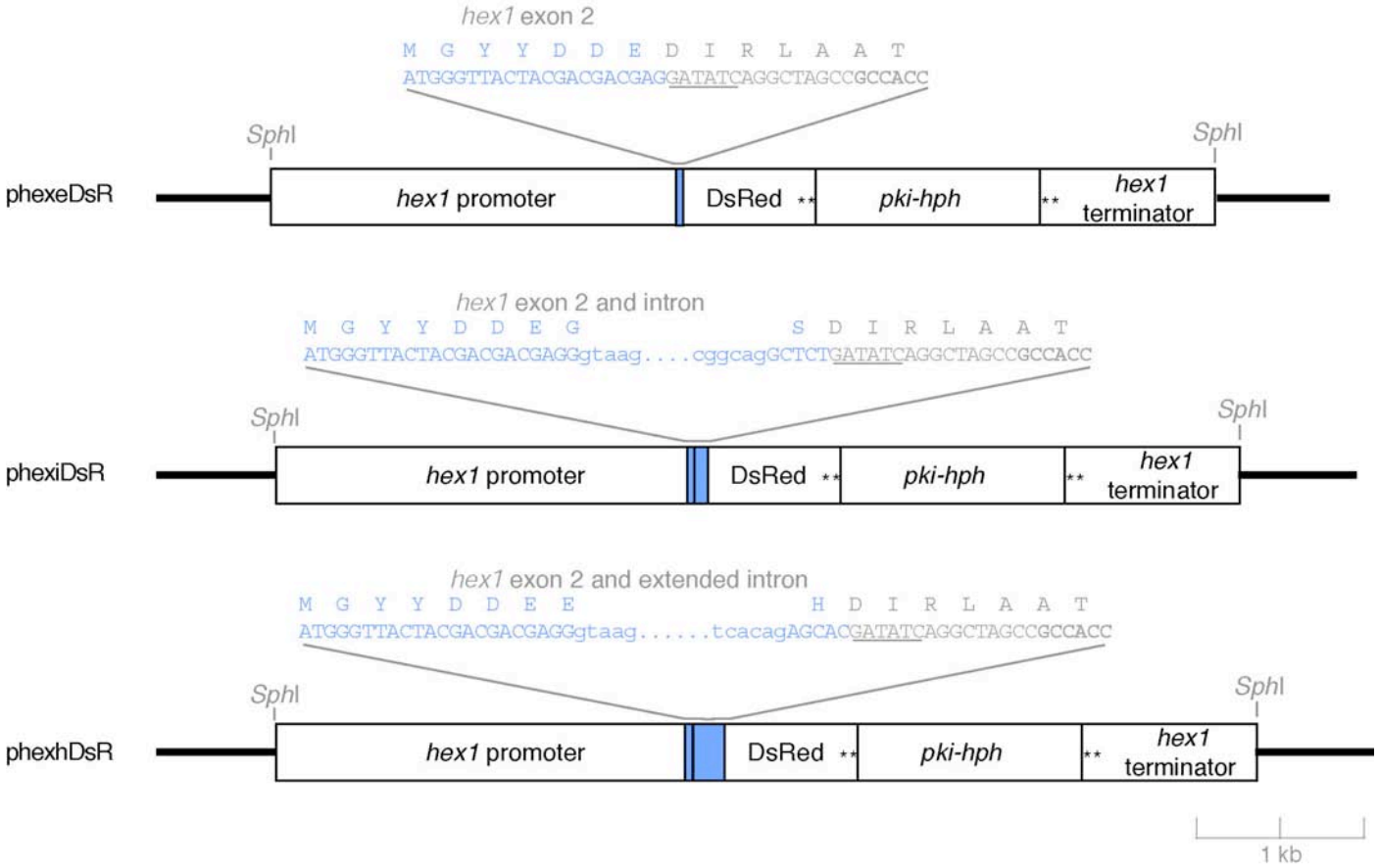


Figure 5-1. Modified phex vectors incorporating exon 2, intron 2 and the extended intron 2. Regions of the *hex1* ORF are shown in blue. The Kozac sequence is in bold and the *Eco*RI site is underlined. The *hph* gene which imparts resistance to hygromycin B is under control of the *pki* promoter and the position of stop codons are shown as asterisks.

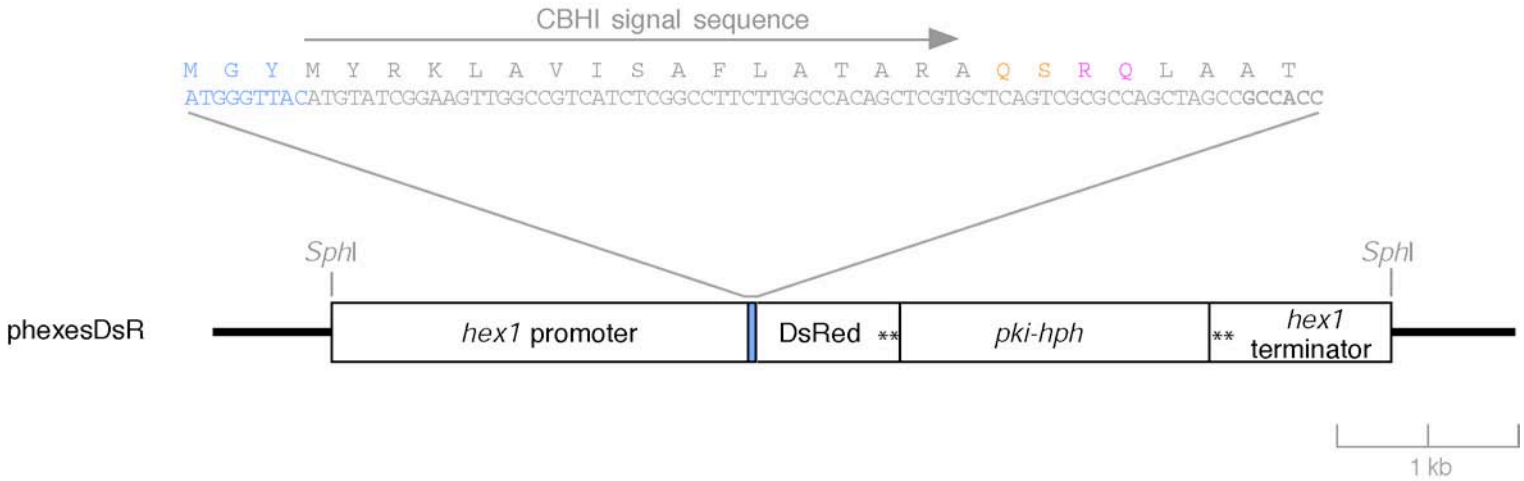


Figure 5-2. The *cbh1* ss was inserted into the phexeDsR expression vector to create phexesDsR. The 5' terminal amino acids of the HEX1 protein are shown in blue, the leading residues of the *cbh1* core are shown in orange and the Kex2-like cleavage site is shown in pink. The *hph* gene which imparts resistance to hygromycin B, is under the control of the *pki* promoter. The Kozak sequence is in bold and the positions of stop codons are marked with asterisks.

hydrophobin, suggesting that splicing may be important. However, in another case involving intron-mediated enhancement of expression of the tryptophan pathway gene PAT1, the actual splicing event was not vital. Splicing of the intron was prevented by point mutations or deletion at the splice sites. Since the rate of transcription remained unchanged but expression was enhanced (Rose *et al.*, 2000), it was suggested that mere association with the splicing machinery, even if splicing didn't occur, was sufficient to enhance expression (Rose, 2002).

Therefore, it is evident that introns can effect gene expression and this is likely to be achieved through a variety of mechanisms. It is possible that the presence of the *hex1* gene introns is vital for efficient expression under the *hex1* promoter.

5.1.2 The construction of fusion expression vectors for improved protein yields

Expression vectors designed for (heterologous) protein production in fungi frequently fuse the coding sequence of the protein of interest to part of an endogenous gene (Gouka *et al.*, 1997) as discussed in Chapter 1 (Section 1.4). This approach has also been used to increase the expression of fluorescent reporter proteins (Gordon *et al.*, 2000b; Rücker *et al.*, 2001). A series of new expression cassettes were designed in order to investigate further the structure of the *hex1* gene and to optimise the level of the DsRed expressed under the *hex1* promoter. The *cbh1* signal sequence was excluded from these constructions in case this sequence was detrimental to DsRed expression. The expression cassettes contained three truncated fragments of the *hex1* gene fused to the DsRed coding sequence (Figure 5-1). The first truncated region included *hex1* gene exon 2, in which the original putative Kozac sequence was intact. The second vector

contained the *hex1* gene exon 2 and the 109 bp intron from within the coding sequence. The third vector contained the *hex1* gene exon 2 and the 109 bp intron with the extended length that encodes the histidine-rich region of the HEX1 protein (refer to Section 3.2.2). The expression cassettes with the incorporation of the intronic sequences, were designed to include any potential regulatory function of these regions which may affect promoter regulation and change the level of DsRed expression.

A further expression cassette containing the *cbh1* ss inserted downstream of a truncated form of *hex1* exon 2 sequence was constructed to determine if the heterologous DsRed protein could be secreted. Within this cassette the *cbh1* ss was N-terminally fused to DsRed resulting in three codons for the *hex1* exon 2 fused to the N-terminus of the ss (Figure 5-2). For the sake of RNA stability and for the incorporation of the full Kozac sequence, these three codons were maintained on the construct although it was unknown if the additional residues at the 5' terminus of the ss would affect the functioning of the secretion signal.

The N-terminus of the HEX1 protein encoded by exon 2 of the *hex1* gene is highly conserved in all HEX1 proteins identified in filamentous fungi. However, this N-terminal region was not included on the molecular structure solved for *N. crassa* HEX1 protein (Yuan *et al.*, 2003), indicating that this peptide is not important for the polymerisation of HEX1 to form the crystalline aggregation of the Woronin body core. The highly conserved nature of this peptide and the potential phosphorylation sites on the tyrosine residues suggest that this region may have another function (discussed in Section 3.2.4). This region may be a targeting signal for HEX1 accumulation in the microbody destined to become a Woronin body (Tenney *et al.*, 2000). If this sequence is

indeed a targeting signal, then a direct fusion to the DsRed protein may disclose this function.

5.2 Results and discussion

5.2.1 *The hex1* fusion expression plasmids

Three new expression cassettes phexeDsR, phexiDsR and phexhDsR, were designed based on the considerations discussed in Section 5.1.1 (Figure 5-1). The fourth expression cassette phexesDsR, was designed with the above considerations and to further investigate the secretion of DsRed when expressed under the *hex1* promoter (Figure 5-2).

5.2.2 Screening of transformants

T. reesei was transformed with the linear expression cassettes (Figure 5-1 and Figure 5-2) by biolistic bombardment and transformants were selected for further analysis based on resistance to hygromycin B as described Section 2.7.1. Genomic DNA was extracted from a sample of the transformants and screened by PCR for integration of the

Table 5-1. Summary of the analysis for transformants carrying the expression cassettes phexeDsR, phexiDsR, phexhDsR and phexesDsR.

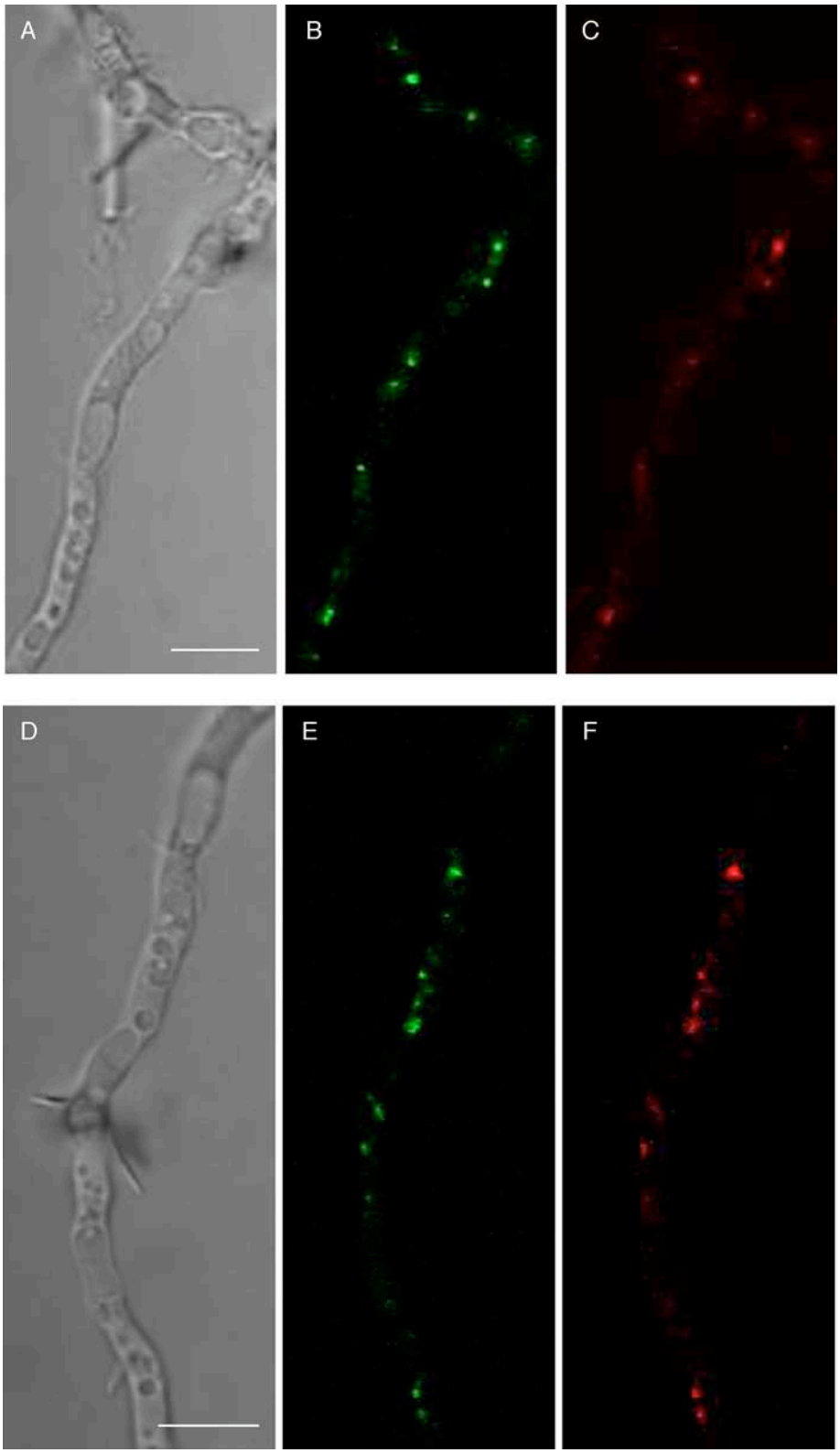
Expression cassette	Number of transformants	Integration of the expression cassette into the genome	Integration of the cassette into the *hex1* locus	Presence of intracellular fluorescence
phexeDsR	15	15	0	6
phexiDsR	12	12	0	6
phexhDsR	20	18	0	8
phexesDsR	13	13	0	6

expression cassette into the genome and for homologous integration into the *hex1* locus. The numbers of transformants screened are shown in Table 5-1. None of the transformants carrying either of the four expression constructs had integration of the transgenic DNA into the *hex1* locus. The level of DsRed expression from the transformants was determined by the intensity of DsRed fluorescence observed by fluorescence CLSM.

5.2.3 Fluorescence microscopy observations of transformants containing the fusion expression cassettes

Transformants from each of the expression constructs were grown in cellulose medium and observed by fluorescence CLSM daily for four days, after which some hyphae of the mycelium appeared dead and fluorescence was no longer visible.

In transformants of the four expression cassettes phexeDsR, phexiDsR, phexhDsR and phexesDsR, the fluorescence appeared within hyphae in the same punctate pattern as in the hyphal cells of transformants containing the phexDsR cassette (Figure 5-3, Figure 5-4 and refer to Section 4.2.3.2). In addition, the cells containing the fluorescence spots were also localised randomly throughout the mycelium for each transformant type (Figure 5-5)(refer to Section 4.2.3). No fluorescence was present at 24 h after sample inoculation, but by day two some faint fluorescent spots appeared. By day three, the number of fluorescent spots within the hyphae had reduced and by day four it was rare to locate a hypha which had any fluorescent spots remaining. Overall, the fluorescence displayed by transformants was very low and sparsely distributed through the hyphae.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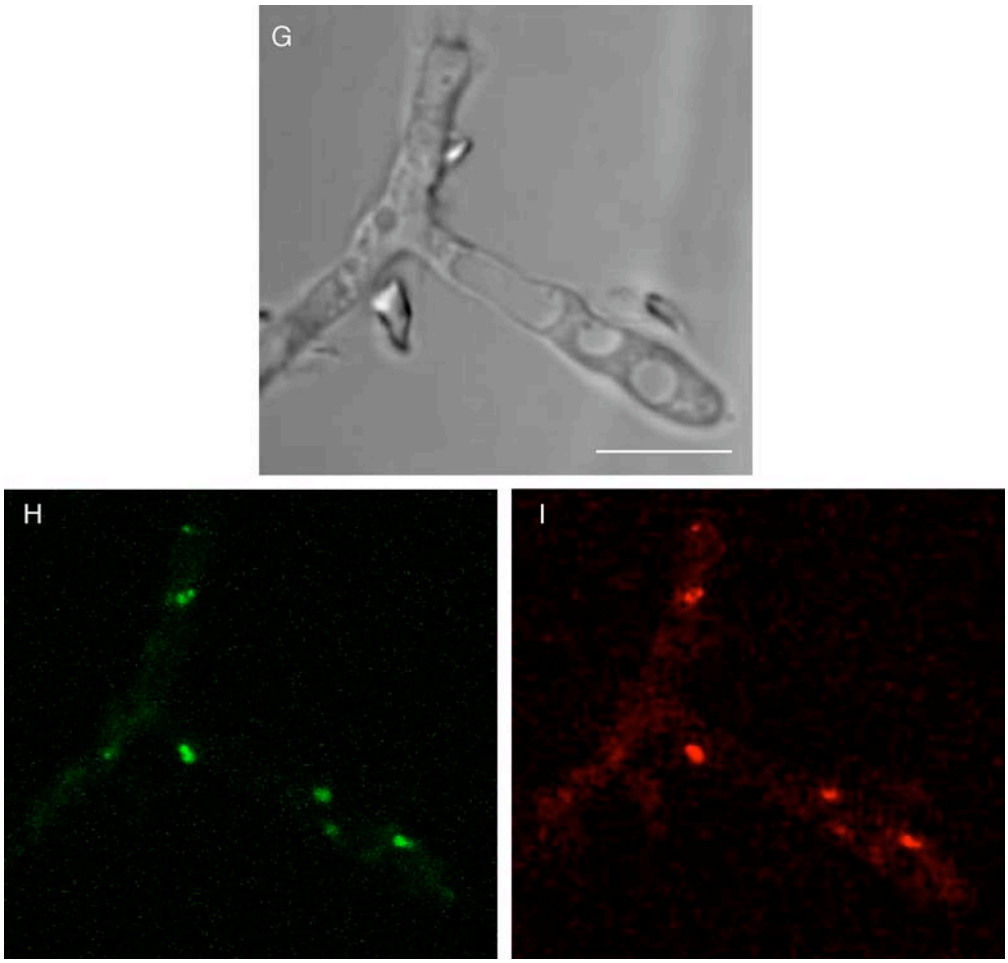


Figure 5-3. Fluorescence CLSM images of fluorescent hyphae typical for transformants carrying the expression cassettes phexhDsR (A-C), phexiDsR (D-F) and phexeDsR (G-I). The hyphae were two days old and grown in cellulase medium. A, D and G are DIC images, B, E and H show green fluorescence when excited by blue light and images C, F and I show red fluorescence on excitation by green light. Images are compiled from z-stacks of 0.2 μm increments. The hyphae shown were grown in cellulose medium for two days. The scale bars represent 10 μm.

