Characterization of GFP Expression Driven by Powdery Mildew Promoters in a *Magnaporthe* Host

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ABSTRACT

Powdery mildew is caused by the ascomycete fungus, Blumeria graminis f. sp. hordei (Bgh). Bgh is an obligate biotrophic pathogen which relies on its host for development and completion of its life cycle. The obligate nature of this fungus has hindered attempts to carry out biochemical and molecular biology analysis. Due to this, characterization of Bgh H4 genes which encodes for histone-4 protein involved in chromatin formation and DNA packaging was carried out in Magnaporthe oryzae, a non-obligate ascomycete fungus, pathogenic on rice. A GFP expression vector, under the control of Bgh H4 promoter was transformed in the wild-type M. oryzae strain. 3 independent replicates were obtained from different transformation attempts and were classified as P7.1, P7.15 and P7.16. The GFP expression was characterized at different time-points throughout M. oryzae life cycle on 4 different surfaces; onion epidermis, barley leaves, glass slides and cellulose membrane. Epi-fluorescence microscope was used to determine GFP expression based on its fluorescent intensity. These results were then compared to the experimental control, pMJK27.2, the primary vector construct which expressed GFP under the control of M. oryzae MPG1 promoter. All P7 transformants exhibit low GFP expression compared to the control. Completion of infection cycle was obtained on onion epidermis and barley leaves whereas on glass and cellulose membrane, the growth of transformants halted following the formation of appresorium. Apart from that, malformed features such as long, branched and multiple germ tubes were also observed on glass and cellulose. Generally, a pattern of decreasing in fluorescent intensity was observed in different developmental structures as time progresses, with P7 transformants exhibited varying fluorescent intensities at each time-points. The GFP expression level of P7 transformants at 0, 4, 8 and 16 hours post inoculation on barley leaves and cellulose membrane were also assessed by performing quantitative-PCR. Based on the analysis, the general trend of GFP expression on these surfaces does not appeared to fit the Bgh H4 expression profile gauged by microarray data analysis carried out in the past.

Keywords: *Powdery mildew, GFP, Magnaporthe oryzae, Blumeria graminis*

Introduction

Powdery mildew

Powdery mildew is one of the major diseases that affect plant species, leading to decrease in yield and quality of agriculture. This disease is caused by economically important pathogen; *Blumeria graminis* f. sp. *hordei* (*Bgh*) which belongs to the *Erysiphaceae* family of the ascomycete fungus (Braun, 1986). These pathogen infect more than 10000 plant species worldwide (Agrios, 2005).

Bgh can reproduce sexually and asexually. This asexual phase has been very well studied since it proceeds in a strictly programmed way and synchronous fashion which offer benefits in understanding the mechanism of its development (Figure 1). Although, this asexual infection cycle of *Bgh* develop synchronously through a highly ordered morphogenetic sequence following contact with host surfaces, this typical developmental sequence of the prepenetration processes is impeded on non host plants and artificial surfaces such as glass and cellulose (Zabka *et al.* 2008). For example, on host plant epidermis, appresorium can normally be observed. On cellulose and glass, however, the development is often halted after the formation of appresorial germ tube. Therefore, it is believed that nature of surface determines the germling development (Tucker and Talbot, 2001).

Analysis of expressed sequence tags (ESTs) of cDNA libraries and microarray analysis result in identification of virulence associated genes in *Bgh* expressed at different developmental stages throughout *Bgh* life cycle (Both *et al.* 2005). The transcript profile of *Bgh* H4 is one of the Co-ordinate Mis-Expressed Genes (CMEG), demonstrated the disparity of H4 expression during development on the host plant and upon non-host

artificial surfaces (Figure 2). H4 gene encodes for H4 protein, one of the main histone proteins involved in the structure of chromatin in eukaryotic cells (Brosch *et al.* 2008).

