HIGH-EFFICIENCY TRANSFORMATION OF THE PLANT PATHOGENIC FUNGUS *MARSSONINA BRUNNEA*

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SUMMARY

Marssonina brunnea is the causal agent of Marssonina leaf spot of poplar. We successfully transformed *M. brunnea* using *Agrobacterium tumefaciens*. A highly-efficient promoter was a key factor in the construction of this transformation system construction. The beta-tubulin gene is stable and highly expressed at almost all stages of the life history of *M. brunnea*. A beta-tubulin gene promoter was obtained based on a genomic sequence using an adapter ligation-mediated PCR. This was then inserted into the pMDC83 plasmid vector for high expression of the inserted gene. A genomic DNA PCR assay and fluorescence observation of transformants authenticated successful transformation and fluorescence intensity indicated the efficacy of the cloned promoter. All *M. brunnea* transformants were mitotically stable. This study provides essential information for the study of the functions of virulence genes and their roles in pathogen-host interactions.

Key words: Agrobacterium tumefaciens-mediated transformation, full length cDNA cloning, green fluorescent protein expression, promoter cloning

INTRODUCTION

Poplars, one of the most important and widely planted woody species in the world, are subject to a number of diseases that affect their productivity, among which Marssonina leaf spot caused by *Marssonina brunnea* (Erickson *et al*., 2004). Plants infected by this fungus show many small brown spots on the leaves and young stems, followed by premature defoliation (Newcombe and Callan, 1997; Cheng *et al*., 2010). In China, *M. brunnea* occurs as two differently specialized strains, i.e. *M. brunnea* f. sp. *monogermtubi* and *M. brunnea* f. sp. *multigermtubi* (Han *et al*., 2000). Although these two forms are widely distributed and cause great economic loss each year, the mechanisms underlying their infection and the role of virulence genes remain unclear. The lack of an efficient transformation system for *M. brunnea* is a key factor in this lack of knowledge as it would have a pivotal role in elucidating the function of the fungal genes involved in pathogenicity and would also provide a useful molecular tool for study of the interaction between the fungus and its host (Liu *et al*., 2010).

Among the currently available protocols for fungal transformation, the one mediated by *Agrobacterium tumefaciens* (ATMT) is a widely used, simple and reproducible strategy for integrating *A. tumefaciens* T-DNA into the recipient's genome. Many transformation systems for plant pathogenic fungi have been established through ATMT, including those for *Magnaporthe grisea*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Guignardia citricarpa* and *Rosellinia necatrix* (Mullins *et al*., 2001; Rho *et al*., 2001; Rolland *et al*., 2003; Weld *et al*., 2006; Figueiredo *et al*., 2010; Kano *et al*., 2011). As reported in the present paper, a similar approach was used for the first time for the transformation of *M. brunnea*.

MATERIALS AND METHODS

Strains and growth conditions. The *M. brunnea* f. sp. *multigermtubi* wild-type strain was isolated from *Populus canadensis*, cultured and maintained at 28°C in potato-dextrose agar medium (PDA) (Cheng *et al*., 2010). *A. tumefaciens* LBA1100 was kindly provided by Professor A.F.J. Ram from Leiden University.

Full length cDNA cloning of the *M. brunnea* beta-tubulin gene. Total RNA was extracted from *M. brunnea* mycelium from liquid cultures using the Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. When peptide sequences of the beta -tubulin gene of *Aspergillus clavatus*, *Botrytis cinerea*, *Sclerotinia sclerotiorum* were downloaded from NCBI and compared using ClustalX2, some conserved regions were found (Fig. S1). Forward primers (Table 1: Beta-tub forward outer and Beta-tub forward inner) used for 3' rapid-amplification of cDNA ends (RACE) were designed using the CODEHOP

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designer based on these conserved regions (Rose *et al.*, 1998). A 3' RACE PCR was performed using a hotstart *Taq* DNA polymerase (Takara, Japan). PCR products were collected and inserted into pMD19-T vectors (Takara, Japan), then custom sequenced (Genscript, China). Reverse primers used for 5' RACE were designed based on the sequencing results (Table 1: Beta-tub reverse outer and Beta-tub reverse inner) and the 5' RACE PCR was done following the manufacturer's instructions (Takara, Japan).