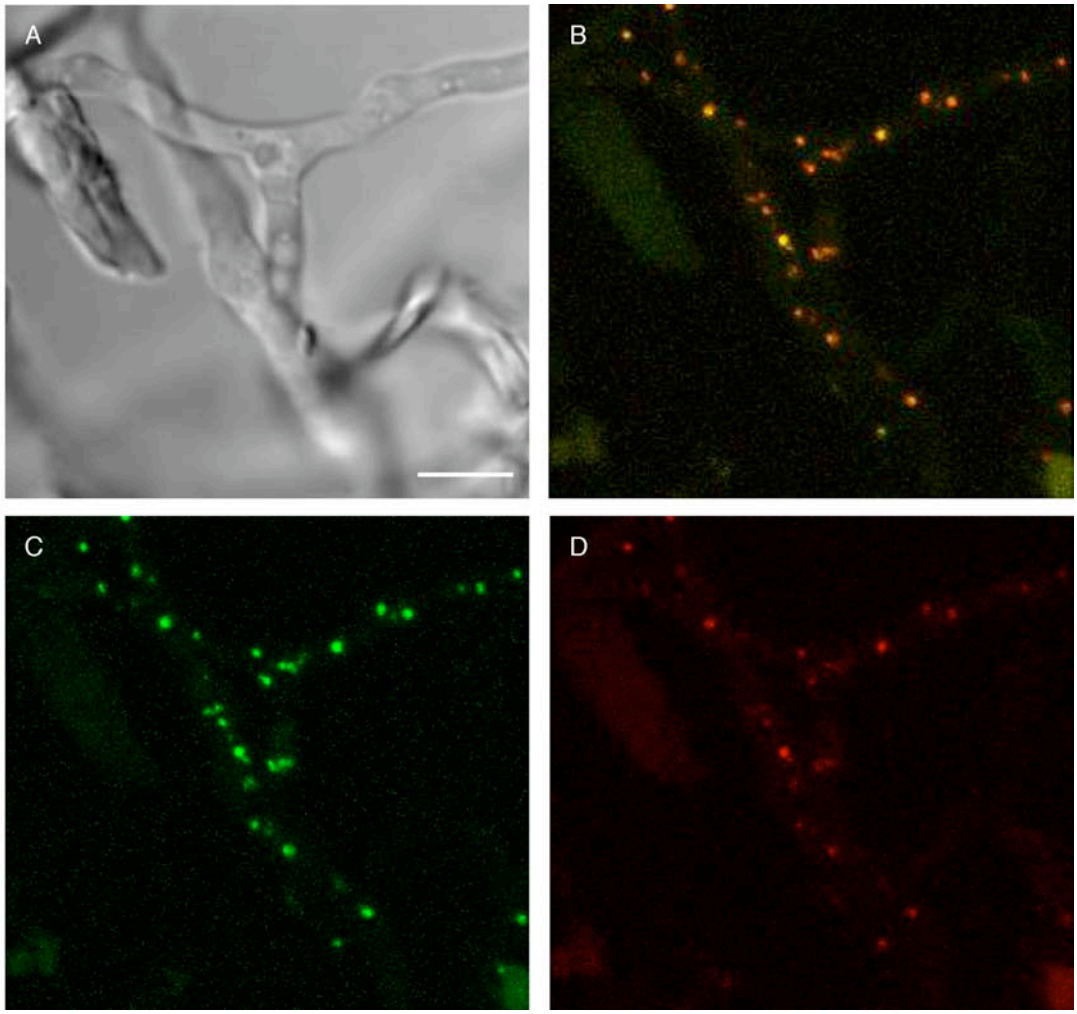


Figure 5-4. Hyphae of a transformant containing the phexesDsR expression cassette after two days growth in cellulose medium. A. DIC image. B. The green and red fluorescent images superimposed. The regions where green and red fluorescence are co-localised are shown in yellow. C. Distribution of green fluorescence. D. Distribution of red fluorescence. Images were compiled from a z-stack series of 0.2 μm increments. The scale bar represents 10 μm.

Only a maximum of 30% of hyphae displayed any fluorescence (Figure 5-5). There was as much variation in the intensity and frequency of fluorescence between individual transformants carrying the same expression cassette, as the variation observed between transformants carrying different expression cassettes (Figure 5-3 and Figure 5-4). Therefore, inclusion of different truncations of the *hex1* gene as a fusion to the DsRed-encoding sequence did not change the intensity of expression of DsRed.

The fluorescence level appeared low within transformants due to a combination of a very sparse distribution of cells displaying fluorescence and the low intensity of fluorescence within these cells. The sparse distribution of cells displaying fluorescence may be due to transcription being localised to these cells, or the translocation and concentration of DsRed to these cells. Whilst fluorescent spots were observed in some apical cells, it was a rare event and not all apical cells contained fluorescent spots. More mature cells were just as likely to contain fluorescent spots which suggests that *hex1* expression does not only occur in apical cells of the hyphae.

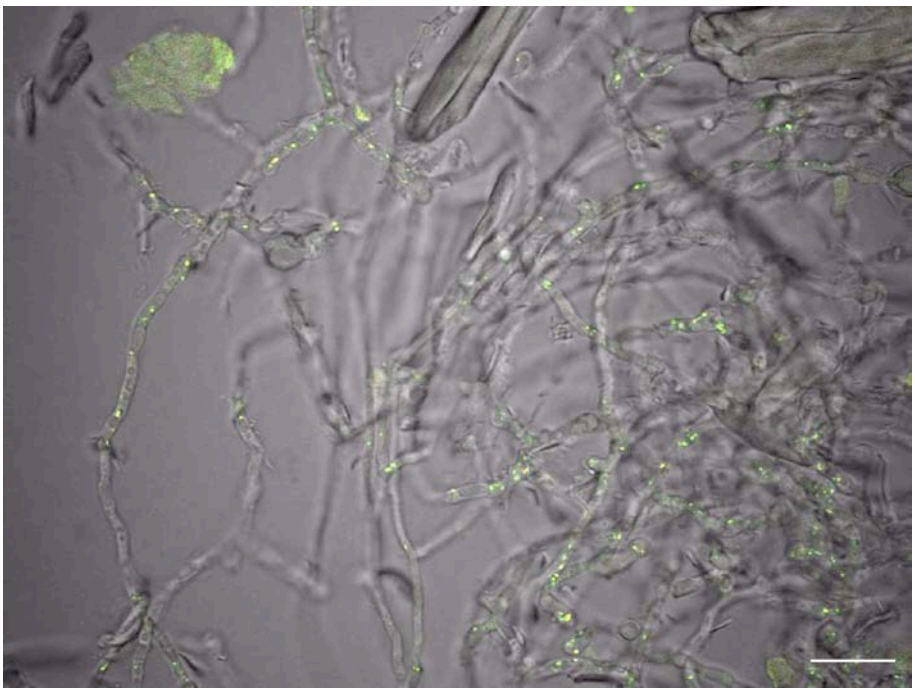


Figure 5-5. Two day old mycelia of a transformant of the phexesDsR expression cassette. The DIC image has been superimposed with the green and red fluorescent images taken under blue or green light excitation respectively. Regions which emitted green and red fluorescence are shown in yellow. The image was acquired by fluorescence CLSM as a single layer scan and Kalman 3. The scale bar represents 25 μm.

When present, the fluorescence was concentrated into small spots throughout the cell, which was the same pattern of fluorescence observed for the phexDsR transformants (Figure 5-3, Figure 5-4 and refer to Section 4.2.3). It was speculated that the

concentration of DsRed into these spots was demonstrative of packaging of the protein into secretory vesicles. However, the fusion vectors phexeDsR, phexiDsR and phexhDsR do not contain the *cbh1* ss. These observations indicate that the *cbh1* ss was not responsible for the concentration of DsRed into these vesicles and that these vesicles were not likely to be secretory granules but perhaps some other microbody.

The punctate pattern of fluorescence between transformants containing the phexDsR cassette and those carrying the newly designed fusion cassettes appeared identical. This common pattern demonstrated that the fusion of the peptide MGYYDDE, encoded by exon 2 of the *hex1* gene, to DsRed did not alter the intracellular localisation of fluorescence. These results suggest that the N-terminus of HEX1 is not likely to be a localisation signal for the HEX1 protein.

In addition, the co-localisation of green and red fluorescence into the same punctate regions (Figure 5-4) indicates that the DsRed was concentrated into these regions before the protein had completely folded into the mature conformation, and that the spots contained a combination of recently produced and more mature protein. Considering that the ratio of red to green fluorescence is a linear relationship over time (Terskikh *et al.*, 2000), the co-localisation of green and red fluorescence suggests that the formation of these concentrations was additive and not a result of discrete packaging of the expressed protein at a particular moment in time, such as the packaging of secretory vesicles.

5.2.4 The secretion of DsRed by transformants carrying a *hex1* fusion expression cassette

Six transformants identified as hes2, hes5, hes6, hes8, hes10 and hes11 carried the phexesDsR cassette and displayed intracellular fluorescent spots. These transformants were chosen for a more detailed study of secretion and the use of the fluorescent reporter DsRed to monitor protein expression in *T. reesei*. Cellulose medium was inoculated with equal numbers of conidia and the supernatants of the cultures were sampled daily. The level of green and red fluorescence was measured on the fluorometer and the fluorescence ratio was calculated as previously described (Section 2.15).

Table 5-2. Fluorescence ratio of supernatants of phexesDsR transformants. Table at top shows the fluorescence ratio under green light excitation and the bottom table shows the fluorescent ratio under blue light excitation.

Transformant	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
2	0.8	0.6	0.5	0.6	0.6	0.9	0.9
5	1.0	1.4	1.0	1.0	1.0	1.1	1.1
6	1.1	1.5	1.0	1.0	1.0	1.0	1.0
8	1.1	1.3	0.9	0.9	0.9	0.9	1.0
10	0.8	1.6	0.9	1.0	1.0	1.0	1.0
11	1.1	1.5	1.2	1.1	1.1	1.1	1.1

Transformant	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
2	0.9	0.9	0.9	0.9	0.9	0.9	1.0
5	0.9	0.8	0.8	0.9	1.0	1.0	1.0
6	0.9	0.9	0.8	0.8	0.9	1.0	1.0
8	0.9	0.8	0.8	0.9	0.9	0.9	1.0
10	0.8	0.9	0.8	0.8	1.0	0.9	1.0
11	0.9	1.0	0.8	0.8	0.9	0.9	0.9

The fluorescence ratio for all of the transformants tested was close to 1 for green and red fluorescence (Table 5-2), indicating that the level of fluorescence detected in the supernatant of the transformant cultures was not different to the level of fluorescence detected in a nontransformant culture. A low level of DsRed in the culture supernatants

was expected due to the low level of intracellular DsRed, but the complete absence of extracellular DsRed was surprising. Since there were no previous known studies on the use of the fluorescent reporter, DsRed, for analysis of protein externalisation in filamentous fungi, the issue of undetectable fluorescence in the supernatant was investigated further to explore the use of DsRed for this purpose.

5.2.5 Secretion of DsRed under the *hex1* promoter determined by Western blotting

The inability to detect DsRed in the culture supernatants may be due to several reasons including proteolysis or denaturation of the protein in the acidic pH (discussed previously in Section 4.2.6.1). As an alternative to demonstrating DsRed secretion by fluorescence intensity, the culture supernatants of a select transformant were analysed by Western blotting.

Transformant hes8, which carried the phexesDsR expression cassette, showed the highest number of fluorescent spots of the greatest intensity of all the transformants from the *hex1* fusion expression cassettes phexeDsR, phexiDsR, phexhDsR and phexesDsR. Hes8 was used to further investigate the secretion of DsRed. Transformant c1, which contained the pHEN54RQDsR expression cassette expressed DsRed under the *cbh1* promoter and had previously demonstrated a high level of intracellular fluorescence comparable to c20 (see Chapter 4), was selected as a comparison to hes8 in the secretion study.

The supernatants of cultures grown in cellulose medium for one to six days were analysed by Western blotting using the commercially available monoclonal anti-DsRed

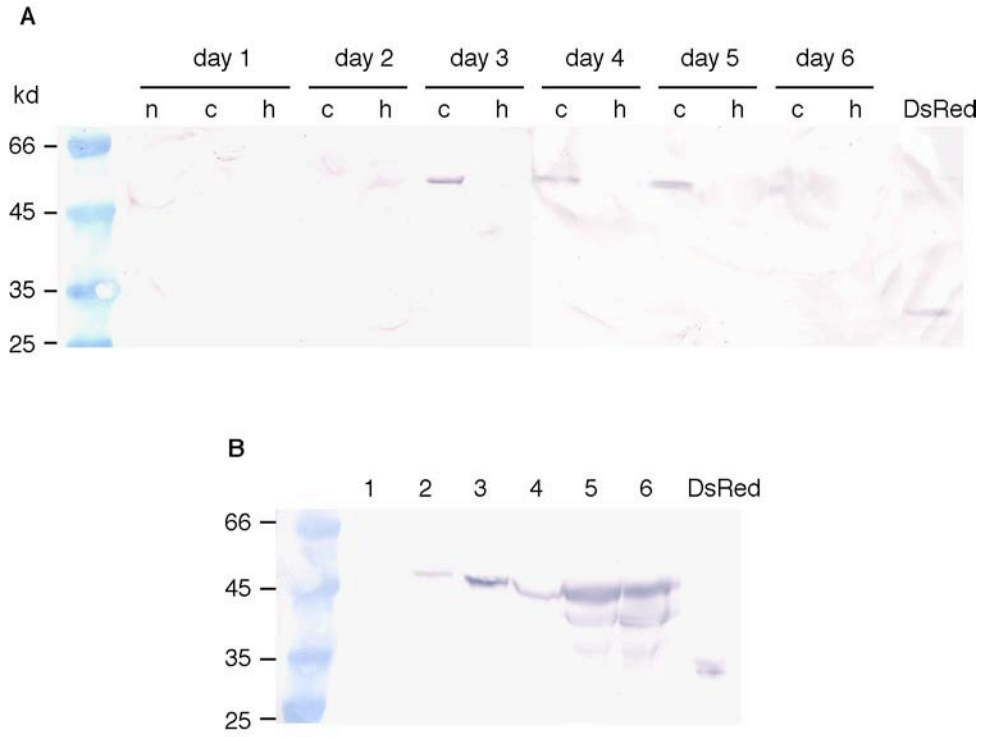


Figure 5-6. Western blots of culture supernatants probed for DsRed. A. nontransformant (n), transformant c1 (c), transformant hes8 (h). B. Supernatant from a nontransformant from day 1 to day 6 conducted in parallel to blot A.

antibody (BD Biosciences Clontech). The crude cell extract of *E. coli*, which expressed DsRed (Section 4.2.1.1), was run alongside the supernatant samples. The expected size of the DsRed protein without the *cbh1* ss was approximately 26 kDa. A band of this expected size did not appear on the Western blots of culture supernatants from transformants c1 and hes8 (Figure 5-6A). However, there was binding of the DsRed antibody to a protein of approximately 50 kDa at day two to three in the supernatants of c1 and the nontransformant cultures (Figure 5-6). This band became progressively stronger on subsequent days of culture until day six. Since this band also occurs in the supernatant of the nontransformant, we can be sure that this protein is not a dimer of DsRed but likely to be a result of the antibody binding non-specifically to the increasingly high concentration of endogenous proteins secreted by *T. reesei* when grown in cellulose medium. Specifically, CBHII, endoglucanase I and endoglucanase

III have molecular masses of 47 kDa, 46 kDa and 42 kDa respectively (Penttilä *et al.*, 1986; Teeri *et al.*, 1987; Saloheimo *et al.*, 1988). The non-specific binding may also be to the dominant secreted protein CBHI, which typically has a molecular mass of 66 kDa (Shoemaker *et al.*, 1983) but may have been running at a slightly lower apparent molecular mass on the SDS-PAGE due to the large quantity of the protein or due to some proteolytic cleavage of the protein.

5.2.6 The stability of DsRed in culture supernatants of *Trichoderma reesei*

An experiment was conducted to determine if the absence of DsRed in the culture supernatants was due to the unsuccessful secretion of the protein or the instability of DsRed in the culture supernatants. The cellular lysate from *E. coli* which expressed DsRed (Section 4.2.1.1) was added to the culture supernatants of c1 and hes8 from day one, day three and day five. The spiked supernatants were incubated overnight with shaking at 28°C to imitate culture conditions. The *E. coli* cellular lysate containing DsRed was also incubated in fresh minimal medium, H_2O or undiluted, under the same conditions. The supernatants were separated by SDS-PAGE and transferred onto a nitrocellulose membrane for a Western blot that was probed for DsRed (Figure 5-7).

A band corresponding to the expected size for DsRed was visible in the day one supernatants but not in the day three or day five supernatants. On days three and five, a band of approximately 50 kDa appeared which corresponded to the nonspecific bands seen previously (Figure 5-6), which are likely to be an extracellular product from *T. reesei*. The DsRed in the controls of fresh minimal medium and H_2O were still detectable in the medium. These results demonstrate that DsRed is not stable in the *T.*

reesei culture supernatants from at least day three onwards. However, these results do not reveal the cause of the DsRed instability. In a previous study the fluorescent reporter GFP was found to be unstable in the culture supernatants of *A. niger* due to the activity of endogenous extracellular proteases (Gordon *et al.*, 2000b).

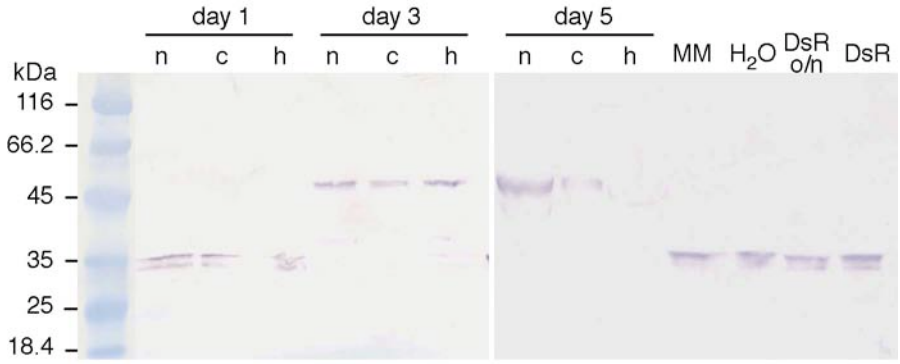


Figure 5-7. Western blot of supernatants spiked with DsRed. Supernatants of cultures of a nontransformant (n) and transformants c1 (c) and hes8 (h) were spiked with a cellular lysate of *E. coli* containing DsRed protein. The cellular lysate containing Dsred was also added to minimal medium (MM) pH 5.5, water or left undiluted (DsR o/n) and incubated overnight under culture conditions. Untreated lysate from *E. coli* expressing DsRed (DsR) was run as a further control.

It was possible that DsRed was degraded by the endogenous proteases of *T. reesei*. To eliminate this possibility, protease inhibitor cocktail tablets (Roche) were added to the culture medium and the experiment was repeated with transformant hes8. Again, no fluorescence above the background level was detected in the supernatants over the course of a seven day culture. Therefore, the action of extracellular proteases may not be the cause for the lack of DsRed detected in the culture supernatants.

The pH of the culture supernatant may have caused denaturation of the DsRed fluorophore (discussed in Section 4.2.6.1). The pH of the cultures was monitored over the course of seven days and was found to peak at pH 6.3 on day three and decreased to

pH 4.1 at day four. The EGFP fluorophore is known to renature and fluoresce in neutral buffer after being denatured by alkaline or acidic conditions (Bokman *et al*., 1981). It is not known if this renaturing can also occur with the DsRed protein but this possibility was tested. The protein within the supernatants was concentrated and underwent buffer exchange with PBS, using Centriprep YM-10 columns (Millipore, USA). The resulting protein solutions were six-fold more concentrated than the original supernatants with an increased pH of 6.5. These concentrated protein solutions were incubated in the dark at room temperature for 24 h to allow protein folding prior to the determination of fluorescence. These concentrated, pH adjusted, samples did not display any fluorescence higher than the background readings. Perhaps the instability of DsRed in culture supernatants could be improved by using a non-acidifying strain of *T. reesei* or an alternative culture medium. However, the effects of these variables were not assessed.