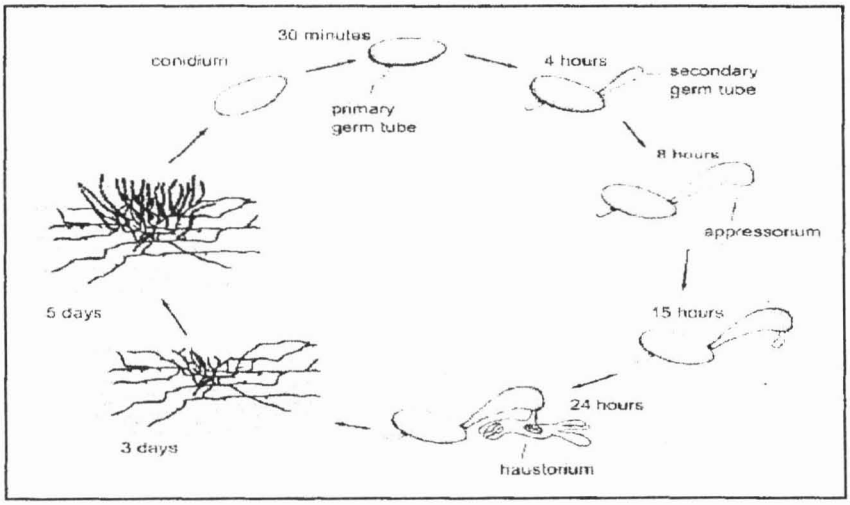

Figure 1: The asexual cycle of *Blumeria graminis* sp. *hordei.* Initial events in the asexual life cycle comprise spore germination, appresorium formation, host cell penetration and the intracellular establishment of its feeding organ, haustorium. Following successful substrate invasion via penetration peg that triggers haustoria development inside the host, the fungal mycelium proliferates on the host and about 5 days post-inoculation onwards, numerous spore carries (conidiophores) emerge to produce massive asexual conidia that are spread to other host plants via the wind (Both and Spanu, 2005).

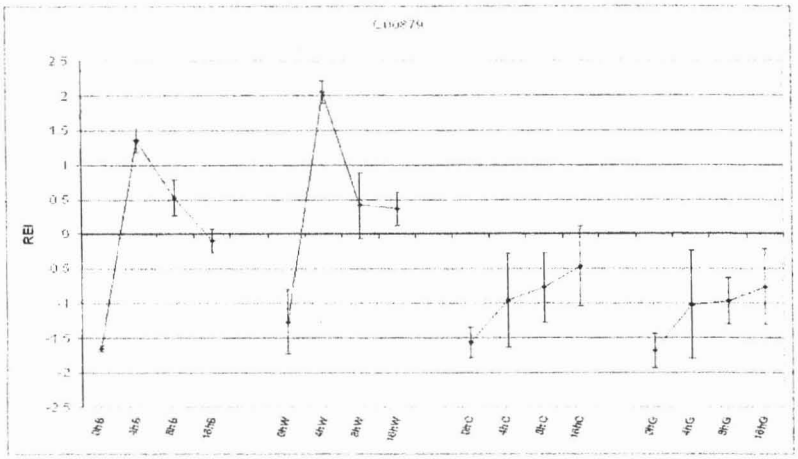

Figure 2: *Bgh* H4 gene expression profiles. The diagram shows relative expression levels of the *Bgh* H4 gene upon barley (B), wheat (W), cellulose (C) and glass (G) surfaces at 0, 4, 8 and 16 hour post inoculation (Both *et al.* 2005).

The studies related to this host-pathogen interation of *Bgh* is however hindered due to the downside of *Bgh* as it exists as an obligate biotroph, which indicates it has lost the ability to grow and complete the life cycle on any other substrate other than their living plant host (Thomas *et al.* 2001). This also means that it is not possible to grow *Bgh* in axenic culture which limits the molecular biology studies can be performed to the process understand its fungal development (Both & Spanu, 2004).

***M. oryzae* – a heterologous expression host**

In order to study *Bgh* fungal development, *M. oryzae*, a nonobligate biotroph was chosen to serve as a heterologous expression host mainly due to its amenability to classical and molecular genetics manipulations, and availability of various genetic resources, genetic maps and cDNA libraries (Valent & Chumley, 1991). *M. oryzae* is a heterothallic ascomycete and the causal agent of rice blast, the most severe diseases of rice throughout the world (Xue *et al.* 2004). In contrast to *Bgh*, *M. oryzae* can easily transformed using abundant of selectable markers available (Talbot, 2002). The advantage of using *M. oryzae* as an expression host is particularly due to the similarity of its life cycle to *Bgh*.

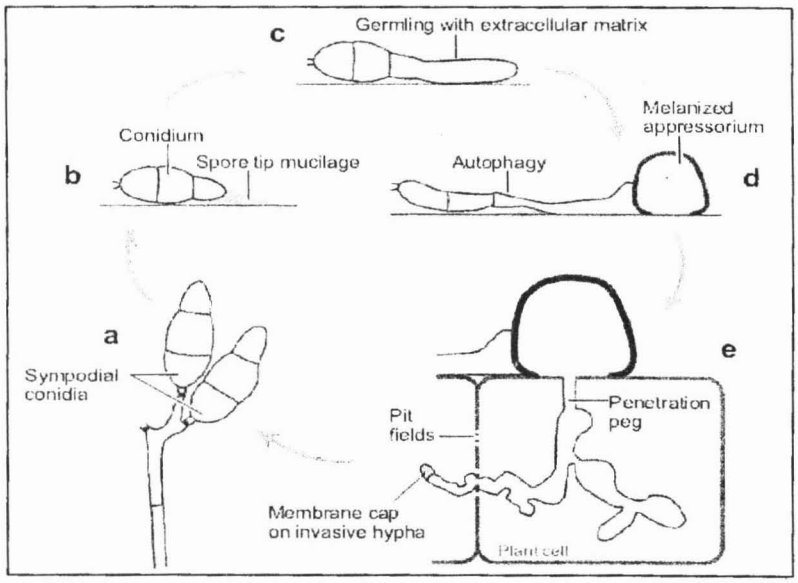

Figure 3: The life cycle of *Magnaporthe oryzae*. (a) Conidia are produced sympodially on aerial conidiophores. (b) Conidia attach to the host surface using mucilage released from the tip of the spores. (c) Conidia germinate and produce a hyphal filament. (d) Formation of melanized appresorium follows with autophagy of conidia and germ tube. (e) Development of penetration peg and host tissue invasion by invasive hyphae. Movement into neighbouring cells occurs at specifically plasmodesmata clusters, pit fields. Invasive hyphae covered with membrane cap that may function in protein secretion into the host cells (Ebbole, 2007).

PEG–mediated protoplast transformation of *M. oryzae* with *Bgh* promoter construct

The plasmid DNA of *Bgh* was introduced to protoplast of *M. oryzae*. This transformation was mediated by polyethylene glycol (PEG). The presence of GFP gene in transformed *Magnaporthe* was detected by polymerase chain reaction (PCR). There were 2 vectors used in this study; pMJK27.2 which performed as a control and pMJK27.2–C00879, derivative of pMJK27.2 plasmid containing *Bgh* promoter from gene C00879 which encodes H4 histone. The vector pMJK27.2 encodes for hydrophobin gene MPG1. Hydrophobin is a small cysteine-rich secreted protein involved in interaction and recognition of host surfaces for appresorium formation and was the first well characterized *M. oryzae* gene (Soanes *et al.* 2002). The tranformants obtained were then used in this study for characterization of the *Bgh* promoter's behaviour by GFP expression. These successful transformants were classified as P7.1, P7.15 and P7.16.

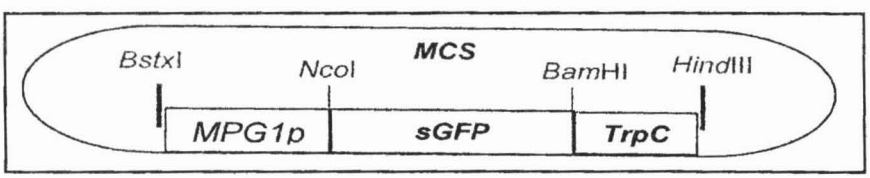

Figure 4: The diagram of pMJK27.2 vector used during *Magnaporthe* transformation. This vector contains a *sGFP* gene with a promoter from the hydrophobin gene *MPG1*, *TrpC* terminator and a Hygromycin B resistance

gene. Whereas, pMJK27.2–C00879 have promoter from *Bgh* H4 histone gene replacing the MPG1 gene promoter (Simpson, 2007).

In this study, characterization of *Bgh* H4 gene transcript dynamics in different developmental stages was assessed in transformed *M. oryzae* strains expressing sGFP reporter gene under the control of *Bgh* H4 promoter (C00879). The characterization of this gene involved microscopy work and qPCR, both aim to detect relative expression of this gene on different surfaces; onion and barley (inductive surfaces) and glass and cellulose (non-inductive surfaces). The level of *Bgh* H4 gene expression depicted by fluorescent intensity was visualized by epi-fluorescence at different stages of *M. Oryzae* life cycle and gene expression level was quantified by qPCR. These findings were then used as a benchmark upon confirming or rejecting the hypothesis that expression level of *Bgh* promoter regions within transformed *M. oryzae* are similar to its development in *Bgh* on barley and other surfaces.