5.2.6.1 Innate features of the DsRed protein

The pattern of intracellular fluorescence in *T. reesei* transformants expressing DsRed and the inability to detect secreted DsRed may also be due, in part, to the innate features of the DsRed protein. At the commencement of this project, the DsRed1-E5 variant used in this study had been made available only recently. The fluorescent reporter DsRed was chosen over the more widely used fluorophore EGFP due to inherent problems with green autofluorescence in *T. reesei.* In addition, the changing spectral properties of the DsRed1-E5 variant offered a greater insight into the functioning of the *hex1* promoter than a fluorophore with a single emission wavelength. However, certain limitations in the earlier developed variants of DsRed include a slow fluorophore maturation time (Terskikh *et al*., 2002) and the disposition to exist as an obligate

tetramer in physiological conditions (Baird *et al*., 2000). This tetramer remains soluble and does not affect the use of DsRed as a reporter of gene expression (Baird *et al*., 2000). However, in one reported case, DsRed expressed in *E. coli* became localised in cells several days old, potentially indicating precipitation or aggregation of DsRed within the cytoplasm (Jakobs *et al*., 2000). It has also been suggested that the fusion of proteins to DsRed can cause further aggregation of the tetramers such that the DsRed becomes insoluble (Lauf *et al*., 2001). However, the aggregates formed from DsRed-ER and vacuole targeting signal fusions did not reveal artefactual or abnormal structures in tobacco cells (Jach *et al*., 2001), suggesting the protein aggregates did not interfere with protein localisation. Based on these previous cases, it is possible that the punctate pattern of fluorescence seen in transformants expressing DsRed under the *cbh1* and *hex1* promoters may be due to aggregation of the DsRed tetramers. This aggregation is likely to have effected the intracellular translocation and the secretion of the protein and is discussed further in Section 7.4.

5.2.7 Further analysis of the expression of DsRed under the *hex1* and *cbh1* promoters

The level of expression and secretion of DsRed from the transformants hes8 and c1 could not be quantified indirectly by the level of extracellular fluorescence or directly on Western blots. However, the expression of DsRed in these transformants has been visualised by fluorescence CLSM. The transformants c1 and hes8 were analysed further by RNA analysis and Southern blotting, in order to further examine the expression of DsRed under the *hex1* and *cbh1* gene promoters.

5.2.7.1 Genomic characterisation of transformants hes8 and c1

To confirm the presence of the pHEN54RQDsR and phexesDsR expression cassettes in the genomes of transformants hes8 and c1, a Southern blot was performed. Genomic DNA was digested with restriction enzymes that cut the expression cassette at least once, but not within the sequence encoding DsRed. The Southern blot demonstrated that at least five copies of the pHEN54RQDsR expression cassette and at least three copies of the phexesDsR expression cassette, had integrated into the genomes of transformants c1 and hes8 respectively (Figure 5-8). PCR analysis (not shown) indicated that the *cbh1* and *hex1* loci were intact and that homologous integration had not occurred in these transformants.

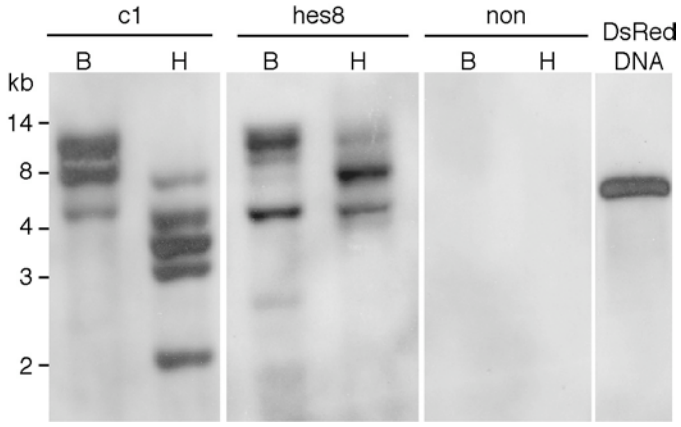


Figure 5-8. Southern blot of genomic DNA from transformants c1 and hes8 digested with *Hind*III (H) and *BamH*I (B), probed for the *dsRed* gene. The DsRed-encoding sequence was not detectable in the genomic DNA of a nontransformant (non).

5.2.7.2 Determination of DsRed expression by RNA analysis

The level of *hex1* and *dsRed* transcripts in transformants hes8 and c1, was determined by northern blotting in order to further investigate the pattern of DsRed expression observed by fluorescence microscopy. Total RNA was extracted from the mycelia

which were grown in the same cultures that underwent analysis of their supernatants in Sections 5.2.4 to 5.2.6.

DsRed message was barely detectable on day one for transformants hes8 and c1 (Figure 5-9) and remained very low in hes8 (*hex1* promoter driven expression) until day three, after which it was undetectable. The level of *dsRed* transcript in c1 (*cbh1* promoter driven expression) appeared to have increased by several thousand-fold by day two, and remained very high until day four. Therefore, the level of *dsRed* transcript produced under the *cbh1* promoter was several thousand fold higher than the level expressed under the *hex1* promoter. The relative levels of *dsRed* transcript between transformants c1 and hes8 was in accordance with the high level of intracellular fluorescence observed in c1 compared to the low intensity and sparse distribution of fluorescent cells observed in hes8.

The total RNA from all the samples was also probed for actin (*act1* gene) as a loading and sample quality control. There was a general decrease in the level of *act1* transcripts from day five and day six. This decrease in *act1* transcripts was consistent with the commencement of cell death within the mycelium and the consequential downgrading of expression.

The level of *dsRed* transcripts expressed from the *hex1* promoter on the phexDsR cassette in transformant hes8 was significantly lower than the level of *hex1* transcripts expressed from the endogenous copy of the *hex1* promoter (Figure 5-9). The relative difference between *hex1* and *dsRed* transcript level could not be quantified by

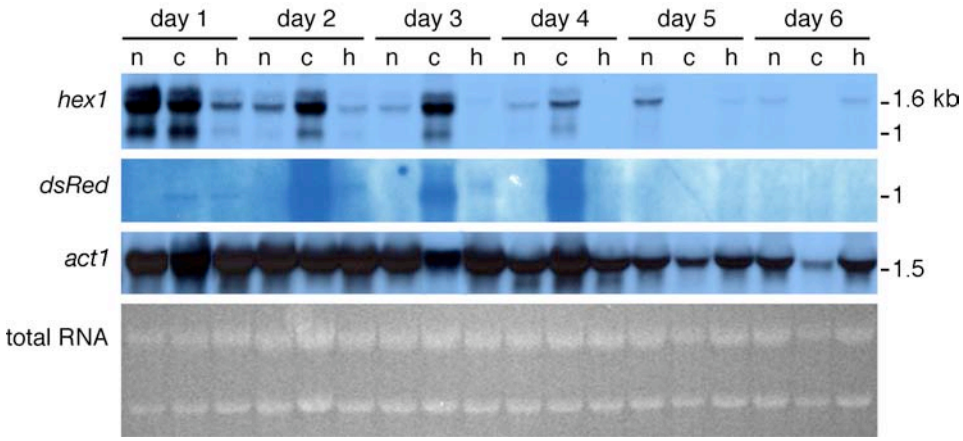


Figure 5-9. Northern blot of total RNA probed for *act1*, *dsRed* and *hex1*. (n) nontransformant, (c) pHEN54RQDsR transformant c1, (h) phexesDsR transformant hes8. Films were exposed for 5 min for *hex1* and *act1*, or 20 min for *dsRed*.

densitometry due to the different exposure times required for the appearance of *hex1* and *dsRed* transcripts on the blots. However, the relative difference between the level of *dsRed* and *hex1* transcripts is indicative of either a low level of accumulation of *dsRed* transcripts or a reduced level of transcription from the *hex1* promoter on the expression cassette compared to the level of *hex1* transcription from the endogenous *hex1* promoter. The rate of transcription from the introduced and endogenous copies of the *hex1* promoter was not determined and a factor which may have affected the stability of the *dsRed* transcripts is addressed below.

The northern blots show that the pattern of *hex1* expression in the nontransformant and in c1 was consistent with the pattern described previously (Section 3.2.6) ie. expression peaked early and then steadily decreased over the culture duration. However, this peak of *hex1* expression was much reduced in transformant hes8 (Figure 5-9). Therefore, in addition to a relatively low level of *dsRed* transcript in hes8, the level of endogenous *hex1* transcript was also low relative to c1 and a nontransformant.

The reduced level of *hex1* transcripts in the hes8 transformant may indicate that endogenous *hex1* expression was affected by the integration of the phexesDsR expression cassette at ectopic locations of the genome. The inhibition of transcript accumulation by the presence of multiple copies of homologous sequences has been observed previously in fungi and is termed quelling (Cogoni *et al*., 1994).

5.3 Post-transcriptional gene silencing

The quelling phenomenon is a form of post-transcriptional gene silencing because the genes involved are transcribed but the mRNAs are targeted for degradation (Romano *et al*., 1992). Some key proteins of this mechanism which have been identified are highly conserved between fungi, plants and animals (Cogoni *et al*., 1999; Fagard *et al*., 2000; Catalanotta *et al*., 2004). It is now believed that quelling in fungi shares a common pathway to the processes of co-suppression in plants and RNA interference (RNAi) in animals (reviewed by Agrawal *et al*., 2003; Pickford *et al*., 2003). It is possible that the reduced *dsRed* and *hex1* expression in hes8 may be due to quelling as discussed below.

5.3.1 The mechanism of quelling or interference RNA

Quelling is a form of post-transcriptional gene silencing whereby specific RNAs are targeted for destruction. The mechanism of RNAi, portrayed in Figure 5-10, commences with the appearance of double stranded RNA (dsRNA) which can originate from several sources that will be discussed later. These dsRNAs are recognised by Dicer, a multidomain protein with RNAse III activity, which cleaves the dsRNA into short interfering RNAs (siRNAs) 21-25 nucleotides in length with characteristic 2-3 nucleotide 3' overhangs (step i, Figure 5-10). The dsRNA is uncoiled and the single strand is passed directly to the RNAi silencing complex (RISC). RISC binds the siRNA

Chapter 6 Expression of DsRed from the *hex1* locus and the phenotypic characteristics of a *hex1* deletion mutant

6.1 Introduction

The analysis of the expression and regulation of the *hex1* promoter has shown that it is complex. Expression cassettes phexeDsR, phexiDsR, phexhDsR and phexesDsR, where the *hex1* promoter was fused with a variable length of the 5' region of the *hex1* ORF, were designed to optimise the expression of DsRed. However, only very low levels of DsRed were expressed under the *hex1* promoter. These results indicated that other factors were influencing the low level of transcript, including the possibility of quelling. The removal of the endogenous *hex1* gene would allow further examination of the biological role of *hex1* in *T. reesei.*

As discussed in Chapter 4 (Section 4.2.5), hyphal cells seemed to be able to differentiate between endogenous and ectopically integrated copies of the *hex1* promoter and consequently alter which transcript type was expressed and of what intensity is was transcribed. It may be necessary to integrate the expression cassette into the *hex1* locus to obtain the equivalent levels of endogenous *hex1* expression for the heterologous protein. In addition, the insertion of an expression cassette into the homologous locus has been found to increase the level of expression in some cases (Harkki *et al.*, 1991), due to the influence of the local primary and secondary structure of the chromosomal loci (Diallinas *et al.*, 1989) on transcription. Therefore, in order to optimise the expression of the heterologous protein DsRed under the *hex1* promoter, numerous transformants containing the phexesDsR cassette were screened to identify a

transformant with integration of the cassette at the *hex1* locus. The expression of DsRed in this *hex1* knockout was investigated.

6.1.1 The application potential of *hex1* knockouts and leaky hyphae

Homologous integration of an expression cassette at the *hex1* locus may improve the level of gene expression under the *hex1* promoter. In addition to the integration of *dsRed* at the *hex1* locus, the removal of the *hex1* coding sequence would prevent the expression of the HEX1 protein and the formation of Woronin bodies in the transformant. Woronin bodies plug septal pores in the event of hyphal damage to prevent the loss of protoplasm from the interconnected mycelium (see Chapter 1 Section 1.7.1). The absence of Woronin bodies may offer other advantages to the *T. reesei* protein expression system.

The secretion of heterologous proteins from fungi is commonly inefficient and limits the yields of protein. For example, investigation of a GFP:glucoamylase fusion expressed in *A. niger* demonstrated that a portion of the fusion protein was retained in the cell wall (Gordon *et al.*, 2000a). In *T. reesei,* up to 80% of extracellular □-glucosidase activity remained associated with the cell wall (Kubicek, 1981). Improved externalisation of proteins expressed in *T. reesei* would enhance the protein production potential and this may be achieved by the deletion of the *hex1* gene. The removal of the *hex1* gene from *N. crassa* resulted in hyphae which were unable to prevent extensive cytoplasmic loss after physical damage or hypotonic shock (Jedd and Chua, 2000). Therefore, work described within this Chapter explores the physiological properties of a *hex1* deletion mutant in *T. reesei*.

6.1.2 Transformation of *Trichoderma reesei*

A homologous recombination event with the transgenic DNA cassette at the *hex1* locus is required in order to knockout the coding sequence of the *hex1* gene. Two transformation methods were applied and the efficiency of the biolistic bombardment transformation procedure to attain homologous recombinants, was compared to that achieved by protoplast transformation.

Transformation of protoplasts is the most commonly used method for gene transfer in filamentous fungi (reviewed by Mach, 2004). Protoplast transformation has been shown to yield transformants in which the transgenic DNA has integrated at the homologous locus. The efficiency of homologous integration was reported to be as high as 63% when using an expression cassette containing 2.2 kb and 0.75 kb of 5' and 3' flanking sequences respectively, homologous to the site of integration (Karhunen *et al.*, 1993). However, the numbers of homologous integration events is typically approximately 2% in *T. reesei* (Seiboth *et al.*, 1992; Mach *et al.*, 1995). The length of the flanking sequence homologous to the site of integration can affect the efficiency of homologous integration and a length of at least 1 kb at either side of the gene to be integrated is considered to be a minimum requirement.

The protoplast transformation protocol involves the degradation of the cell wall of young hyphae by incubation in a mixture of chitinases, cellulases and proteases derived from *T. harzianum*. As points in the cell wall are weakened, the cell membrane protrudes and buds off, taking various cellular contents with it. The protoplasts are maintained in osmotic stabilisers and DNA uptake occurs during the polyethylene glycol (PEG) induced fusion of protoplasts (Timberlake *et al.*, 1989). Since hyphal cells

are multinucleate, a protoplast may contain more than one nucleus, which can result in heterokaryotic transformants. In *T. reesei,* these heterokaryotes can be sorted into homokaryotic transformants via the selection of individual colonies derived from at least one generation of conidiation. The potential generation of heterokaryons and the fragility of the protoplasts are the main disadvantages of this procedure. An alternative transformation procedure available for *T. reesei* is transformation by biolistic bombardment in which uninucleate conidia are bombarded with tungsten or gold particles coated in DNA. However, the efficiency of this procedure in attaining homologous recombinants has not been determined.

6.2 Results and discussion

6.2.1 Generation of *hex1* deletion mutants

The integration cassette phexnull (Figure 6-1) was constructed to create a *hex1* knockout mutant to investigate hyphal physiology and protein secretion in the absence of Woronin bodies. This cassette contained 1.48 kb of the *hex1* promoter region and the *hex1* gene coding sequence divided into two fragments by the insertion of the *pki-hph* fragment. The inclusion of fragments of the *hex1* coding sequence was designed to increase the length of the flanking sequences on the cassette to favour homologous integration.

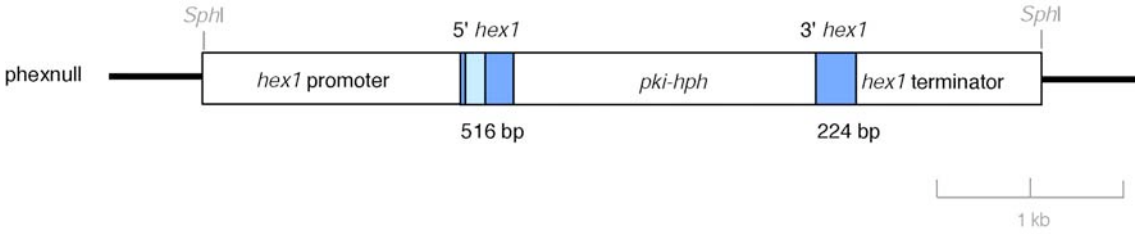


Figure 6-1. The integration cassette phexnull. This cassette was designed for the replacement of the coding region of the *hex1* gene with a truncated form of the gene and the *pki-hph* fragment that confers resistance to hygromycin B. The dark blue regions are the coding sequence of the *hex1* gene and light blue represents the intronic sequence.

The phexnull cassette was excised from the plasmid by digestion with *Sph*I. *T. reesei* was initially transformed with the linear phexnull cassette by biolistic bombardment. Genomic DNA was extracted from transformants, which had survived two rounds of selection on hygromycin B. They were further screened by PCR using the primers hexprobe2.fwdpr and hexprobe2.revpr (Figure 6-2). A fragment of approximately 606 bp in length would be amplified from the uninterrupted *hex1* gene whilst a transformant with a potential homologous integration event would not have amplification of the 606 bp fragment.

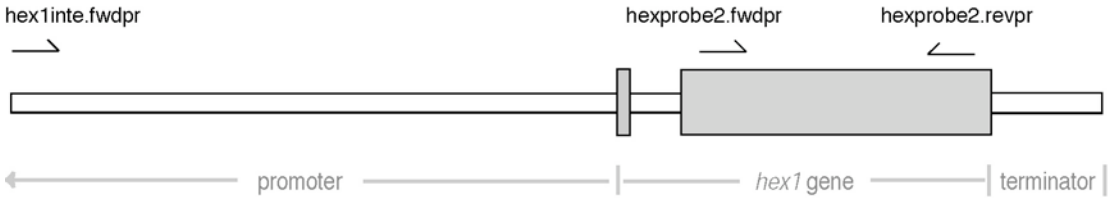


Figure 6-2. The location of primers used in PCR to screen for the interruption of the endogenous *hex1* gene.

If the endogenous *hex1* fragment did not amplify from a transformant, a second PCR was carried out to demonstrate integration of the expression cassette into the *hex1* locus. This second PCR used a primer that annealed to the *hex1* promoter region upstream

from the sequence included on phexnull (hex1inte.fwdpr), and a primer internal to the *pki-hph* fragment of phexnull (cbh1hphint.revpr). The amplification of a fragment of approximately 4.3 kb in length indicated that a homologous integration event had occurred.

A total of 172 transformants generated by biolistic bombardment, were analysed by the first round of PCR screening. In all transformants the *hex1* locus appeared to be intact which indicated that ectopic integration of the phexnull cassette had occurred. The biolistic bombardment procedure was then modified to increase the likelihood of homologous integration.

6.2.2 Modification of the biolistic bombardment method

Homologous integration is dependent upon the alignment of the transgenic DNA with like sequences in the genome when recombination occurs (Shibata, 2001). The earlier failure to generate a transformant with homologous integration of the phexnull cassette using the biolistic bombardment procedure may have been due to the nature of the DNA in the host. Freshly harvested conidia used for biolistic bombardment are in a relatively dormant transcriptional state and their DNA is highly condensed into nucleosomes and chromatin. This compaction of DNA was visible in DAPI stained conidia as a single dense concentration of localised fluorescence (not shown). A dividing cell that is transcriptionally active has a much higher percentage of decondensed or completely unravelled chromatin for DNA replication. It is possible that the highly condensed nature of the DNA within conidia renders the *hex1* locus physically inaccessible to the transgenic DNA. Therefore, the biolistic bombardment procedure was modified to circumvent the potential limitations of highly condensed DNA.

Freshly harvested conidia were incubated for 6.5 hours in PD broth to a pre-germination stage. Over this period, the conidia began to swell but had not yet germinated or commenced nuclear replication. Staining of the conidia with DAPI revealed a less defined concentration of fluorescence, indicating relaxation of the highly condensed genomic DNA into a more transcriptionally-active conformation. It was suspected that the pre-germinated conidia would be less resilient to bombardment at high velocity so the bombardment was carried out at a range of lower pressures (Table 6-1). Following bombardment, the pre-germinated conidia were allowed to recover for 16 hours at 22°C prior to overlay with hygromycin B in PDA.

Table 6-1. Summary of the conditions and outcomes of biolistic bombardment of pre-germinated conidia at a range of pressures.