Materials and Methods

Characterization of *Bgh* promoters behaviour within transformed *M. oryzae* on different surfaces by assessing GFP expression via microscopic visualization

GFP expression in *M. oryzae* transformants were assessed in terms of fluorescent intensity by using epi-fluorescence microscope. The *Bgh* promoters behaviour was initially observed by performing mycelia crushes. A small piece of fungal colony was pressed against glass slide. The slide was viewed using an epi-fluorescence microscope. This observation indicated its behaviour at 0 hour. To further analyze development and GFP expression of transformants at 4, 8, 16 and 31 hour post inoculation, four surfaces; onion, glass, cellulose membrane and barley leaves were utilized as a substrate for germination test. For each time-point, 2 weeks old colonies were rubbed in 100 µl of filtered sterile water using a sterile L-shaped spreader. Serial dilutions were performed to deduce uniform spore inoculums in each sample. On each substrate, 100 µl solutions was added and allowed to germinate at 25°C under light. After incubation, approximately 10 spores were assessed for its germination states and GFP expression. Substrates were placed on microscope glass slides, covered with cover slip and viewed under epifluorescence microscope (Zeiss Axioscop 2 plus microscope with HAL-100 fitting). Brightfield and fluorescent images were captured with 'Zeiss Axiocam Fitting camera'.

Quantification of GFP expression within transformed *M. oryzae* by qPCR (Quantitative PCR)

Collection of fungal RNA

Barley and cellulose were utilized as substrates to collect fungal RNA at 0, 4, 8 and 16 hour post inoculation. 3 independent replicates were assembled for each substrate at all time-points. Preceding inoculation, fungal spores were collected by gently rubbing the upper surface of transformed *M. oryzae* cultures and suspended in sterile distilled water. A spore suspension of 2.5 X 10^6 spores/ml was sprayed on 1 week old barley pot. While for cellulose, 2.5 X 10^4 spores/ml were sprayed on 3 trays of cellulose. At 0, 4, 8 and 16 hour post inoculation, the surfaces were dipped in 5% (w/v) cellulose acetate in acetone. After drying, the cellulose acetate was stripped off and stored at -80°C.

RNA extraction and purification, cDNA creation and qPCR analysis

Isolated fungal material was ground in liquid nitrogen. 3250 µl 4M GTC (Guanidine thiocyanate), 3250 µl saturated phenol (pH4.5) and 7 µl mercaptoethanol were added to the fungal matter in 50 ml polypropylene tube. The mixture was thoroughly vortexed for 5 min. 0.2 volumes of chloroform was added and mixed. The mixture was then centrifuged at 4000 g for 25 min (4°C). The top phase was transferred to a new tube; an equal volume of chloroform was added, mixed and centrifuged again for 4000 g at 25 min (4°C). Subsequently, the top phase was added with 3M sodium acetate pH5.2 to 0.3M final concentration and precipitated with an equal volume of isopropanol. The mixture was then left at -20°C overnight. After incubation, the mixture was centrifuged at 4000 g for 30 min (4°C). The supernatant was discarded and spin again shortly to collect all liquid. The remaining liquid was discarded and the pellet was air-dried for 10 min. The pellet was eluted with 70 µl diethyl-pyrocarbonate (DEPC) water. Next, the RNA collected was purified using AGENCOURT® RNA CLEAN™ kit following manufacturer's protocol (Beckman Coulter). The purified RNA then converted to cDNA using

Superscript™ III Reverse Transcriptase. The cDNA used as a template in qPCR reaction. The qPCR analysis was performed following manufacturer's protocol (Platinum® Quantitative PCR SuperMix-UDG with ROX dye). qPCR reactions were performed upon ABI systems 7500 real time PCR system. All analysis was carried out using Applied Bio-system Fast System SDS software version 4.1 and Microsoft Excel©. All primers used in qPCR were designed using Primer3 Input™ and manufactured by Sigma-Genosys Ltd. The primers used were as follows:

Number	Primers designation	Targeted gene encodes	Product size	Primer sequence
1	MagC00479	60S ribosomal protein L18	186bp	**Left:**GTTCTCCGTCGGTTGCTCGT **Right:**CTTGGCGTCCATCTCGTCCT
2	MagdCont1	Oxidoreductase gene	211bp	**Left:** TTCAGGCCGCCTTACACCAT **Right:**CGATGTCGTAGCGGGTTGTG
3	GFP	GFP	180bp	**Left:**CGGCAAGCTGACCCTGAAGT **Right:**AAGATGGTGCGCTCCTGGAC

Table 1: The properties of qPCR primers designed using Primer3 Input™ to amplify fungal RNA.

Results and Discussion

Characterization of *Bgh* promoters behaviour within transformed *M. oryzae* on different surfaces by assessing GFP expression via microscopic visualization

In this study, the primary focus is to characterize the GFP expression under control of *Bgh* H4 promoter in three independent *M. oryzae* transformants (P7.1, P 7.15 and P7.16). The majority of transformants were fluoresced in contrast to wild type when viewed with an epi-fluorescence microscope. However, fluorescent intensity was varied amongst the tranformants at all time-post inoculation on different surfaces. These fluorescent intensity observed indirectly depicted the expression level of *Bgh* H4 gene and *MPG1* gene within transformed *M. oryzae*; an increase in fluorescent intensity observed within fungal structure indicate the expression level of gene was also increased.

As an experimental control, PMJK27.2 expressing GFP under control of *M. oryzae* MPG1 promoter was also assessed. At 0 hour, the spores and some parts of hyphae of all transformants fluoresced at 0 hour. However, fluorescent intensity is different among the transformants. The fluorescent intensity of PMJK27.2 transformants are stronger compared to P7 transformants. Among of the P7 independent replicates, P7.16 appears to have the highest GFP expression, followed by P7.15 and P7.1 respectively.

At 4 hour post inoculation on barley and onion, most all of the germinating spores displayed high intensity of GFP expression. This observation correlates to previous experiment performed by Soanes *et al.* (2002) where he observed a strong *MPG1* expression in conidia at early stages of development. Similarly, high levels of H4 expression were also observed. This gene is essential in transcriptional activation of genes in MAP kinases signaling cascades or cAMP and PKA signaling pathway which play a role in surface signal perception (Both and Spanu, 2004). However, this observation was slightly delayed on glass and cellulose where high intensity of GFP expression spotted only at 8 hour post inoculation. This has been previously described by Simpson (2007) where the growth of *Bgh* on glass and cellulose was slightly impeded.

During appresorial development, high GFP intensity observed within developed appresorium compared to the spore (Figure 5a). The migration of fluorescence from spore to appresorium resembled to process termed autophagy in the life cycle of *M. oryzae*. In this process, conidia undergo cell death whereby cytoplasmic contents are drained to germ tube and appresorium and shifting carbon to appresoria to developed turgor pressure (Veneault-Fourrey and Talbot, 2007). The intense appresorium fluorescent presumably attributed to the accumulation of cytoplasmic nutrients in the structure to developed high turgor pressure for penetration into the host. Completion of infection cycle was achieved on barley and onion membrane with the formation of invasive hyphae, whereas on cellulose and glass, the growth halted following appresorium formation. Development on cellulose and glass surfaces progressed slowly with appresoria developing at 16 hours time-point post inoculation and malformed germ tubes were observed, in which the germ tubes were long, multiple and branching (Figure 5c and 5d).

The behaviour of these transfomants was similar to the developmental growth observed from the wild type *M. oryzae*. However, at this later stage it was hard to localize and characterize GFP intensity. This was due to the half life of GFP and interference with autofluorescence background. At 31 hour post inoculation, invasive hyphae formed on onion and barley exhibit low fluorescent intensity and partially masked by the strong autofluorescence from barley and onion membrane (Figure 5b). GFP fluorescence did not seem to migrate into this structure. Weakness in GFP signals might due to its half life, recently reported to be about 18 hours (Kavita and Burma, 2008).