Bombardment pressure (psi)	**Number of plates**	**Number of transformants (per plate)**
650	3	0
900	3	0
1100	3	0
1350	3	0
1350[c]	3	9, 12, 16

[c] Control transformation using freshly harvested conidia.

No transformants were generated by bombardment of conidia at the pre-germination stage at any of the pressures tested. Therefore, even though pre-germinated conidia may have decondensed genomic DNA, making them more susceptible to homologous recombination events, they did not survive the biolistic bombardment procedure.

All expression cassettes used in previous studies with *T. reesei* were readily integrated into the genome of *T. reesei* conidia. Accordingly, it was assumed that this method was

suitable for producing transformants with targeted integration. The transformation and screening of numerous transformants in this and in other work has indicated that biolistic bombardment is not an efficient transformation technique to achieve homologous integration in *T. reesei*. Therefore, the more laborious protoplast transformation method was adopted to produce a *hex1* knockout mutant.

6.2.3 Transformation and homologous recombination efficiency in protoplast transformation

6.2.3.1 Generation of a *hex1* deletion mutant

Protoplasts were transformed with the linear phexnull cassette following the protocol detailed in Section 2.7.2. On homologous integration, the phexnull cassette would interrupt the *hex1* gene coding sequence by the insertion of the *pki-hph* fragment (Figure 6-1). Transformation of protoplasts resulted in approximately 900 potential transformants, which survived the first round of selection on hygromycin B. Genomic DNA was extracted from 72 transformants and screened by PCR as outlined above (Section 6.2.1). The coding sequence for the *hex1* gene failed to be amplified from one of the transformants, suggesting disruption of the *hex1* coding sequence. A second PCR screen, which aimed to amplify the phexnull expression cassette from within the *hex1* locus (Section 6.2.1), successfully produced a band of approximately 4.3 kb which corresponded with the expected sized product from a homologous integration event (Figure 6-3). This transformant, with the phexnull cassette integrated at the *hex1* locus, was identified as the *hex1*$^{-}$ mutant. The efficiency of homologous integration of the phexnull cassette into the *hex1* locus using protoplast transformation was 1.4%.

6.2.3.2 The replacement of the *hex1* coding sequence with the *dsRed* coding sequence

New transformants of the phexesDsR expression cassette (Figure 5-2) were generated by protoplast transformation in order to express DsRed from the endogenous *hex1* locus. A total of 87 potential transformants survived a second round of selection on hygromycin B. Genomic DNA was extracted from 13 potential transformants and screened by PCR as described above (Section 6.2.1). Amplification of the *hex1* coding sequence using primers hexprobe2.fwdpr and hexprobe2.revpr failed in two transformants, hes4 and hes6, indicating the possible disruption of the endogenous *hex1* gene.

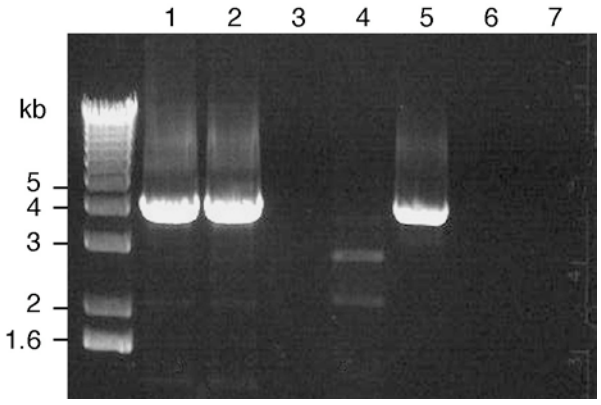


Figure 6-3. Screening of transformants by PCR for integration of the phexesDsR or phexnull cassette into the *hex1* locus. PCR was carried out with the primers hex1inte.fwdpr and cbh1hphint.revpr. 1. Genomic DNA of hes4. 2. Genomic DNA of hes6. 3. Genomic DNA of hes9. 4. Linear phexesDsR DNA cassette. 5. Genomic DNA of *hex1*$^-$. 6. Linear phexnull DNA cassette. 7. Genomic DNA from nontransformant strain.

The second PCR screen using primers hexinte.fwdpr and cbh1hphint.revpr (Figure 6-2), amplified a band of approximately 3.8 kb (Figure 6-3) indicating that the phexesDsR expression cassette had integrated correctly at the *hex1* locus in hes4 and hes6. The efficiency of homologous integration of the phexesDsR cassette into the *hex1* locus in *T. reesei* using protoplast transformation was a relatively high 15.4% compared with

1.4% for phexnull. Different homologous integration efficiencies for different DNA constructs is not unusual (Mach, 2004) and may be attributed to the longer 5' flanking sequence on the phexexDsR cassette compared with the phexnull cassette (Figure 5-2 and Figure 6-1).

6.2.3.3 Further characterisation of the *hex1* knockout mutants

To confirm that the *hex1* coding sequence had been replaced, the *hex1* knockout strains hes4, hes6 and *hex1*$^-$ were analysed for the expression of the *hex1* gene. Strains hes4 and hes6, expressing *dsRed* under the *hex1* promoter, were grown in cellulose medium for 54 h and RNA was extracted for analysis on a northern blot. The absence of *hex1* transcripts in hes4 and hes6 was indicative of a non- functioning endogenous *hex1* gene (Figure 6-4).

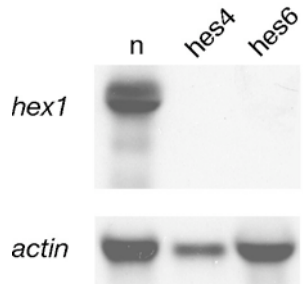


Figure 6-4. Northern blot demonstrating the absence of *hex1* transcript in *hex1* knockout mutants hes4 and hes6 compared to the nontransformant (n). Total RNA was extracted from 54 h old cultures grown in cellulose medium.

The *hex1*$^-$ mutant was analysed by northern blotting to confirm that no expression of *hex1* was occurring. No *hex1* transcripts were detected (Figure 6-5A) which confirmed the elimination of a functional *hex1* gene. To investigate the effect of *hex1* deletion on growth, a growth curve was generated on the neutral carbon source glycerol, to prevent any influence on growth by upregulated protein expression.

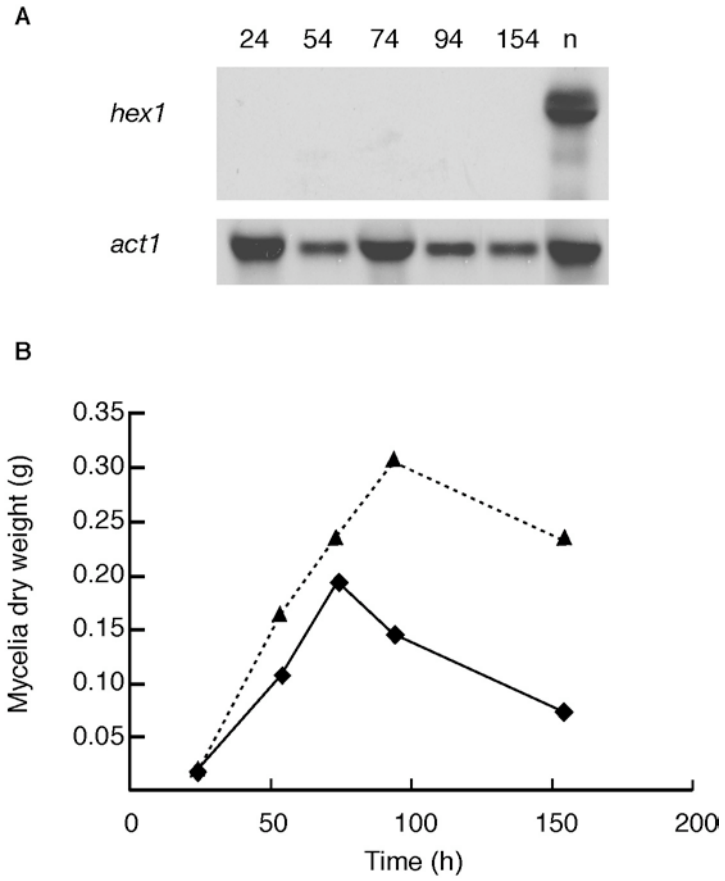


Figure 6-5. Analysis of the expression of *hex1* and growth pattern in the *hex1*$^-$ strain. A. Northern blot of total RNA extracted after 24 h, 54 h, 74 h, 94 h and 154 h probed for *act1* and *hex1*. B. Growth curve of a nontransformant (dotted line) and the *hex1*$^-$ mutant (solid line) in glycerol medium.

The rate of growth of the *hex1*$^-$ strain was very similar to that of the nontransformant until approximately 74 h from inoculation, after which the dry weight of the mycelium decreased (Figure 6-5B). At the peak of growth during the culture periods, the total mycelial mass developed in the *hex1*$^-$ strain was approximately 18% less than the mycelial mass to the parental strain.

When grown on solid PDA medium, the *hex1*-, hes4 and hes6 strains were indistinguishable to the nontransformant as the rate of growth and conidiation appeared normal (not shown). However, when grown on PDA containing hygromycin B, the *hex1*

knockout strains favoured hyphal growth within the medium over the growth of aerial hyphae.

6.2.4 DsRed expression in the absence of endogenous *hex1*

With the generation of two *hex1* knockout mutants, hes4 and hes6, which contained the phexesDsR expression cassette at the *hex1* locus, it was possible to study the expression of DsRed without the influence of the endogenous gene product. To be consistent with previous experiments (Section 5.2.7), transformants hes4 and hes6 were grown in cellulose medium and total RNA was extracted and subjected to analysis by northern blotting (Figure 6-6). The level of expression of DsRed remained low as an exposure time of 20 min was required to visualise the bands compared to 2 min for *cbh1,* and 5 min for the detection of *act1* transcripts. However, expression of *dsRed* from the *hex1* locus occurred at a relatively constant rate from day two to day five in hes4 and hes6. This constant level of *dsRed* expression in the absence of the endogenous gene, was

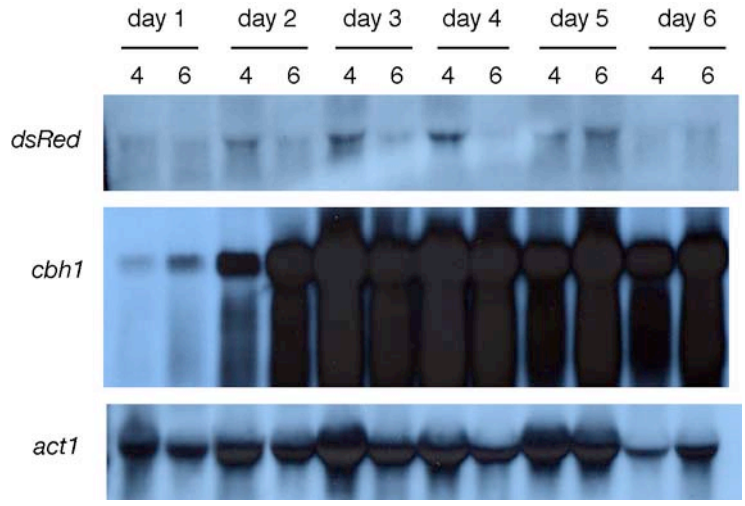


Figure 6-6. Northern blot showing the expression of DsRed from the *hex1* locus in *hex1* knockout strains hes4 (4) and hes6 (6). Total RNA was extracted daily from cultures grown in cellulose medium. The blots were probed with *dsRed, cbh1* and *act1*. The level of the transcripts is not directly comparable between blots due to the different exposure times required for the visualisation of the bands.

different to the pattern of *hex1* and *dsRed* expression observed previously ie. high expression early in the culture period followed by a decrease to undetectable levels by day four (Section 3.2.6). These results suggest that the *hex1* gene is under a form of self-regulation by (negative) feedback of the gene product.

Even though some mechanism of down-regulation of the *hex1* promoter seemed to have been removed by knockout of the *hex1* gene, the level of *dsRed* transcript was significantly less than the native *hex1* transcript levels seen previously (Figure 3-6 and Figure 5-9). This low level of *dsRed* transcripts may be due to reduced mRNA stability or a reduced rate of transcription. It is recognised that the expression of foreign genes as a fusion (Gouka *et al*., 1997) and from the homologous locus of the promoter (Nyyssönen *et al*., 1995), improves transcript levels. However, it is not unusual for the level of heterologous transcript to be reduced compared with the native transcript when expressed from the homologous locus of the promoter within the expression cassette. For example, in the expression of the light chain of Fab antibody fragments under the *cbh1* promoter from the *cbh1* locus in *T. reesei*, the level of light chain mRNA was approximately three to four orders of magnitude less than the level of *cbh1* mRNA produced by the parent strain under the same conditions (Nyyssönen and Keränen, 1995). Therefore, it is unlikely that the level of *dsRed* transcript expressed from the *hex1* locus would be equivalent to the level of *hex1* transcripts. However, the outcomes of the above example were based on the transcript levels determined by northern blotting and do not distinguish between the rate of transcription from ectopic or homologous integration sites, and the stability of the heterologous transcripts. It remains possible that the heterologous transcripts were unstable.

The *dsRed* transcripts are approximately 2 kb in size which is similar to that seen previously for transformant h1 (Figure 4-7). A transcript of this size could result from transcription commencing at the dominant *tsp* 1 and the inefficient splicing of the intron within the 5' UTR, but this explanation was not investigated.

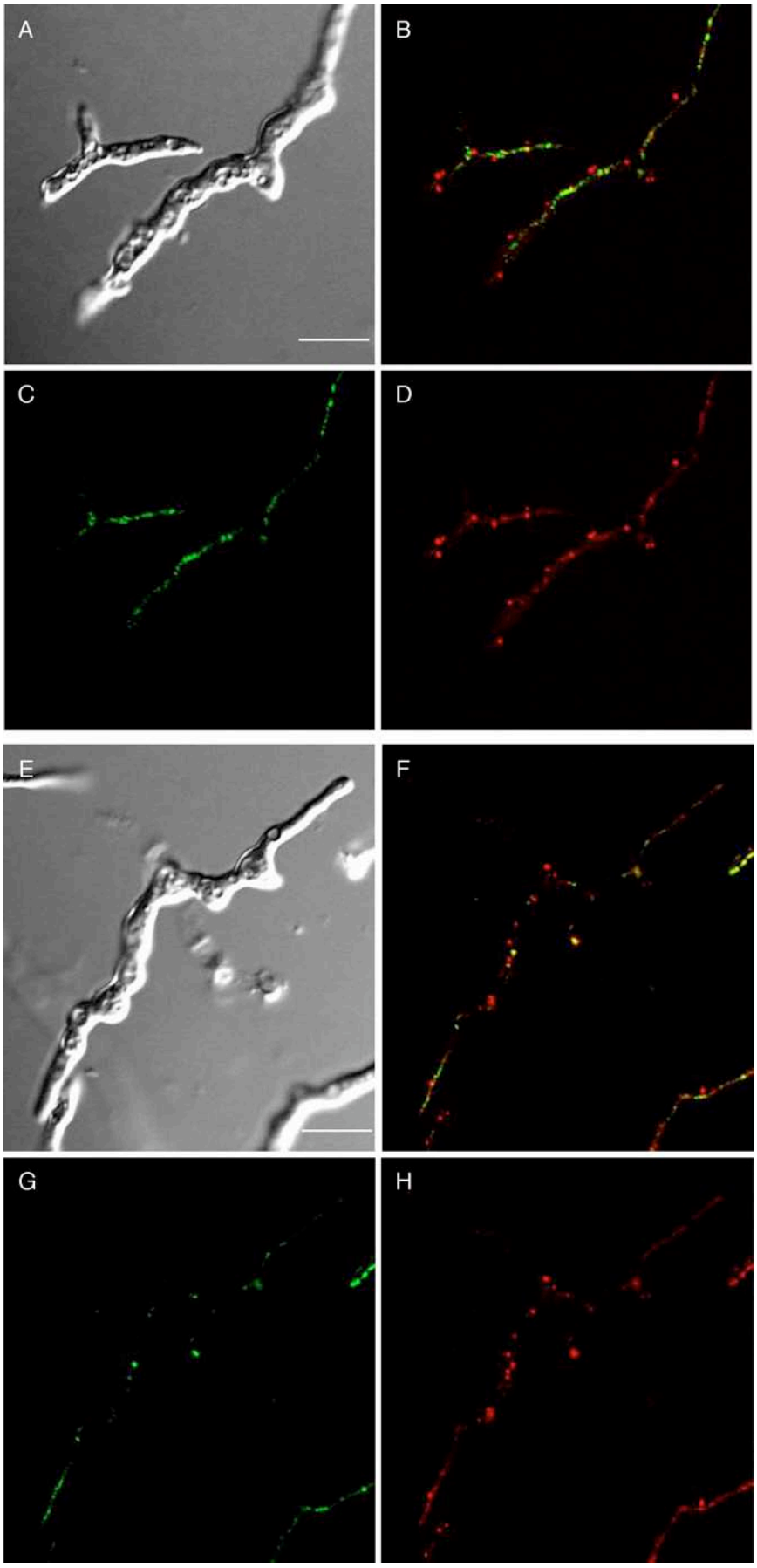
6.2.5 Fluorescence microscopy observations of transformants expressing DsRed from the *hex1* locus

The mycelia of transformants hes4 and hes6 were observed daily by fluorescence CLSM over the course of seven days when grown in cellulose medium. The constant level of *dsRed* expression indicated by the northern blot (Figure 6-6) was reinforced by the pattern of fluorescence. The fluorescence was again concentrated into small spots throughout the hyphae and appeared on day two. However, the fluorescence remained strong until day five and up to 90% of the hyphal cells were fluorescent at any one time over the four day period. Beyond five days, the cells which had died no longer fluoresced and the distinction between living nonfluorescent cells and dead nonfluorescent cells was difficult and made counting of the cells unreliable. Previous observations of *T. reesei* which possessed an intact copy of the endogenous *hex1* gene and expressed DsRed under the *hex1* promoter demonstrated a peak of fluorescence on day two where, at most, only 30% of hyphae displayed the fluorescent spots (Section 5.2.3). These previous transformants also displayed short-lived fluorescence by which the appearance of intracellular fluorescence became increasingly rare beyond day three. The observations of fluorescent hyphae in transformants hes4 and hes6 also suggested that the mechanism for the downregulation of *hex1* promoter activity had been removed with the removal of an intact *hex1* gene.

In transformants hes4 and hes6 the pattern of green and red fluorescence co-localisation was different to that seen in transformants with an intact *hex1* gene. Previously, the transformants demonstrated co-localisation of green and red fluorescence in the discrete concentrations (Figure 5-4). However, in hes4 and hes6 the green fluorescence appeared diffuse as well as in the typical punctate pattern probably due to the constant expression of the protein and the gradual accumulation into discrete spots as the protein matured. This mode of accumulation is also evident by the fact that the larger concentrations of fluorescence were red and the co-localisation of new and mature DsRed (shown as yellow) occurred in the smaller spots (Figure 6-7).

The fluorescent spots only appeared in a few isolated cells in transformants with an intact *hex1* gene. It was suggested previously (Section 5.2.3) that these fluorescent cells were either targeted by the translocation of the DsRed protein or that transcription of *dsRed* was only localised to these cells. In transformants hes4 and hes6, green and red fluorescence were present concurrently within single cells, indicating that newly translated and mature protein were co-localised. This observation suggests that the DsRed protein is not translocated through hyphae to these specific cells. Therefore, the isolated cells displaying fluorescence were likely to have resulted from localised transcription of *dsRed*. Furthermore, these results reveal that not only was the *hex1* promoter downregulated by negative feedback from the gene product, but the downregulation of the *hex1* promoter is spatially regulated through the mycelium.

Figure 6-7. Fluorescence CLSM images of hyphae typical for hes4 at day 3 (A-D) and day 5 (E-H). A and E are DIC images, B and F are an overlay of the green and red flug4orescent images where yellow regions signify colocalisation of green and red fluorescence. C, D, G and H are extended view images of z-stacks of 0.2 μM increments. The scale bars represent 10 μm.


A
B
C
D
E
F
G
H

6.2.6 Protein secretion by the *T. reesei hex1*$^-$ mutant

The *hex1*$^-$ strain was created to explore the effect of the absence of Woronin bodies on protein externalisation. It was of interest to determine if the *hex1*$^-$ strain leaked a higher level of protein than the strain with the *hex1* gene intact. Shake flask cultures were carried out in quadruplicate in cellulose medium and the supernatant was sampled daily.

There was no significant difference in the level of protein in the supernatants of *hex1*$^-$ and the parental strain (Figure 6-8) over four days even though the mean total protein

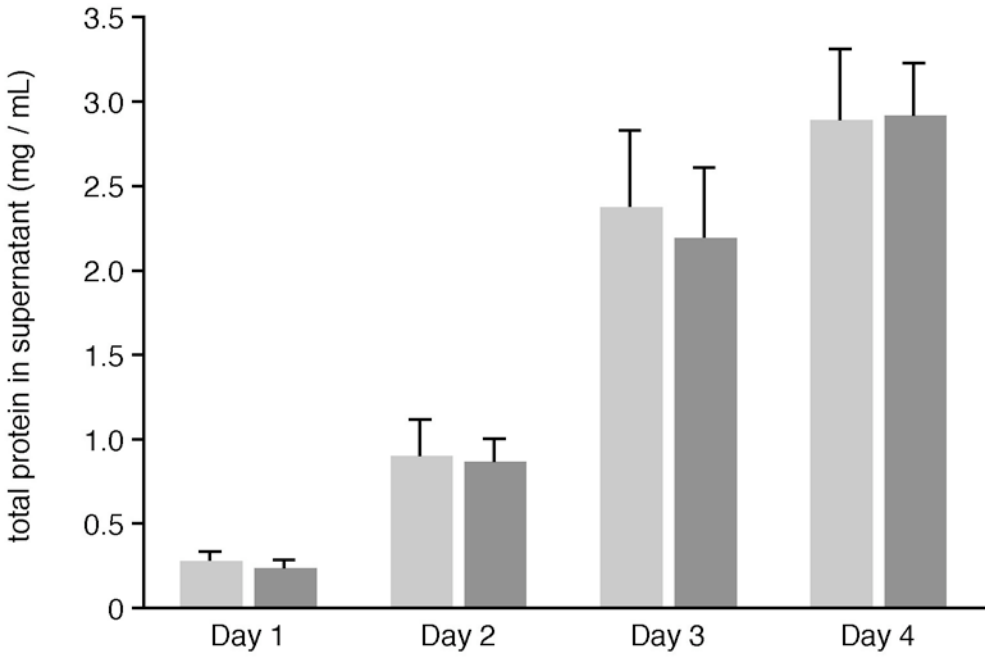


Figure 6-8. Total protein in the supernatants of *hex1*$^-$ (light grey) and VTT-D-79125 (dark grey) and cultures grown in cellulose medium. Error bars show the standard deviation.

concentrations of the supernatants for the *hex1*$^-$ strain were slightly above the means for the parental strain during the active growth phases between day one and day three. By day four the nutrients largely have been exhausted and microscopic examination of the

mycelium showed that cell death was occurring causing cellular debris to accumulate in the culture medium.

Therefore, under normal growth conditions the *hex1*$^-$ strain did not secrete significantly more protein into the supernatant than the parental strain when grown in shake flask culture. This result may be because a shake flask culture at 240 rpm was not sufficiently destructive to the mycelium. Possibly, if a flask with baffles or a fermenter tank with mixing blades were used, the physical damage to the mycelium would be exacerbated resulting in a higher level of protein externalised. In order to challenge the cellular integrity of the *hex1*$^-$ mutant, it was subjected to hypotonic shock by the replacement of the culture medium with water.

The determination of the total protein within the supernatants of cultures that had undergone hypotonic shock did not demonstrate a significant difference between the *hex1*$^-$ mutant and the parental strain (Figure 6-9). However, after 24 h from the time of hypotonic shock, the *hex1*$^-$ mutant displayed a higher mean and maximum of total secreted protein compared to the parental strain (Figure 6-9). The minimal difference in total protein secreted between the *hex1*$^-$ and the parental strain following hypotonic shock was surprising. The deletion of the *hex1* gene from *N. crassa* resulted in approximately six times more protein secreted from the *hex1* deletion mutant after hypotonic shock (Jedd and Chua, 2000).

The mycelia subjected to hypotonic shock were observed by microscopy at each of the sampling time points. After 15 min, the hyphae of the two strains appeared different. The *hex1*$^-$ mutant appeared similar to the untreated control. However, the

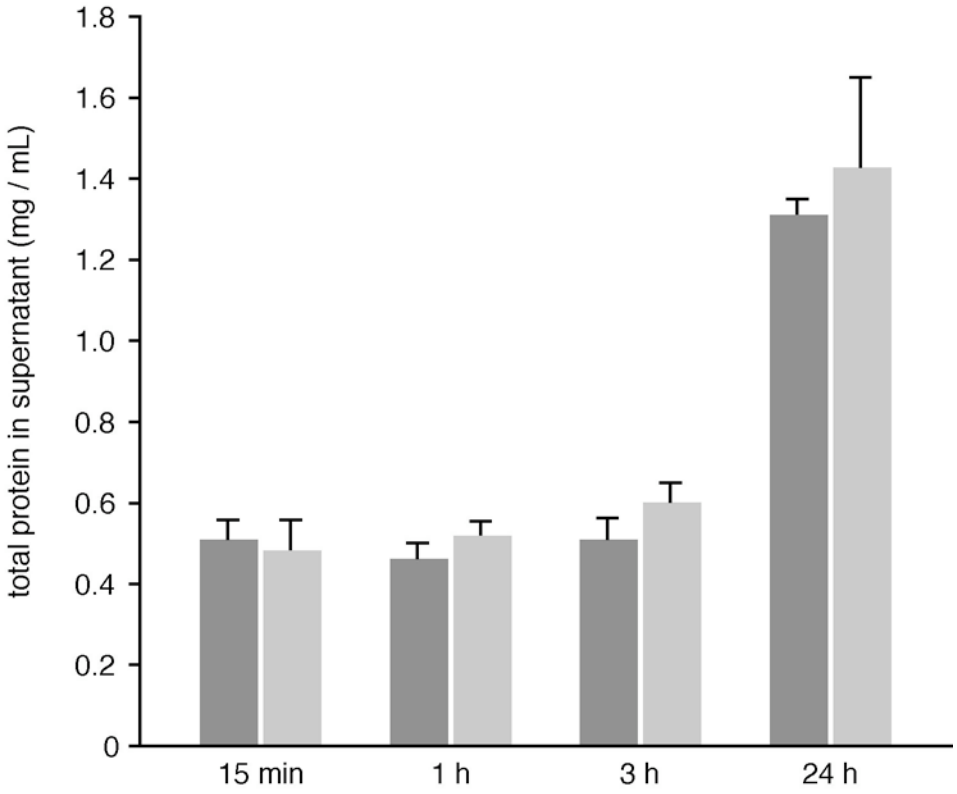


Figure 6-9. Total protein in the supernatants of *hex1*$^-$ (light grey) and VTT-D-79125 (dark grey) cultures after hypotonic shock. The growth medium of three day old cultures was replaced with water and the supernatant was sampled after 15 min, 1 h, 3 h and 24 h. Error bars show the standard deviation.

hyphae of the parental strain appeared to be bulging along its length (Figure 6-10). This observation was the same after 1 h and 3 h from the time of hypotonic shock, but after 24 h both the *hex1*$^-$ and parental strain had bulging hyphae. After 24 h there was also numerous independent spherical sections of hyphae present. It appeared as if the bulges along the hyphae had completely budded off from the filament (Figure 6-10). It is possible that the bulges along the hyphae of the parental strain resulted from the sudden increase in osmotic pressure within the hyphae, which occurred on the transfer of the mycelium into water. The *hex1*$^-$ mutant would also have suffered the same sudden increase in osmotic pressure but instead of bulging at the tips and along the length of the hyphae, the pressure may have been relieved by the bursting of the hyphae and an

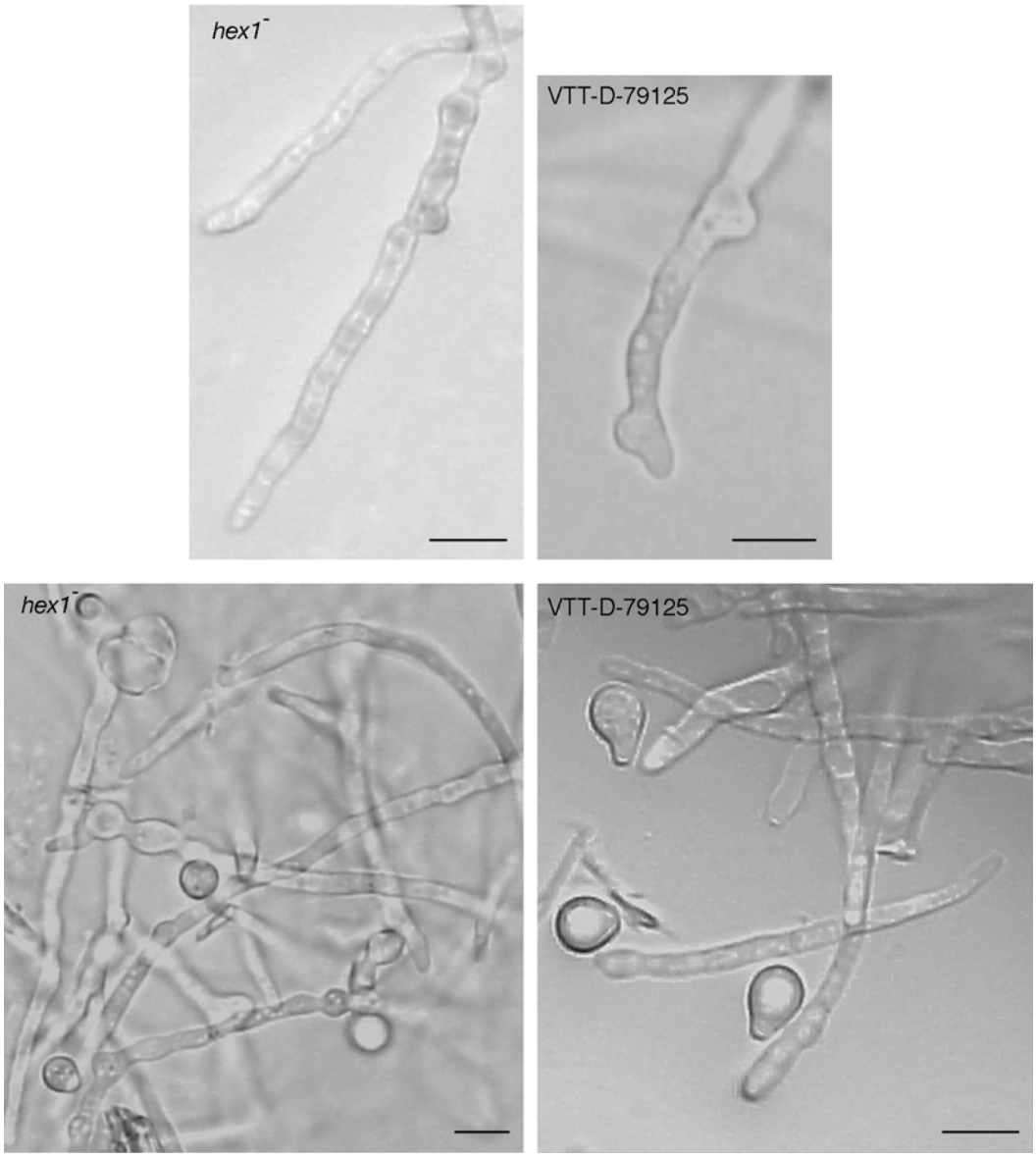


Figure 6-10. The appearance of hyphae following hypotonic shock. The top panel shows the appearance of hyphae 15 min after hypotonic shock and the bottom panel shows the hyphae 24 h after hypotonic shock. The scale bars represent 10 μm.

outpouring of the cellular contents. The bursting hyphae would also have occurred in the parental strain but the flow of protoplasm from the damaged hyphae would have been blocked at the nearest septum by a Woronin body and the osmotic pressure would have remained within the hyphae. It was difficult to distinguish between lysed hyphal tips and healthy tips by light microscopy in order to support the above explanation. As a result, these microscopic observations gave no indication of the degree of hyphal tip

lysis due to hypotonic shock. Therefore, the susceptibility of the hyphae to hypotonic shock was assessed with cultures grown on solid medium to make microscopic observation more effective.

6.2.7 The vulnerability of *hex1* deletion strains to cellular damage

To assess the susceptibility of *T. reesei* hyphae to hypotonic shock more closely and to investigate the degree of cytoplasm leakage from burst hyphae, the hyphae were challenged in two ways. The *hex1*$^-$ mutant and parental strain were grown on PDA on a microscope slide as described in Chapter 2 (Section 2.16.1). The colony was either flooded with water or the edge of the colony was cut with a scalpel blade. The treated colony was observed immediately using light microscopy.

6.2.7.1 The effects of hypotonic shock on *Trichoderma reesei*

On the addition of water, the lysis of the hyphal tips was immediate and explosive due to the release of turgor pressure within the hyphae. Burst hyphae of the *hex1*$^-$ mutant resulted in a balloon of protoplasm at the site of the burst tip (Figure 6-11). In the parental strain this resulted in a small amount of granular matter around the burst tip, presumably from the emptying of the apical cell only. The flow of protoplasm from the hyphae was stopped at the nearest septum (Figure 6-12) probably by the plugging of the septal pore with a Woronin body.

As a colony was flooded with water, the number of burst hyphal tips along the colony periphery were counted. A total of 881 tips were counted for the *hex1*$^-$ strain from four slide preparations. Of these, 3.78% ± 1.55% of tips had burst indicating that the

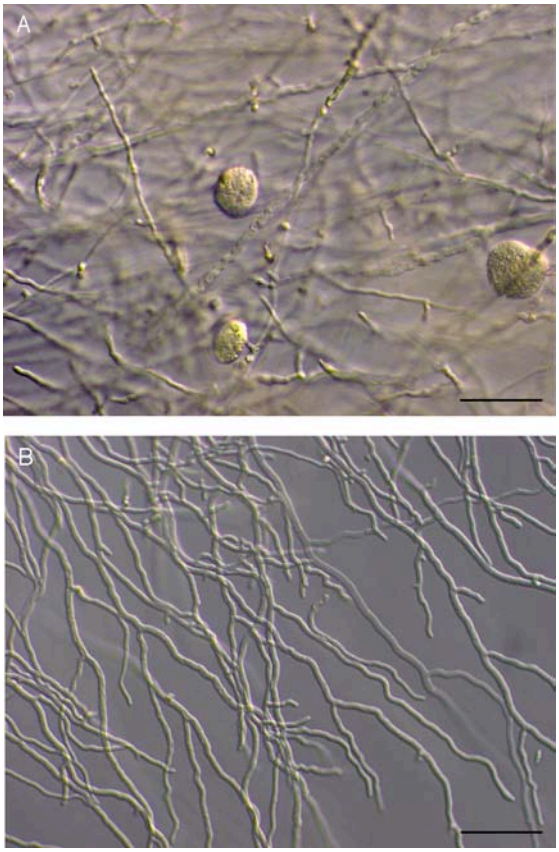


Figure 6-11. The appearance of hyphae, grown on PDA medium, immediately after flooding with water. A. *hex1*$^{-}$ mutant. B. VTT-D-79125. The scale bars represent 50 μm.

hyphae of *T. reesei* are quite resilient to hypotonic shock. The small number of burst hyphae compared with the parental strain (4% ± 2.9%), explains the negligible difference in protein externalised from the *hex1*$^{-}$ strain (Figure 6-9). However, the burst hyphae were more difficult to identify in the parental strain as the volume of leaked cellular contents was greatly reduced compared to the *hex1*$^{-}$ mutant and required observation at 40x magnification opposed to 10x magnification. Any bias in the determination of burst hyphal tips in the parental strain would be towards an underestimate. Therefore, deletion of the *hex1* gene does not make hyphae more susceptible to lysis under hypotonic shock. In *N. crassa,* the number of burst hyphal tips from hypotonic shock varied between 0% and 50% depending on the strain used (Trinci and Collinge, 1974). This difference between strains may also apply to *T. reesei.*

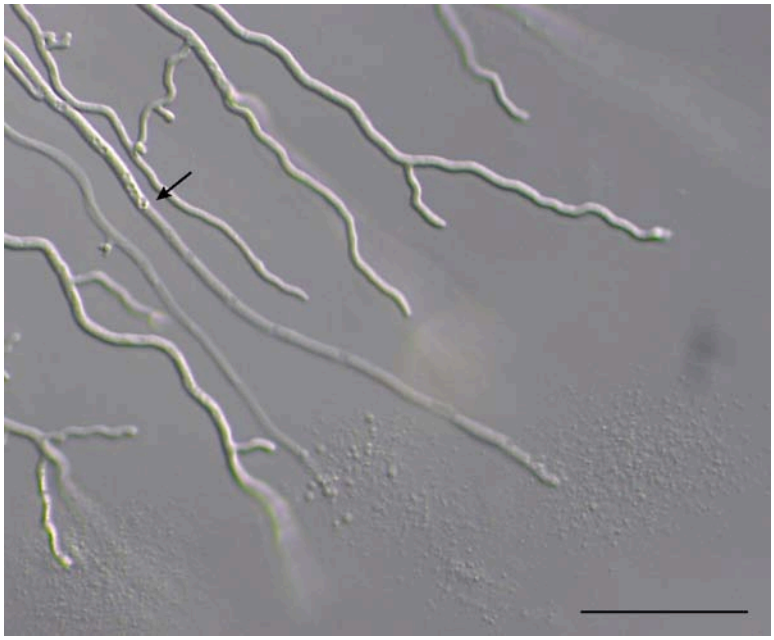


Figure 6-12. Hyphal tips of *T. reesei* strain VTT-D-79125 after flooding with water. The arrow indicates the presence of a septum which is preventing the flow of protoplasm into the damaged cell. DIC image with a scale bar representing 50 μm.