On the whole, fluorescence of P7 transformants were weaker compared to PMJK27.2 on all surfaces. The difference in the intensity of GFP could be due to mutational effect resulting from transformation. Since homologous recombination rates in *M. oryzae* is low, therefore DNA mediated transformation could results in random integration of the plasmid construct in the genome. Thus the level of GFP expression in each transformants might be affected by the position where plasmid integrated or by the copies number integrated in the genome (Soanes *et al.* 2002). Generally, as time progressed, the similar trend of decreasing in GFP expression observed in all the transformants possibly indicating the reduced of *H4* and *MPG1* gene expression level.

Quantification of GFP expression within transformed *M. oryzae* on different surfaces by qPCR

The level of GFP expression was assessed using qPCR. Prior to that, the RNA purity was investigated following spectophotometric analysis. The value of A260/A280 that was not ranging in between 1.8-2.0 indicated the presence of carryover contaminants during phenol-chloroform RNA extraction. Apart from that, the low RNA yield obtained in some samples might result from several possibilities. First of all the spore suspension of 2.5 x 10^6 spores/ml used to inoculated on barley leaves might be too low. However, 2.5 x 10^4 spores/ml spore suspension inoculated on cellulose seems to work well giving good RNA yield.

In general, the qPCR was successfully performed. However, there are some samples with disparity in the average of primers disscociation temperature (T_M) value indicating the impurities of cDNA created from the RNA samples. This also might due to lack of cDNA presence in the plate as a result of pipetting error. There were 3 primers used in qPCR which consisted of 2 control primers and 1 GFP primers. The 2 control primers, MagCont1 and MagD00725 encoded oxidoreductase and GCIP-interacting protein P29 respectively. They were chose as a control since this gene is expected to constantly express throughout development.

The analysis of qPCR shows successful amplification of sGFP products of fungal RNA matters. The scattergraphs show relative sGFP expression of P7.1 (Figure 6a), P7.15 (Figure 6b) and P7.16 (Figure 6c) at 0, 4, 8 and 16 hour post inoculation on barley leaves. For P7.1 transformants, the curve is relatively flat with a significantly decrease of GFP expression at 8 hour post inoculation. As for P7.15, there was a drop of GFP expression at 4 hour post inoculation and started to increase at 8 and 16 hour post inoculation. The GFP expression of P7.16 was not understandable as there was no RNA samples obtained at 8 hour post inoculation. Overall, the *Bgh*'s H4 gene expression levels within transformed *M. oryzae* displayed by the GFP is not similar to its expression in *Bgh* upon barley gauged by microarray data (Figure 2).

While on cellulose membrane, P7.1 and P7.15 transformants show a similar trend in which there is fluctuation in GFP expression observed at all time-points (Figure 6d and 6e). As for P 7.16, highest GFP expression spotted at 4 hour post inoculation with a significant drop at 8 hour post inoculation. However, the curve shows an increasing of GFP expression at 16 hour post inoculation (Figure 6f). This GFP expression trend of transformed *M. oryzae* which indirectly show the *Bgh* H4 gene expression is not similar to expression profile of H4 gene within *Bgh* upon cellulose that demonstrates a constantly increase in gene expression at 0 to 16 hour post inoculation (Figure 2).

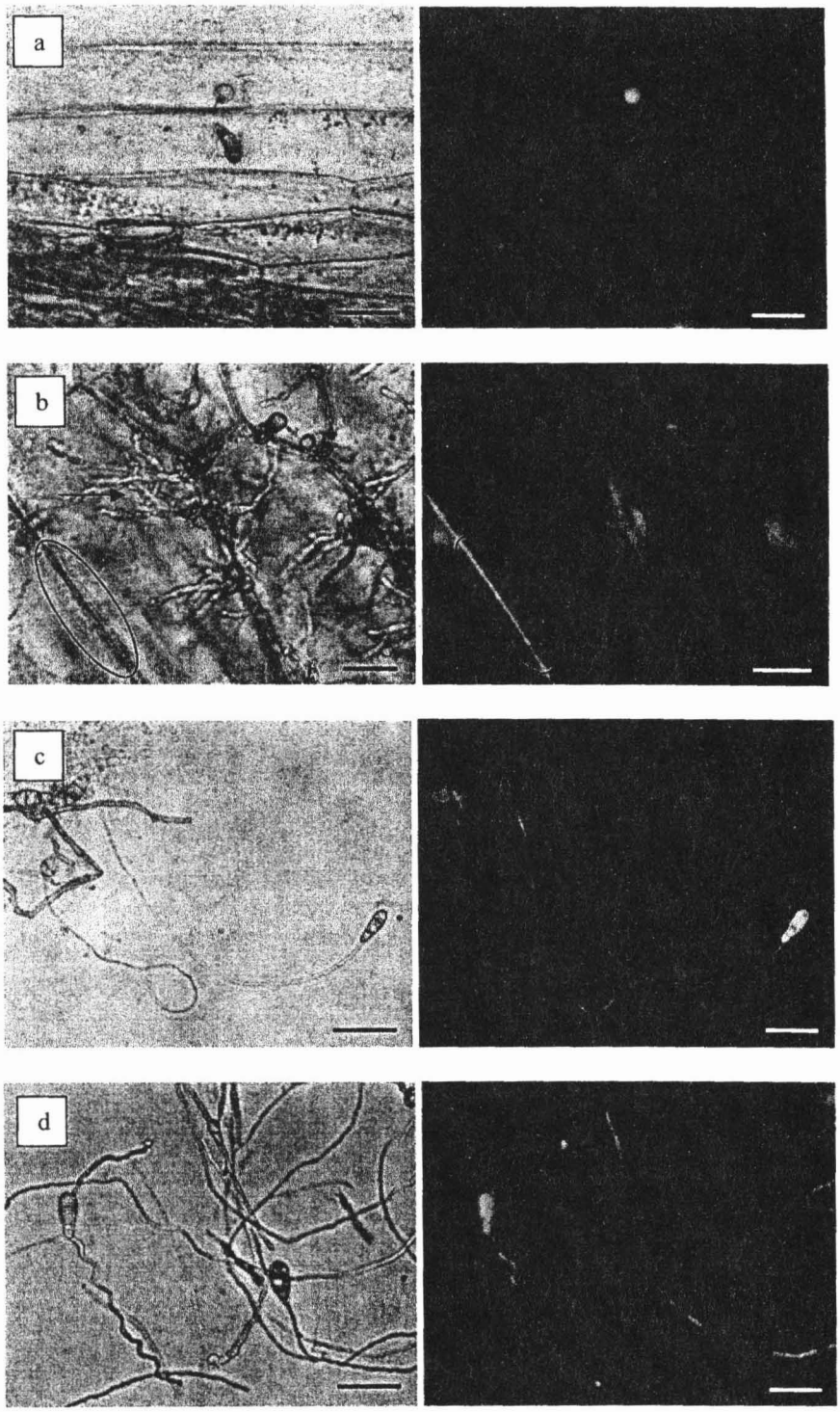

Figure 5: The developmental growth and level of GFP expression in *M. oryzae* transformants. Bright-field and epi-fluorescent images were captured. (a) P7.1 transformants on barley leaves at 8 hour post inoculation. (b) P7.15 transformants on onion membrane at 31 hour post inoculation. The arrow shows fluoresced invasive hyphae and the area in the oblong shape indicates there is an autofluorescence background from the onion membrane. (c) PMJK27.2 transformants on glass surface at 16 hour post inoculation. (d) P7.16 transformants on cellulose membrane at 4 hour post inoculation. (Scale bar: 10 μm)