6.2.7.2 The effects of physical damage on *Trichoderma reesei*

Other methods of hyphae damage were investigated since hypotonic shock was not sufficient to cause a large degree of protein externalisation. Colonies of *hex1*$^-$ and the parental strain VTT-D-79125, were grown on a thin layer of PDA on a microscope slide. The edge of the colony was cut with a scalpel blade and the hyphae were viewed immediately by light microscopy. Very large pools of cytoplasm collected at the cut site in the *hex1*$^-$ mutant (Figure 6-13). The cytoplasm continued to leak for up to 40 seconds but the rate of leakage slowed over time. The septum was not a barrier to the flow of leaking cytoplasm (Figure 6-14 and supplementary DVD), which continued to pass through until a large organelle became lodged in the pore. In the parental strain VTT-D-79125, there was no pooling of cytoplasm at the site of the cut and no movement of protoplasm through the hyphae towards the site of damage was observed (Figure 6-13). The difference between the leakiness of the *hex1*$^-$ mutant and

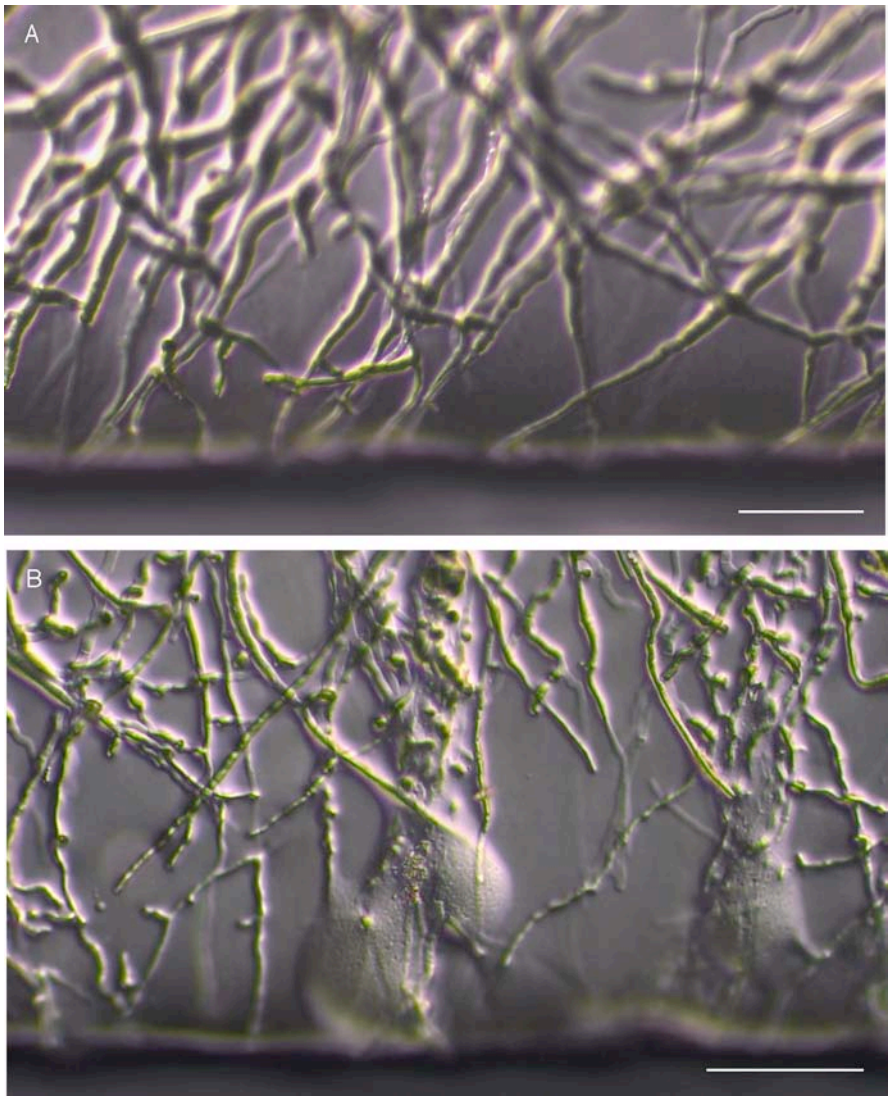


Figure 6-13. Hyphae of VTT-D-79125 (A) and the *hex1*$^{-}$ mutant (B), immediately after slicing the edge of the colony with a scalpel blade. The slice site is along the bottom edge of the images. The leaking protoplasm is also presented in a movie on the supplementary DVD. Scale bar represents 50 μm.

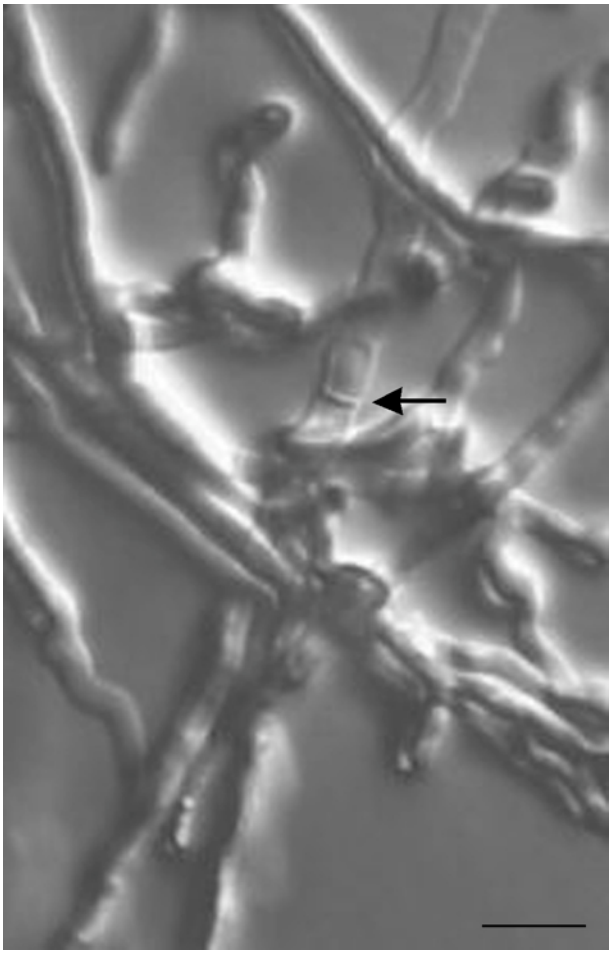


Figure 6-14. The passage of protoplasm through a septum of the *hex1*$^{-}$ mutant. The hyphal tips were cut with a scalpel blade resulting in the leakage of protoplasm from the tips. The arrow indicates a septum which provided no barrier to the passage of protoplasm. The movement of protoplasm through the septum is shown in the supplementary DVD. The DIC image was amplified using Image J software. Scale bar represents 10 μm.

the parental strain demonstrates the role and effectiveness of Woronin bodies in plugging septal pores to conserve the integrity of the mycelium in the advent of hyphal damage.

6.2.8 The potential implications of leaky hyphae

It was found that the deletion of the *hex1* gene does not make the hyphae more sensitive to damage under cultivation conditions but does prevent the sealing of hyphae in the advent of damage. *T. reesei* was quite resilient to lysis induced by hypotonic shock

which limited the level of protein externalised from the *hex1*$^-$ mutant. However, when the hyphal tips were removed by slicing with a scalpel blade, the *hex1*$^-$ mutant lost a large amount of protoplasm whilst the strain VTT-D-79125 was able to instantly block the septal pores adjacent to the site of damage so that the leakage of protoplasm was limited. These results indicate that physical damage to the hyphae is required for a significant increase in the quantity of protein externalised from the *T. reesei hex1*$^-$ mutant derived from the strain VTT-D-79125.

Ultrastructural examination of *Penicillium chrysogenum* from chemostat cultures showed a large number of septa plugged with Woronin bodies (Collinge *et al.*, 1978). This observation suggests that fermentation culture causes sufficient damage to *P. chrysogenum* hyphae to induce blocking of hyphal pores. It may be that *T. reesei* also suffers from extensive hyphal damage in fermentation culture to warrant the blocking of septal pores by Woronin bodies, and is likely to succumb to leaky cells and a high level of passive protein secretion.

Therefore, cytoplasmic leakage may be an advantage if the goal is for externalisation of a protein that may be difficult to externalise via the secretory pathway. It may be possible to express a protein of interest in a *hex1*$^-$ strain of *T. reesei* and then subject the mycelium to cellular damage to maximise the yield of protein attained.

6.3 List of main findings

- The biolistic bombardment transformation method was not efficient in generating transformants with homologous integration of the phexnull or phexesDsR cassettes into the *hex1* locus.

- The protoplast transformation technique generated a transformant with homologous recombination of the phexnull cassette at the rate of 1.4% and for homologous integration of the phexesDsR cassette at the rate of 15.3%.

- The deletion of the *hex1* gene did not affect the conidiation or rate of growth of the mycelium but the total mycelial mass was reduced when grown on glycerol medium.

- In the absence of endogenous *hex1* expression, the mechanism of *hex1* promoter downregulation was lifted such that the level of DsRed expression under the *hex1* promoter was relatively constant (albeit at a low level) over the course of five days.

- *T. reesei* strain VTT-D-79125 is not susceptible to high levels of cellular lysis induced by hypotonic shock.

- Deletion of the *hex1* gene prevented the formation of Woronin bodies and disabled the emergency plugging response of hyphae in the advent of cellular damage.

- Physical damage to the hyphae of the *hex1* deletion mutant leads to a high level of protoplasmic leakage from the mycelium and thereby higher amounts of protein released from the hyphae.

Chapter 7 Summary and concluding discussion

There is a need for an increase in the number of promoters available to regulate expression in a range of cultivation conditions relevant for protein production in filamentous fungi applied as efficient protein expression systems. Several approaches have been used to identify such promoters as discussed in Section 1.6.1. Using a proteomic approach, the HEX1 protein was discovered to be an abundant protein of the cell envelope of *T. reesei* when grown in conditions conducive to cellulase expression as well as in conditions that repress cellulase expression. The abundance of the HEX1 protein signified an important biological role, and the apparently uninhibited expression of the gene on different carbon sources warranted further investigation into its promoter activity. This thesis is an exploration into the *hex1* gene promoter of *T. reesei* and an investigation into the potential for this promoter to drive protein expression in an industrially-relevant filamentous fungus. In addition, the physiological importance of the *hex1* gene product in the maintenance of mycelium integrity was addressed.

7.1 Isolation and transcriptional regulation of the *hex1* gene

The discovery of a large quantity of the HEX1 protein within the total protein extracts of *T. reesei* cell envelope inspired the idea for the discovery of novel strong promoters based on the protein profiles of cultures grown under a set of specific conditions. This method was based on the hypothesis that strong promoters drive the production of large quantities of protein and was used to correctly identify HEX1 as a highly expressed protein when *T. reesei* was grown on various carbon sources. However, the findings of two dimensional gel protein profiles must be considered in a wider biological context

including the intracellular location and stability of the protein, the duration of expression and any undue enrichment of the sample in processing.

The abundance of HEX1 protein separated from total protein extracted from the *T. reesei* cell envelope (Lim *et al.*, 2001) also suggested that HEX1 is biologically important to *T. reesei.* The *hex1* gene was isolated and sequenced from the fungus *T. reesei* and from the fungus *O. floccosum*, which is currently being developed as an expression host (Nevalainen *et al.*, 2004b). The coding region of the genes isolated from these fungi showed a high degree of similarity to the *hex1* genes of other filamentous fungi (Figure 3-5) indicating that the biological role of the protein is probably highly conserved.

In addition, the dominance of HEX1 in cell envelope extracts from mycelia grown in cellulose and glucose media suggested that the *hex1* promoter was a strong promoter that may be constitutively expressed. In order to investigate these predictions further, the expression profile of *hex1* on several carbon sources was addressed.

7.1.1 The expression profile of *hex1*

The results of Chapter 3 indicated that the *hex1* gene was expressed under conditions inducive to *cbh1* expression (CLS medium), in conditions that repress *cbh1* expression (glucose medium) and on neutral medium in relation to expression of cellulases (glycerol medium). These observations were consistent with the discovery of HEX1 protein in cell envelope extracts from mycelium grown in glucose and CLS medium by Lim *et al.* (2001). Therefore, this study has provided further evidence that *hex1* expression is not directly regulated by the carbon source.

The comparison of *hex1* transcript level with the rate of growth revealed that *hex1* expression was correlated to growth phase. The level of *hex1* transcripts was highest at 24 h in CLS medium and 54 h in glycerol medium, which corresponded to the active growth period of the culture (Section 3.2.6). This brief peak in expression was followed by a sudden decrease and the continuation of a very low level of transcript. The pattern of *hex1* expression was in contrast with the expression profile seen for the strong inducible *cbh1* promoter. The *cbh1* promoter was expressed strongly only in the presence of cellulose, in which, the level of expression was very high for the duration of the culture period. Therefore, the *hex1* promoter is a growth-regulated promoter with a brief window of strong expression during the active growth phase.

The expression profile of the *hex1* gene from *O. floccosum* was not investigated in this work due to time constraints. However, future work into the functioning of *hex1* in *O. floccosum* should be carried out in order to evaluate the value of the *hex1* promoter for protein expression in the *Ophiostoma* system.

7.1.2 The *hex1* gene produced alternative transcripts

The analysis of *hex1* RNAs also revealed the presence of multiple transcripts for the *hex1* gene (Section 3.2.2). Further analysis identified three alternative transcript types that were not affected by the carbon source. The *hex1* promoter region contained two regions that were used as transcriptional start sites. They were separated by a 506 bp intron with the dominant *tsp* being 5' to the 5' UTR intronic sequence. A second intron within the *hex1* coding sequence has an alternative 3' splice site such that an unconserved stretch encoding for a histidine-rich peptide was either included or excluded from the *hex1* transcript. The dominant *hex1* transcript type did not contain the

coding sequence for this unconserved histidine-rich stretch, but did include the intron within the 5'UTR which contained several putative regulatory motifs. The presence of two transcript types from the *hex1* gene of *M. grisea* has recently been confirmed (Soundararajan *et al.*, 2004). One of these transcripts also contains an intron within the 5' UTR and has alternative splicing of the intron within the coding sequence such that the gene encodes two forms of HEX1 protein. It has recently been discovered in *M. grisea* that the differential regulation of the alternative forms of HEX1 protein occurs at the level of transcription and splicing in response to nitrogen starvation (Soundararajan *et al.*, 2004). The expression of *hex1* in *T. reesei* was found to be carbon-source insensitive but the effects of nitrogen limitation were not assessed in this study. In light of the recent results presented by Soundararajan *et al.* (2004), it would be worthwhile to further investigate the pattern of *T. reesei hex1* expression under nitrogen limiting conditions to gain a broader understanding of the regulation of alternative transcripts for *T. reesei hex1* gene. Nonetheless, the discovery of multiple transcripts early in this study indicated that the regulation of expression of *hex1* had additional layers of complexity.

7.2 Expression of a heterologous protein under the *hex1* promoter

The expression of a foreign protein under the *hex1* promoter was explored using the gene encoding the fluorescent reporter, DsRed, within a series of expression plasmids (Chapter 4 and Chapter 5). The simplest expression cassette, phexDsR, included the *cbh1* ss fused to the DsRed-encoding sequence under the control of the *hex1* promoter. The second expression cassette, phexesDsR, contained the leading three codons of the *hex1* gene followed by the *cbh1* ss and the DsRed-encoding sequence, to ensure that the

putative Kozac sequence adjacent to the start codon of the endogenous *hex1* gene was intact within the cassette.

The importance of the regions of *hex1,* which differ between the alternative transcript types, upon promoter regulation and gene expression, were investigated further using a series of fusion expression cassettes. The *cbh1* ss was excluded from these constructions and the expression cassettes contained a truncated form of the 5' *hex1* gene sequence fused to the DsRed encoding sequence. The first truncated region included *hex1* gene exon 2, in which the original putative Kozac sequence was intact, to make plasmid phexeDsR. The second plasmid (phexiDsR) contained the *hex1* gene exon 2 and the 109 bp intron from within the coding sequence. The third plasmid (phexhDsR) contained the *hex1* gene exon 2 and the extended length intron, which encoded the histidine-rich region of the HEX1 protein. The *cbh1* ss was re-introduced into the fourth plasmid, phexesDsR, downstream from three codons of exon 2 to further investigate the secretion of a heterologous protein expressed under the *hex1* promoter.

When DsRed was expressed from the ectopically integrated phexesDsR cassette, the level of endogenous *hex1* transcript was reduced due to RNA instability or a decrease in transcription. The possible involvement of quelling was addressed only briefly and further investigation would open a whole new research area for *T. reesei*, especially in relation to protein expression. To clarify if quelling had occurred it would be necessary to determine the rates of transcription from the endogenous copy of the *hex1* promoter and the introduced copy by using nuclear run-on assays, whereby the level of *dsRed* and *hex1* RNA actively transcribed within the nucleus is determined.

The intensity and pattern of DsRed fluorescence was indistinguishable between transformants of the different plasmids and provided no clues to the functionality or relevance of these regions on the 5' end of the *hex1* gene. Therefore, the variable regions of *hex1* transcripts did not seem to play a role in gene expression when the expression cassette was ectopically integrated into the genome. Following from these results, the expression of DsRed from the homologous *hex1* locus was investigated as described later.

In future work, an additional expression cassette should be constructed to determine the importance of the 5' UTR intron on *hex1* promoter activity. This intron contains several putative regulatory motifs as described previously (Section 3.2.5) and is spliced from the dominant *hex1* transcript type, an observation suggestive that the sequence may be important for efficient expression. An investigation into this intron could also be extrapolated to the few other fungal genes, which also contain introns within the 5' UTR (Section 3.2.5.1). The proposed new plasmid would include the full *hex1* 5' noncoding sequence, without the 5' UTR intronic sequence, fused to the fluorescent reporter DsRed. The pattern of expression would be monitored by transcript analysis and intracellular fluorescence for comparison to the data acquired throughout this work. Once the importance of the 5' UTR intron was established, point mutations could be introduced into the putative regulatory boxes to clarify a functional role.

7.2.1 Localised expression of *hex1*

The expression of DsRed under the *hex1* promoter was investigated and monitored by observing intracellular fluorescence and determining transcript levels. This study was the first to use any mutant form of DsRed in filamentous fungi and clearly showed that

DsRed is a functional reporter protein in this organism. The additional advantages to the use of the DsRed1-E5 mutant in this study were the ability to localise the site of expression within the mycelium and to determine the recent history of promoter activity by the differential observation of green and red fluorescence.

The typical pattern of DsRed expression under the *hex1* promoter demonstrated a peak in fluorescence at day two to three, which was consistent with the pattern of expression observed previously by northern blot analysis. Fluorescence occurred in isolated cells scattered throughout the mycelium. However, the green and red fluorescence were co-localised to the same cell and apical cells frequently did not exhibit fluorescence. The observation of non-fluorescent apical cells is not in accordance with previous suggestions that Woronin bodies are produced in growing hyphal tips then passed along to the mature cell as the septum forms (Momany *et al.*, 2002). However, it could be argued that due to the delay in folding of DsRed into the mature fluorescent conformation, the time from transcription to the appearance of fluorescence would allow several replications of cells at the hyphal apex such that the fluorescence finally appeared in a cell far removed from the hyphal apex. Either way, the expression of *hex1* only in apical cells or within isolated distal cells clearly demonstrates that the activity of the *hex1* promoter is regulated locally within the mycelium.

The expression of DsRed under the *cbh1* promoter demonstrated fluorescence in cells throughout the mycelium including the older, non-growing cells. These observations are in accordance with the previous detection of *cbh1* RNAs and CBHI protein throughout the mycelium by *in situ* hybridisation (Nykänen *et al.*, 1997). The spatial differences in expression of the *hex1* and *cbh1* genes demonstrate that the fungal mycelium is not a

uniform mass but is subject to locally regulated gene expression that may result from the position of the hypha in relation to its position in the mycelium and to the immediate physical environment.

7.3 Expression of DsRed from the *hex1* locus

Additional information on the regulation of the *hex1* gene promoter was provided by the analysis of the transformants hes4 and hes6, which carried the expression cassette phexesDsR integrated at the *hex1* locus. In these transformants, the *hex1* promoter remained active over the duration of the culture period, as demonstrated by the sustained presence of *dsRed* transcripts and a consistent level of intracellular fluorescence over five days (Section 6.2.4). The constant activity of the *hex1* promoter in the absence of the native gene product suggests that the endogenous *hex1* gene is under a form of regulation through a negative feedback mechanism.

It is not clear from the data collected if the feedback mechanism arises from the transcript of the endogenous gene, the spliced introns of the transcript or the HEX1 protein itself. However, the regulation is likely to occur at the level of transcription or RNA stability. More work is required to investigate the mode of *hex1* promoter down-regulation. In order to test if the presence of HEX1 protein affects the regulation of the *hex1* promoter, transformants hes4 or hes6 could be co-transformed with *hex1* cDNA under the control of an alternative promoter. A return of the growth-regulated pattern of DsRed expression may suggest that the HEX1 protein is involved in the down-regulation mechanism of *hex1* promoter activity.

7.4 Secretion of DsRed into the culture supernatants

DsRed fused to the *cbh1* ss was expressed constantly from the *hex1* locus over a five day culture period yet extracellular DsRed could not be detected indirectly by fluorescence, or directly by Western blotting. Extracellular fluorescence was detectable in the supernatant of one transformant, which expressed DsRed under the *cbh1* promoter. However, this result may need to be interpreted cautiously since DsRed is an intracellular protein of the coral *Discosoma* sp. and exists as obligate tetramers. In addition, to our knowledge, this protein has not been previously successfully secreted from fungi. Further investigation into the secretion of DsRed from this positive transformant could not be carried out because it did not survive storage at –20°C.

The properties of DsRed in fungal culture, including the effects of pH and protease activity on the stability of extracellular DsRed, were investigated in the course of this work (Section 5.2.6). The results were open-ended and it remains possible that the endogenous proteases and low pH of the supernatant may be at least partially responsible for the instability of DsRed in culture supernatants.

In the course of this work I also addressed the tendency of DsRed to form insoluble aggregates within the cell as discussed in Section 5.2.6.1. Intracellular fluorescence appeared as discrete spots throughout the cell that were not demonstrative of packaging of the protein into secretory granules since there was no ss to target the DsRed for secretion. Consequently, an experiment based on a method by Jach *et al*. (2001) was devised to ascertain if the fluorescent spots were due to aggregation. The total protein was extracted from a mycelium in two steps. The soluble components were extracted in citrate buffer (Section 2.12.1) before the pellet was subjected to a more vigorous

extraction method using powerful denaturants (Section 2.12.2) to extract the insoluble component. A Western blot was carried out (not shown) for the two fractions but the DsRed protein was not identified in either, probably due to the demonstrated low affinity of the monoclonal antibody for this particular DsRed mutant (BD Biosciences Clontech). It may be that DsRed is a suitable reporter of promoter activity in fungi but is not useful for monitoring protein secretion.

7.5 Leaky hyphae in the absence of Woronin bodies

Polymers of the HEX1 protein form Woronin bodies that plug septa to prevent excessive cytoplasmic loss from damaged hyphae (Tenney *et al.*, 2000). It was proposed in this thesis that leaky hyphae would be beneficial for increased protein externalisation from *T. reesei* and thus, a *hex1* gene knockout mutant (*hex1*$^-$) was generated by the interruption of the *hex1* coding sequence with the *pki-hph* fragment (Section 6.2.3.1).

The *hex1*$^-$ strain had the same growth rate as the parent strain VTT-D-79125, but the total mass of the mycelium was reduced. This reduction may have been due to the inability to seal damaged or dead cells that arose in the mycelium after four days, from the living cells of the mycelium. This inability would have resulted in excessive leakage of protoplasm from otherwise healthy hyphae and limited the continued growth of the mycelium. Therefore, the *hex1* gene and Woronin bodies are crucial for efficient growth and colonisation of filamentous fungi.

Hypotonic shock was used to induce hyphal damage to the *hex1*$^-$ strain but the hyphae proved to be largely resistant to the challenge as less than 4% of hyphal tips burst.

However, when the edge of a colony was cut with a scalpel blade, the protoplasm poured out from the damaged hyphae (refer to the movie on the supplementary DVD). Therefore, physical damage to hyphae may be a potential way of externalising proteins of interest from fungal cells.

Previous studies in *N. crassa* indicated that hyphae grow from the site of a blocked septum (Trinci and Collinge, 1974) but not from a burst hyphal cell in a *hex1* deletion mutant (Jedd and Chua, 2000). Therefore, once a hypha of *hex1*$^{-}$ has been damaged there is not likely to be further growth. Consequently, for the maximisation of protein externalisation in a fermenter cultivation, it may be advantageous to allow the culture to generate a maximal level of protein before purposely damaging the mycelium and harvesting the proteins as a way of "milking" the fungus. Trials should be conducted in the future to test this method of harvesting protein.

7.6 Conclusions

The work conducted for this thesis was the first characterisation of the *hex1* gene and promoter from an industrially important fungus. This study revealed that whilst the *hex1* promoter was insensitive to the carbon source, it was regulated by growth phase. In addition, the pattern of expression was multi-layered and complex as shown by the presence of alternative transcript types and a negative feedback regulatory mechanism. However, the *hex1* promoter proved to be a strong promoter with a narrow window of high activity. As it stands, the use of the *hex1* promoter for efficient protein expression in fungi would require the development of a suitable fermentation system to maintain the active growth phase.

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Isolation, characterization and expression of the *hex1* gene from *Trichoderma reesei*

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Abstract

Polymers of the HEX1 protein produce Woronin bodies in filamentous fungi. We have isolated and sequenced the *hex1* gene and flanking regions from the industrially exploited fungus *Trichoderma reesei*. Multiple transcription start sites (TSS) and the 5′ untranslated region (UTR) were identified by 5′ RACE PCR. There are three *hex1* transcript types, two of which originate from two TSSs at approximately − 320 and − 1335 from the start codon, which are separated by a 500-bp intron within the 5′ UTR. The third transcript type results from alternative splicing of the intron within the coding sequence at the 3′ end, which results in the inclusion or exclusion of an unconserved histidine-rich coding region. The three transcripts code for two forms of HEX1 protein. N-terminal sequencing of HEX1 separated by 2D gel electrophoresis confirms that there are two forms of HEX1 protein which are modified further by alternative cleavage of the N-terminus. The dominant form of HEX1 is coded by a cDNA with TSS at position − 1335. Expression of *hex1* on cellulase-inducing medium peaks strongly within 24 h of growth but the protein is expressed at a lower and more consistent level in medium containing glucose. This is the first investigation of expression of the *hex1* gene encoding a protein unique to filamentous fungi.

Keywords: *hex1*; Woronin body; Multiple transcription start sites; Alternative splicing

1. Introduction

Woronin bodies are vital in the maintenance of mycelial integrity of filamentous fungi by plugging septal pores to prevent cytoplasmic bleeding in the event of hyphal damage (Markham and Collinge, 1987). The HEX1 protein is the major component of Woronin bodies (Jedd and Chua, 2000; Tenney et al., 2000) and high amounts of the protein are seen as electron dense spheres at the hyphal tip and in very close association with the septa (Markham and Collinge, 1987; Momany et al., 2002). This visual evidence and our previous work using a proteomic analysis of cell-envelope associated proteins of *Trichoderma reesei* (Lim et al., 2001) indicate that HEX1 is an abundant protein. In our earlier study, where cell envelope extracts were separated by 2D gel electrophoresis and a range of spots identified by mass spectrometry, approximately 50% of the total protein extracted from the cell envelope was HEX1 from mycelia grown in glucose medium.

There is a need for strong promoters insensitive to catabolite repression for the expression of valuable gene products in industrially important fungal hosts (Nevalainen et al., 2003). Since plentiful quantities of HEX1 are synthesised on glucose, the promoter driving *hex1* expression would be a good candidate.

The *hex1* gene has been previously isolated and characterized from some other fungi. *Neurospora crassa hex 1* (Genbank Accession No. AF001033) contains a single

Abbreviations: TSS, transcription start site; UTR, untranslated region; DIG, digoxigenin; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase PCR.

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intron and codes for a 19-kDa protein that appears to form dimers and tetramers of HEX1 (Jedd and Chua, 2000). In addition to *N. crassa*, the *hex1* gene has been isolated from the plant pathogenic species *Botrytis cinerea* (Genbank Accession No. AL114136) and *Magnaporthe grisea* (Genbank Accession No. AY044846), and *Aspergillus nidulans* (Genbank Accession No. AF239659). The HEX1 protein sequences deduced from the respective gene sequences are highly homologous amongst these fungi, except for a region of variable length and composition that lies seven residues internal to the N terminus, as previously described (Tenney et al., 2000). *N. crassa* and *M. grisea hex1* genes have an intron following the conserved seven residues of the N-terminus and the variable length of the unconserved region is a result of an alternative 3′ splice site in the introns. Two types of cDNA clones for the *M. grisea hex1* gene have been reported (Tenney et al., 2000). Deducing from this data, these two forms differ by alternative splicing of 66 bp at the 3′ end of intron 1, which codes for a histidine-rich unconserved region. The two transcripts theoretically could be translated into a 19.9- and a 22.7-kDa form of the HEX1 protein. However, only a single protein of an approximate molecular weight of 22 kDa was detected when cell extracts of *M. grisea* were probed with anti-HEX1 antibody (Jedd and Chua, 2000) and there is no further evidence in the literature of multiple forms of HEX1 in *M. grisea*.

The work published so far on *hex1* has reported the gene sequence and concentrated on the role of the HEX1 protein in the physiology and functioning of the Woronin body. Until now, no molecular analysis of a fungal *hex1* promoter or its regulation by growth phase or media composition has been reported. In this paper, we describe the isolation and characterisation of the *hex1* cDNA and the chromosomal *hex1* gene with its regulatory sequences from *T. reesei*. Time-dependent expression of *hex1* on cultivation media, which either promote or repress the synthesis and secretion of hydrolytic enzymes by the fungus, is also reported.

2. Materials and methods

2.1. Culture conditions and growth curve

T. reesei strain VTT-D-79125 (Bailey and Nevalainen, 1981) was grown on potato dextrose agar (PDA) for 7 days and spores were harvested in 0.9% (w/v) NaCl, 0.01% (v/v) Tween 20. To compare *hex1* expression under different growth conditions, two types of medium were used for cultivation. These contained mineral salts (Lim et al., 2001) with 2% (w/v) glucose (glu) or 2% (w/v) glycerol as the sole carbon source, or mineral salts with 1% (w/v) cellobiose, 1% (w/v) lactose and 3% (v/v) soy hydrolysate extract (CLS) (Lim et al., 2001). Three 250-ml conical flasks containing 50 ml of each medium were inoculated with 3×10^8 spores for each sampling point. Cultures were grown at 28 °C and 240 rpm for 24, 54, 74, 94, and 154 h. Mycelia were collected on preweighed GF filter paper (Whatman, England) by vacuum, washed with 1 volume H_2O, then freeze dried. Mycelial dry weight was calculated by subtracting the weight of the filter paper.

2.2. Genomic DNA isolation

T. reesei was grown on cellophane discs placed on PDA plates for 3 days. Mycelia were harvested and ground under liquid nitrogen, then incubated in lysis buffer (50 mM Tris–HCl, 50 mM EDTA, 3% (w/v) SDS and 1% (v/v) 2-mercaptoethanol) for 1 h at 65 °C. Organic extraction of genomic DNA was carried out using Eppendorf (Hamburg, Germany) 15-ml phase lock gel tubes as recommended by the manufacturer. DNA concentration was determined by absorbance reading at 260 nm.

2.3. Isolation of T. reesei hex1 by chromosome walking PCR

Degenerate primers hex1fwd.pr 5′-ACATCTTC-CAAAATGGGNTAY and hex1rev.pr 5′-ACGGGGCCG-CACATNGTYTGNAC were designed based on translation of conserved HEX1 sequences from *T. reesei* and *A. nidulans* (Lim et al., 2001) at positions 2488 and 3015, respectively (Fig. 1). The PCR mixture contained one unit of heat activated AmpliTaq Gold Polymerase (Perkin Elmer, USA), 100 ng of each of the above primers, 3 mM $MgCl_2$, 12.5 mM dNTP's, approximately 10 ng of *T. reesei* genomic DNA and H_2O to 50 μl. Amplification conditions were [1 ×] 94 °C, 10 min [5 ×] 94 °C, 30 s; 40 °C, 30 s; 72 °C 30 s [35 ×] 94 °C, 30 s; 50 °C 30 s; 72 °C 30 s. This PCR yielded a 550-bp product.

Chromosome walking (Morris et al., 1995) was applied to isolate the remaining 5′ and 3′ coding regions and flanking sequences. *Hex1* specific primers used for chromosome walking PCR are shown in Table 1 with the same reaction components as above. Amplification conditions were [1 ×] 94 °C, 10 min, [35 ×] 94 °C, 30 s; 60 °C 30 s; 72 °C 2 min. Chromosomal walking PCR products were inserted into the TA cloning vector (Invitrogen, USA) as instructed or were purified and sequenced directly using the ABI Prism 377 DNA Sequencer (Applied Biosystems, USA).

2.4. Southern blotting

The *hex1* hybridization probe was generated from chromosomal DNA by PCR using the PCR DIG labelling mix (Roche, Germany) as instructed by the manufacturer with the same reaction components as in Section 2.3. The primers for amplification of the 616-bp probe were hexprobe2.fwdpr 5′-CCTCAAGCACGGCGTCGCC and hexprobe2.revpr 5′-CCTTCATCTCAACAGCGAGC. Amplification conditions were [1 ×] 94 °C, 10 min, [35 ×] 94 °C, 30 s; 56 °C 30 s; 72 °C 1 min.

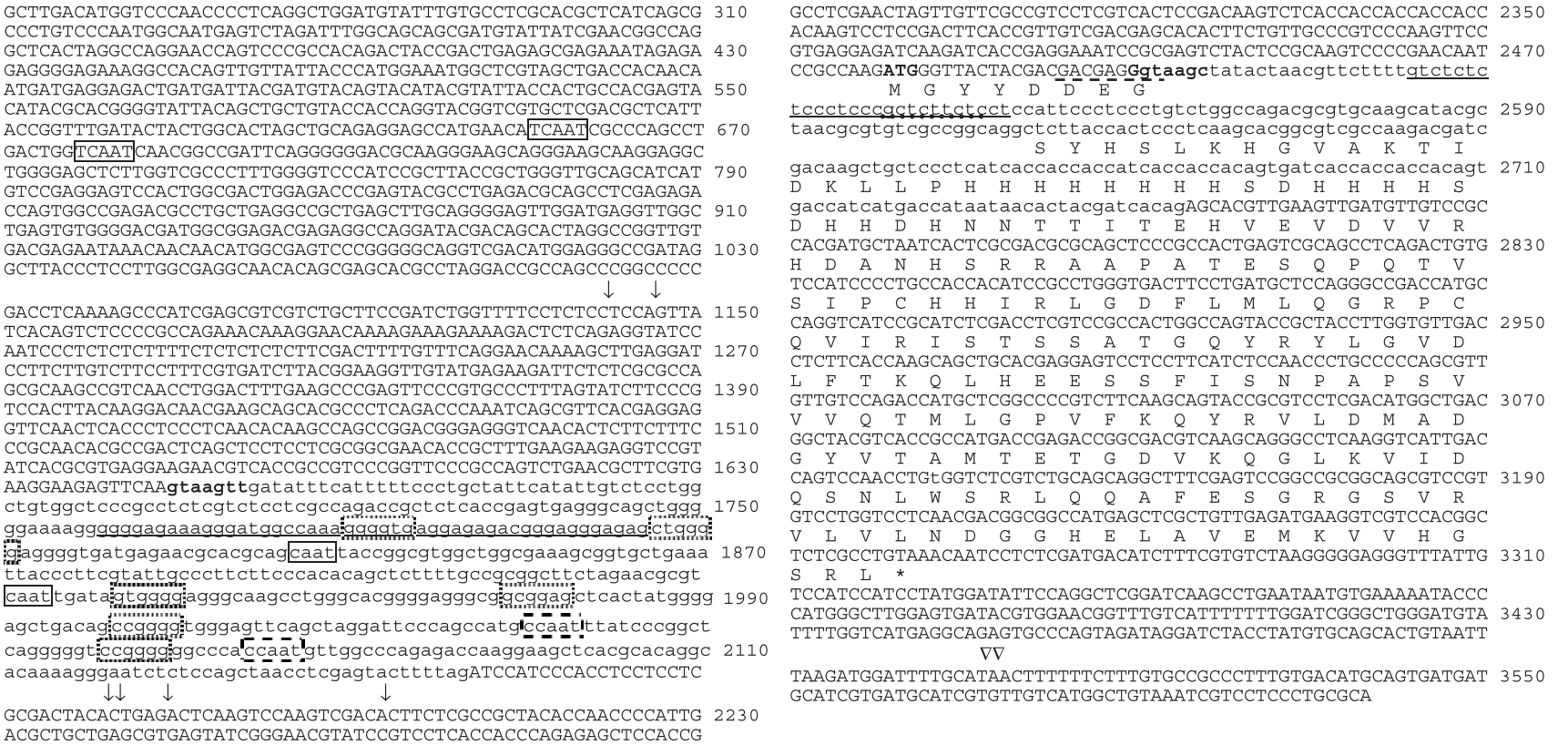

Fig. 1. *T. reesei hex1* gene sequence with the 5′ and 3′ flanking regions and deduced amino acid sequence. Lower case indicates intron sequence. Transcription start sites are marked with arrows. The underlined sequences within the two introns are highly complementary to each other. Dashed underline marks the Kozac sequence. Start codon is shown in bold. Conserved intron splice sites are bold and the lariat sequence is dot underlined. Putative CAAT boxes are boxed with a solid line and putative CCAAT boxes are boxed with a dashed line. The putative CREA boxes are boxed with a dotted line. Triangles mark the polyadenylation sites. Further sequence upstream and downstream can be accessed at Genbank Accession No. AY288289.

Table 1
Hex1 specific primers for chromosome walking PCR

Primer	Sequence 5′ to 3′
hex1rev2.gwpr	GCGTATGCTTGCACGCGTCTG
hex1rev3.gwpr	GCAACAGAAGTGTGCTCGTCG
hex1rev4.gwpr	GTGTAGCGGCGAGAAGTGTCG
hex1rev5.gwpr	GGTTGACGGCTTGCGCTGGC
hex1rev6.gwpr	TAGGCGTGCTCGCTGTGTTGC
hex1rev7.gwpr	GTGGACTCCTCGGACATGATGC
hex1fwd1.gwpr	CTCCAACCCTGCCCCCAGC
hex1fwd2.gwpr	GCTACGTCACCGCCATGACCG
hex1fwd3.gwpr	CAGTACACGTTGCCTCTTGACG
hex1fwd4.gwpr	GAGATAACATGTAGGTAGCGCG
hex1fwd5.gwpr	GGCATGTAAGCAATGAGGACCG

Aliquots of 2.5 μg genomic DNA were digested with *Eco*RI, *Bam*HI and *Not*I (Roche, Germany) in the appropriate buffers and separated by electrophoresis on a 1% (w/v) agarose gel. Digested genomic DNA was transferred onto positively charged nylon membrane (Roche, Germany) using the BioRad vacuum blotter (BioRad, USA) following the manufacturers instructions, then fixed by baking at 120 °C for 20 min. Hybridization and detection was carried out using the DIG system (Roche, Germany). The membrane was hybridized with 20 ng/ml DIG-labelled probe in prehybridization buffer (Roche, Germany) at 55 °C overnight.

2.5. Total RNA isolation

Mycelia from liquid cultures described in Section 2.1 were collected by centrifugation (4400 × *g* for 10 min at 4 °C), washed in cold DEPC-treated 0.9% (w/v) NaCl, and frozen under liquid nitrogen. Frozen mycelia were ground under liquid nitrogen and RNA extracted using Trizol reagent (Invitrogen, USA) following manufacturer's instructions. An additional centrifugation at 12,000 × *g* for 10 min at 4 °C was performed prior to chloroform extraction to remove the large amount of cell debris. RNA concentration was determined based on absorbance at 260 nm.

2.6. Northern blotting

Hybridization probes were generated from chromosomal DNA by PCR as described in Section 2.4. The primers for amplification of the full length actin gene as an internal control were actin.fwdpr 5′-TCAGTCCCATGGAGGGTACGTGCACCTGTC and actin.revpr 5′-CTGTATCCCATGGTCCTTAGAAGCACTTG. The 720bp *cbh1* probe was generated with the primers TSTR.pr 5′-TTCTACGGGTTATGAACGGG and cbhIfusion.seqpr 5′-CAAGCAGCTGACTGAGATG. The *hex1* probe used is described in Section 2.4. The DIG Luminescent Detection Kit for Nucleic Acids (Roche, Germany) was used to detect gene-specific mRNAs following the protocol supplied by the manufacturer.

Total RNA (20 μg) was denatured and electrophoresed on a 1.2 % (w/v) agarose/formaldehyde gel as in Sambrook et al. (1989). Transfer and detection are described in Section 2.4. The hybridization temperature was 60 °C. The ChemiImager 4400 (Alpha Innotech, USA) was used for detection of chemiluminescent signal on blots and the accompanying Alpha Ease software used for image analysis. The intensity of expression was normalized against actin RNA levels.

2.7. RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) PCR

Total RNA was extracted from a 54-h-old *T. reesei* culture grown in CLS medium as described for total RNA isolation. Rapid amplification of 5′ and 3′ cDNA ends was carried out using the First Choice RLM-RACE Kit (Ambion, USA) according to the manufacturer's instructions with the following exceptions. For the reverse transcription step in the 5′ RLM-RACE, 16 pmol of the primer hexprobe.revpr 5′-CGTGCAAGCATACGCTAACG was used in place of the recommended 100 pmol random decamers to reduce the level of smearing in subsequent PCR steps. The *hex1* cDNA generated was amplified in a single PCR step rather than nested PCR. The PCR reaction contained 1 unit of Qiagen Hotstart Taq Polymerase, 10 pmol hex5'RACE.inpr 5′-CGTCGCGAGTGATTAGCATC, 10 pmol 5′ RACE outer primer (Ambion, USA), 1 × Qiagen Hotstart buffer, 20 μM dNTP's, 2 μl RT reaction and H_2O to 50 μl. The PCR conditions were [1 ×] 95 °C, 15 min, [35 ×] 94 °C, 30 s; 60 °C 30 s; 72 °C 1 min 30 s [1 ×] 72 °C, 7 min.