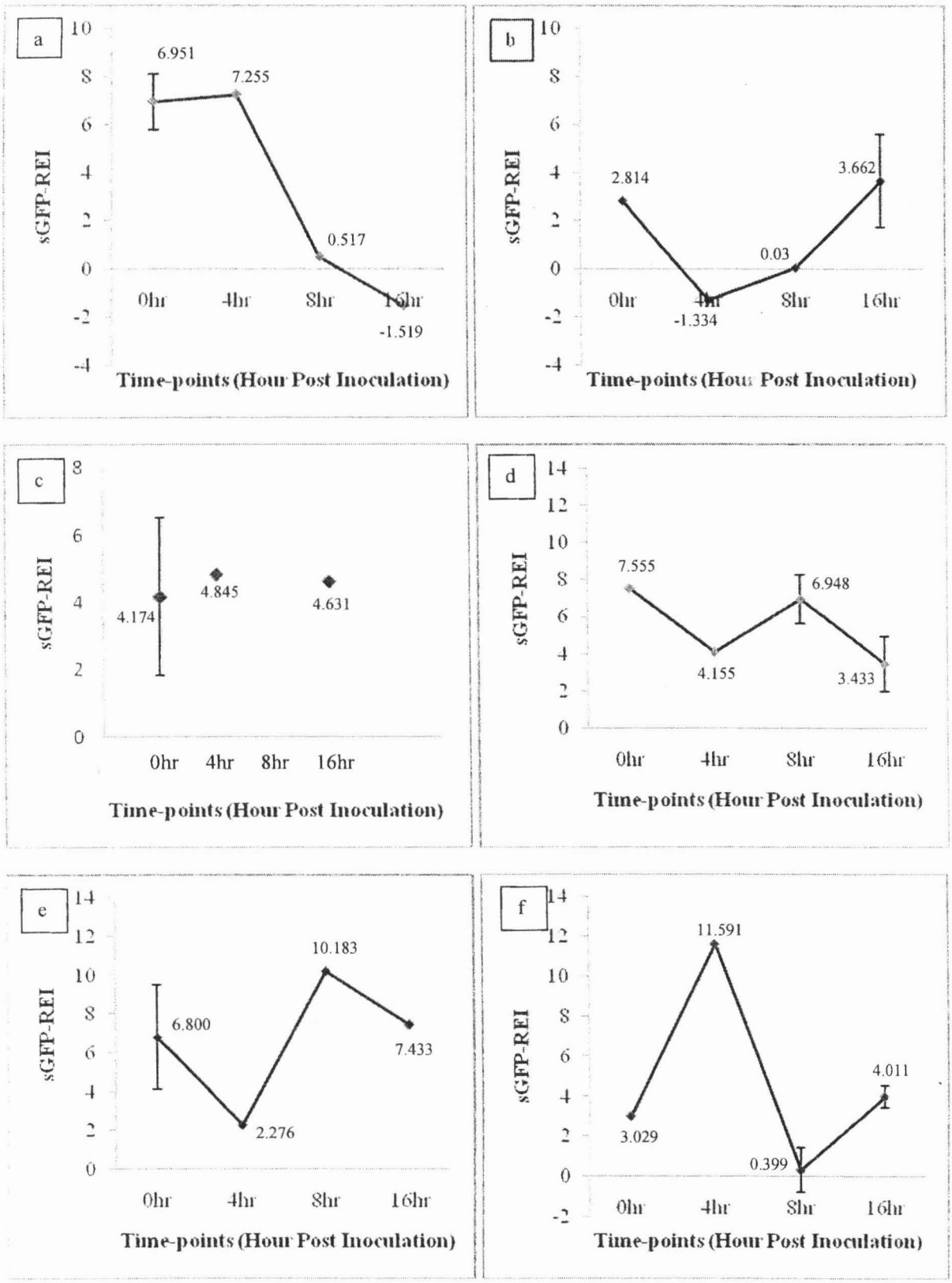

Figure 6: The relative sGFP expression of P7.1 (a), P7.15 (d) and P7.16(c) transformants at 0, 4, 8 and 16 hour post inoculation on barley and also the relative sGFP expression of P7.1 (d), P7.15 (e) and P7.16(f) transformants at 0, 4, 8 and 16 hour post inoculation on cellulose.

Conclusion and Recommendations

To conclude, *Bgh* H4 gene expression level within transformed *M. oryzae* during development does not appeared to be similar with its expression in *Bgh* as shown by the difference expression profiles obtained from qPCR analysis and microarray data carried out in the past. Besides, the GFP expression trend observed by epi-fluorescence microscopy was not corresponded to the microarray data analysis. Therefore, it can be concluded that these genes are not properly expressed within transformed *M. oryzae* compared to its original host, *Bgh*. This might due to the transcriptional system of *M. oryzae* that does not recognize the *Bgh* H4 gene promoter with the same efficiency performed within *Bgh*. There are many possibilities leading to this result, in biological context, there might be a mutational effect resulting from transformation while based on the technical perspectives, there might be some experimental errors occurred during the study such as pipetting errors, operation failure of growth room and others. Therefore, this result indicates an optimization is required for this study before rejecting the hypothesis that expression level within transformed *M. oryzae* is similar to *Bgh*. For future work, RNA collection of fungal matters should be designed properly by using an effective spore concentration to prevent self inhibition during their development on different surfaces. Apart from that, downstream processes following transformation should be carried out to determine gene localization and integration copy number to verify that there is no mutational effect resulted from the transformation.

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