The nested PCR conditions for 3′ outer PCR were as recommended with 10 μM gene-specific outer primer hex3′RACE.outpr 5′-CTTCAAGCAGTACCGCGTCC, 1 unit of Qiagen Hotstart Taq Polymerase and 1 × Qiagen Hotstart buffer. The conditions were [1 ×] 95 °C, 15 min [35 ×] 94 °C, 30 s; 60 °C 30 s; 72 °C 30 s [1 ×] 72 °C, 5 min. The 3′ inner PCR was carried out, as recommended, with 10 μM gene-specific inner primer hex1fwd2.gwpr using the same conditions as for the 3′ outer PCR. PCR products were cloned using the TA cloning kit (Invitrogen, USA), and recombinant plasmids were isolated and sequenced.

2.8. N-terminal sequencing of HEX1

Cell envelope proteins of *T. reesei* grown in CLS medium were previously separated by 2D gel electrophoresis (Lim et al., 2001). Two spots identified as HEX1 were cut from the gel, eluted in gel elution buffer at 37 °C overnight, transferred to PVDF and washed using a ProSorb cartridge. The sample was then subjected to 10 cycles of N-terminal sequencing at the Australian Protein Analysis Facility (APAF, Macquarie University, Sydney, Australia).

3. Results and discussion

3.1. Analysis of the T. reesei hex1 sequence

The *T. reesei hex1* coding sequence and flanking regions were isolated using degenerate primers and chromosome walking PCR (Fig. 1). The 783-bp coding region contains a single 109-bp intron and shows considerable sequence similarity to other known *hex1* genes. The sequence of the gene isolated in this work is available on Genbank (Accession number AY288289).

Southern blot analysis was conducted on *T. reesei* genomic DNA digested by *Bam*HI, *Eco*RI and *Not*I. A single band resulted from each digest, indicating that *hex1* exists as a single copy gene in the genome (Fig. 2).

The entire 2.4-kb 5′ flanking region of *hex1* from *T. reesei* was compared to the 5′ flanking regions of *hex1* from *N. crassa* and *M. grisea* for small conserved sequences using the DNA-pattern programme at the site http://embnet.cifn.unam.mx/rsa-tools/. *T. reesei*, like *N. crassa* and *M. grisea*, has a conserved putative ribosome binding site adjacent to the start codon (GCCAAGATGG). Promoter analysis failed to recognize any TATA-like box upstream of the start codon. There are two putative CAAT boxes at positions 657 and 678, which are approximately 460 and 480 bp upstream of the major TSS. On locating the TSS of *hex1*, an additional intron was identified within the 5′ UTR (see below). Several putative CAAT boxes were also located within this 5′ UTR intron (Fig. 1). There are also three putative AGAA boxes and six putative consensus sites for CREA binding which lie within the 5′ UTR intron yet upstream from the minor TSS (Fig. 1). These sites are involved with promoter regulation by *cis* acting motifs (Mach and Zeilenger, 2003) and metabolism of not readily metabolised carbon sources (Kulmburg et al., 1993), respectively. The CREA sites may contribute to the different pattern of expression seen between cellulase inducing medium and glucose medium (Fig. 5). Within the 5′ UTR intron are also two putative CCAAT motifs (Fig. 1), which have been shown to induce transcription and enhance promoter activity (Narendja et al., 1999; Steidl et al., 1999). The high concentration of putative fungal promoter regulatory motifs suggests the 5′ UTR of *hex1* is important for regulating expression.

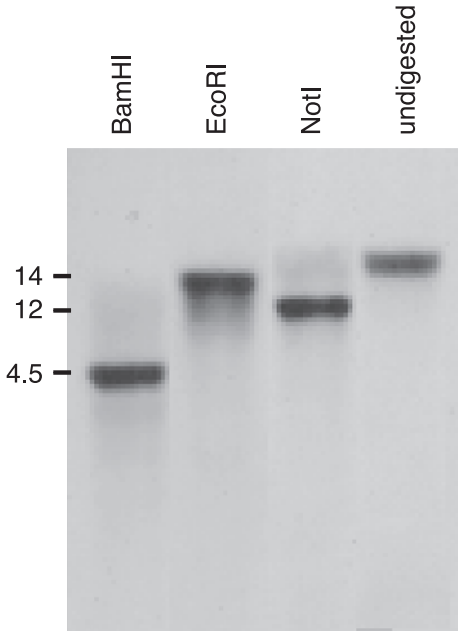

Fig. 2. Southern blotting analysis. Genomic DNA was digested with *Bam*H1, *Eco*RI and *Not*I and hybridized with a DIG-labelled *hex1* probe. The approximate sizes of the bands are shown in kilobase.

Analysis using BioManager (http://bn1.angis.org.au) MEME programme of 48 introns from fungal genes showed conserved features in the *hex1* introns. The conserved splice site GTAHGYT marks the 5′ boundary of both *hex1* introns (Fig. 1). The intron within the coding sequence also contains the highly conserved UACUAAC box, found in *Saccaromyces cerevisiae* (Parker et al., 1987) and *T. atroviride* (Donzelli et al., 2001). This is the recognition site for binding of snRPS that leads to Lariat formation and splicing of the intron.

The presence of introns, containing promoter regulatory motifs, within the 5′ UTR of fungal genes is not unusual. There is a 116-bp intron within the 5′ UTR of *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (Punt et al., 1988), in the endoglucanase III gene of *T. reesei* (Saloheimo et al., 1988) and in the 1,3-β-glucosidase gene from *T. atroviride* (Donzelli et al., 2001). The 188-bp intron within the 5′ UTR of 1,3-β-glucosidase gene from *T. atroviride* is 78 bp upstream of the start codon and contains two putative TATA boxes as well as transcription factor binding sites such as STRE motifs, Cre1 binding elements, conidiation responsive elements, and four CAAT boxes (Donzelli et al., 2001). These 5′ UTR introns are likely to play a role in the regulation of gene expression. Further discussion on the *hex1* coding sequence is included in Section 3.3.

3.2. Transcription start points and sequencing of partial cDNAs

We used RT-PCR to identify the TSS and polyadenylation site of the *hex1* transcripts. This approach resulted in several differently sized products which were sequenced to reveal two general TSS regions and alternative splicing of the transcript resulting in three different isoforms of *hex1* mRNA (Fig. 3). The TSS of several cloned cDNAs was in the range between −327 and −275 upstream of the start ATG. In these cloned cDNAs, the transcript had undergone alternative splicing at the 3′ end of the intron within the coding sequence. Cloned transcript n198 (TSS −288) (Genbank Accession No. AY517641) had a 109-bp intron spliced out and contains the coding sequence for a histidine-rich unconserved region of HEX1 (Fig. 4). The intron spliced from cloned transcript n197 (TSS −285) (Genbank

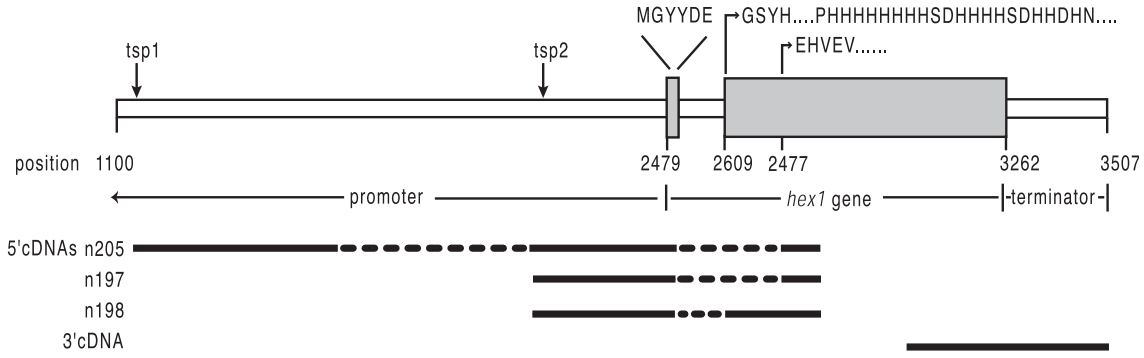

Fig. 3. Structure of the *hex1* gene and partial cDNAs. The three types of *hex1* transcripts identified are represented by the cDNA recombinants n205, n197 and n198, transcribed from two TSSs labeled as TSS1 and TSS2. The positions labeled correspond to those in Fig. 1. The coding region of the gene is shaded and the regions spliced from the transcripts are shown as dotted lines. The protein sequence of exon 1 is given and the two alternative starts to exon 2 are also shown.

Accession No. AY517640) has an alternative 3′ splice site resulting in an extended 243-bp intron that encompasses the histidine-rich coding region. This transcript could code for a protein that excludes most of the unconserved region of HEX1 (see below). The third type of transcript, represented by cloned cDNA n205 (Genbank Accession No. AY517642), is different in that the TSS is at −1337 and it had a 506-bp intron spliced from the 5′ UTR. In *N. crassa hex1*, the TSSs are concentrated 360–420 bp upstream of the start codon with a single intron within the coding region for which no alternative splicing has been reported (Tenney et al., 2000). The *N. crassa* gene layout is most similar to the *T. reesei hex1* transcript type n197.

The three transcript types code for two isoforms of the HEX1 protein in *T. reesei*. One form is 225 aa in length with a predicted M_w of 25 kDa, and the other form is 180 aa in length with a predicted M_w of 19 kDa. The HEX1 proteins identified from *N. crassa*, *M. grisea*, *B. cinerea* and *A. nidulans* all have a highly homologous first exon followed by a variable region that leads into the conserved remainder of the protein (Tenney et al., 2000). The two forms of *T. reesei* HEX1 share this pattern, with the length of the variable region depending on the alternative splicing of the internal intron (Fig. 4). HEX1 from transcript type n198 contains a long histidine-rich stretch. HEX1 from *A. nidulans* and one reported form of *M. grisea* HEX1 (Tenney et al., 2000) also have an extended variable region that is histidine rich (Fig. 4). A *M. grisea hex1* cDNA recombinant also has a 111-bp region spliced from the 5′ UTR of the transcript (Genbank Accession No. AF170544). This transcript codes for HEX1 without the histidine-rich unconserved region and this splicing pattern is similar to *T. reesei hex1* transcript n205. The conservation of this pattern of expression and splicing suggests it has functional importance but it is unknown why these alternative forms occur.

Two *T. reesei* recombinants showed the polyadenylation site to be 243- and 244-bp from the translation stop codon (Fig. 1) (Genbank Accession No. AY517639).

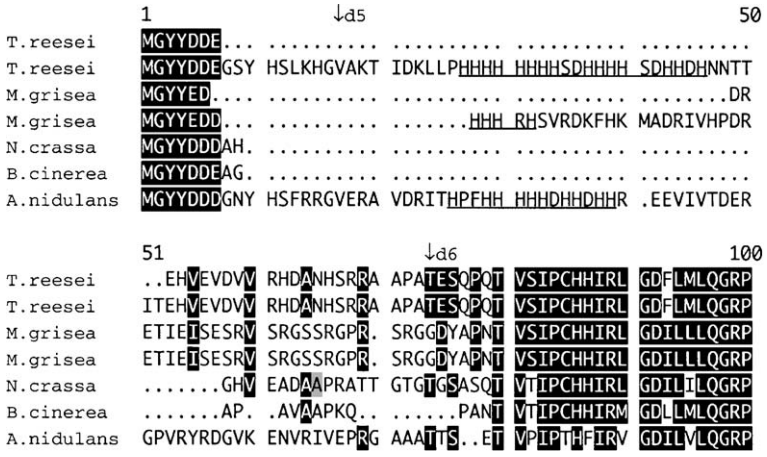

Fig. 4. Alignment of the N-terminus of HEX1 protein from different species, including the alternative isoforms from *T. reesei* and *M. grisea*. This region shows the highly conserved region translated from exon 1 (residues 1 to 7) and the highly variable region from positions 8 to 79. Arrows mark the alternative posttranslational cleavage sites of *T. reesei* HEX1 from spots d5 and d6 (see Section 3.3). Grey highlight marks the N-terminal cleavage site for *N. crassa* HEX1 (Tenney et al., 2000). The histidine-rich regions of *T. reesei*, *M. grisea* and *A. nidulans* are underlined. The regions of similarity are highlighted in inverse font.

3.3. N-terminal sequence of the HEX1 protein

We extracted two spots, which had previously been identified as HEX1, from a 2D gel (Lim et al., 2001) for N-terminal sequencing to identify if they were derived from alternatively spliced transcripts. HEX1 of spot d5 has an approximate p*I* of 6.4 and M_w of 19 kDa. Spot d6 has an approximate p*I* of 6.6 and M_w of 22 kDa. The N-terminus of HEX1 from spots d5 and d6 are shown in Fig. 4. Each spot represented an alternative form of HEX1 with a differently cleaved N-terminus from the translated protein, demonstrating multiple forms of HEX1 are translated from the differentially spliced messages. HEX1 of spot d5 must have been derived from transcript type n198 with post-translational cleavage of approximately 18 residues from the 25 kDa histidine-rich form. HEX1 of spot d6 may have been derived from either type of transcript by cleavage of 28 residues from the 19 kDa HEX1 or alternatively, cleavage of 73 residues from the histidine-rich 25 kDa HEX1. There is no recognized conserved cleavage sequence at these two sites. Nonetheless, this shows that *T. reesei* HEX1 exists in at least two forms due to post-translational cleavage of the N-terminus. *N. crassa* HEX1 is a 19-kDa protein that polymerizes to form Woronin bodies (Yuan et al., 2003), but it also undergoes cleavage of 16 residues from the N-terminus to yield a 17-kDa form of HEX1 with an unknown function (Tenney et al., 2000).

T. reesei HEX1 has a peroxisome targeting signal (type 1, SRL) at the C-terminus which is a highly conserved feature of HEX1 across fungal species (Jedd and Chua, 2000). Since HEX1 is so highly conserved throughout filamentous fungi, the reported mechanism of Woronin body formation in *N. crassa* (Yuan et al., 2003) is likely to be applicable across species. However, *N. crassa* does not have an extended variable region of unusual composition like the histidine-rich stretch in *T. reesei*. This histidine stretch contains eight consecutive histidine residues with another seven histidines in close proximity (Fig. 4) making this region strongly polar and unlikely to be folded within the protein. *A. nidulans* and *M. grisea* HEX1 also have an extended variable region and it is unclear how these HEX1 proteins fit into the structural model described for *N. crassa* Woronin body formation. Direct interaction between residues His39, Arg41, Asp44, Arg68, Glu81, Ser84, Gln49, Ser61, Gln127, Val125, Ile56 and Gln134 of *N. crassa* HEX1 enables crystallization of HEX1 into the dense lattice structure of the Woronin body (Yuan et al., 2003). Residues within the N-terminus of *N. crassa* HEX1 are not directly involved in the intermolecular interactions required for HEX1 polymerization in Woronin body formation, neither does the N-terminus fold into the helices or β-strands of the HEX1 secondary structure (Yuan et al., 2003). An explanation for the poor conservation of the N-terminal variable region of varying length in *T. reesei*, *A. nidulans* and *B. cinerea* HEX1 is that it may not be important for Woronin body formation. Alternatively, since *T. reesei* and *M. grisea* HEX1 are differentially expressed with and without this region it suggests there is a yet unknown functional role for this region.

3.4. Growth related expression of the T. reesei HEX1 gene

Woronin bodies have been identified in hyphae of *A. nidulans* shortly after germination and prior to branching of the hyphae (Momany et al., 2002). In order to explore the expression of *hex1* in relation to growth and culture medium, RNA was extracted from *T. reesei* cultures grown in (i) medium (CLS) supporting the synthesis of secreted enzymes such as the main cellulase, (ii) minimal glucose medium, which typically represses synthesis of enzymes regulated by carbon catabolite repression and (iii) minimal glycerol medium, which is considered a neutral carbon source that has no effect on the expression of cellulases (Ilmén et al., 1997). Northern blotting showed expression of *hex1* to be very high in the first 24 h of growth in CLS medium and very high after 54 h of growth in glycerol medium (Fig. 5A). After these peaks in expression the level of message decreased over time (data not shown). *Hex1* is more rapidly and more intensely expressed at these early time points under the conditions tested as compared to expression of the main cellobiohydrolase gene, possessing one of the strongest

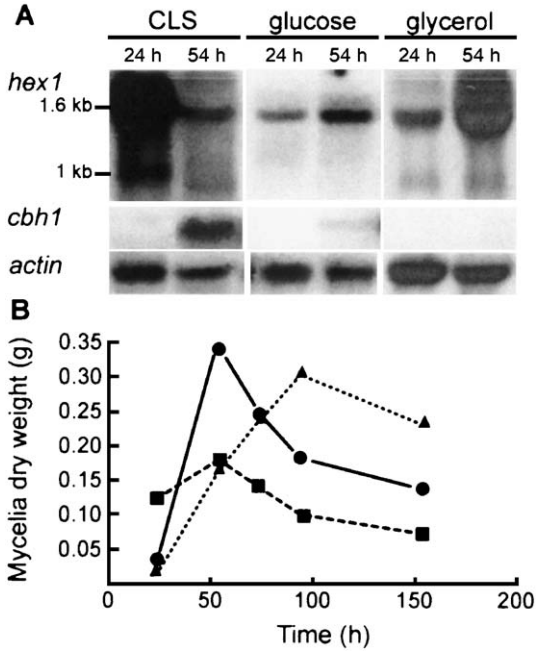

Fig. 5. (A) Expression of *hex1* and *cbh1* after 24 and 54 h of growth analyzed by northern blotting. Total RNA was extracted from mycelia grown in CLS, glucose medium or glycerol medium. The sizes of the alternative transcripts of *hex1* are marked and a Northern blot of actin is shown for the normalization of the expression levels. (B) Growth curve of *T. reesei* in CLS (dashed line), glucose medium (solid line) and glycerol medium (dotted line). Mycelial dry weight is plotted against culture time.

inducible promoters known for filamentous fungi. The high expression of *hex1* at 24 h in CLS and at 54 h in glycerol medium coincides with a period of exponential growth (Fig. 5B). The highest *hex1* expression on glucose was also recorded at 54 h; however, this time point is past the exponential growth phase, therefore, the actual *hex1* expression may have been even higher at some point between 24 and 54 h. The early peak of *hex1* expression reflects the early appearance of Woronin bodies in fungal development (Markham and Collinge, 1987; Momany et al., 2002). Possibly, once the cell has produced sufficient numbers of Woronin bodies the level of *hex1* expression is downregulated.

The dominant *hex1* message expressed in CLS, minimal glucose medium and minimal glycerol medium runs at approximately 1.6 kb (Fig. 5A). This corresponds to transcript n205 with a predicted size of 1617 bp (Fig. 3). This transcript codes for the 19-kDa form of HEX1, without the histidine rich region. A second band on the *hex1* Northern blots ran at approximately 1 kb and was expressed less intensely than the 1.6-kb message on CLS and glycerol media. This 1 kb mRNA transcript coincides with cloned transcript type n197, which also codes for the 19-kDa HEX1 but the TSS is approximately at −300 bp (Fig. 3). These expression results suggest that transcription from each *hex1* TSS is differentially regulated and that each *hex1* transcript type is expressed at a different level. Whilst a 1.2-kb band corresponding to transcript type n198 does not appear on these Northern blots, we have previously detected low expression of this transcript encoding the 25-kDa histidine-rich form of HEX1 in CLS medium at a later stage of cultivation (data not shown).

3.5. Conclusions

In summary, we have isolated and sequenced the single copy *hex1* gene and flanking regions from *T. reesei*. Sequencing of the *hex1* cDNAs has confirmed that different isoforms of *hex1* cDNA transcripts exist which vary according to the transcription start sites and splice sites of introns. *T. reesei hex1* cDNA encodes for at least two forms of HEX1 protein, including a 25-kDa form that has an extended variable region similar to *A. nidulans* HEX1 and one form of *M. grisea* HEX1. The biological role of these alternative forms remains to be explored. The *hex1* gene is strongly expressed within the early stages of growth, thus the promoter shows potential for the time-dependent expression of heterologous gene products in *T. reesei*.

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