Cloning and analysis of the tubulin gene promoter. Prior to the cloning of the promoter, *M. brunnea* DNA was amplified to obtain the genome sequence of the beta-tubulin gene, which was found and cloned using an adapter ligation-mediated PCR method (O'Malley *et al.*, 2007). DNA was first extracted using a commercial extraction kit (Qiagen, Germany), then digested and ligated to adapters consisting of long and short strands (Table 1: Long strand of adapter *Hind*III and short strand of adapter *Eco-R*I), The short strand will ligate to the DNA after the two strands of the adapter are hybridized. The adapters were prepared according to O'Malley *et al.* (2007). Primers were designed to amplify the upstream region of the beta-tubulin gene using the adapter primer pairs (Table 1: AP1 and AP2 as forward primers, Beta-tub reverse outer and Beta-tub reverse inner as reverse primers). Finally, after sequencing the PCR products, the upstream region of the beta-tubulin gene was obtained and the promoter elements were analyzed using the TFSEARCH program (threshold score >90) http://www.cbrc.jp/research/db/TFSEARCH.html (Heinemeyer *et al.*, 1998).

Vector construction. The vector described in this study was constructed on the backbone of pMDC83 (Curtis and Grossniklaus, 2003). A fragment containing the hygromycin B-resistance gene under the beta-tubulin promoter was obtained using overlapping PCR. Beta-tub- forward (*Ase*I) and Beta-tub-hyg- reverse were used to amplify the promoter, Beta-tub-hyg- forward and Hyg reverse (*Ase*I) were used to amplify hygromycin B-resistance gene. Beta-tub- forward (*Ase*I) and Hyg reverse (*Ase*I) were used to obtained an overlapping fragment with *Ase*I sites at both end sequences (Heckman and Pease, 2007). This was then ligated to pMDC83 at the *Ase*I site instead of the hygromycin B-resistance gene and the 35S promoter which promotes the transcription of the hygromycin B-resistance gene. In addition, the 35S promoter upstream of the attR site was also replaced by the beta-tubulin promoter at *Hind*III and *Xba*I sites.

Transformation method. Standard protocols with modifications (Rolland *et al.*, 2003; Weld *et al.*, 2006) were used for growth medium and bacterial cultures. A fresh colony of *A. tumefaciens* LBA1100 containing modified binary vector was cultured overnight at 28°C in liquid Luria-Bertani (LB) medium supplemented with 25 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin. The culture was then diluted (1:100) into a minimal medium (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 µM $FeSO_4$, 4 mM NH_4SO_4, 10 mM glucose) supplemented with 25 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin and incubated overnight at 28°C. *A. tumefaciens* cells were diluted to an optical density at 660 nm (OD_{660}) of 0.15 in induction medium (IM) [minimal medium, 40 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.3, 0.5% glycerol (w/v), 200 µM acetosyringone] and cultured at 25°C for 4-6 h with gentle shaking. For co-cultivation, approximately 10^6 conidia of *M. brunnea* were collected and plated onto PDA on the surface of cellophane sheets, and kept at 23°C for two days before transformation. When transformation was carried out, the cellophane was covered with *ca.* 200 µl induced *A. tumefaciens*, the cells were transferred to IM, and were co-cultivated at 23°C for two days in the dark. The cellophane sheets were transferred onto PDA supplemented with hygromycin B (10 g ml^{-1}), cefotaxim (150 µM) and timentin (150 µg ml^{-1}).

Transgene stability in *M. brunnea*. Transgenic colonies selected on PDA containing antibiotics were propagated on the same medium without antibiotics. After incubation at 23°C for 12 days, the colonies were transferred back onto the selective medium. This procedure was repeated three times.

DNA isolation and PCR assays. To detect the hygromycin B-resistance gene in the hygromycin B-resistant isolates, PCRs were performed on the DNA of isolates using the primers specific to the hygromycin B-resistance gene. PCR products were visualized on a 1.5% agarose gel by electrophoresis, then recovered and custom sequenced.

Expression analysis of reporter green fluorescent protein. The green fluorescence emission associated with green fluorescent protein (GFP) was detected using a Leica UV microscope (DMKLB or MZFL111) with the following filter settings: 488 nm excitation and 515 nm emission. All the primers used in this study are listed in Table 1.

RESULTS AND DISCUSSION

Cloning of the *M. brunnea* beta-tubulin gene and its promoter. The expression level of a gene is closely related to many cis-regulatory elements in its upstream promoter and many trans-acting elements. A high efficiency promoter is thus a key factor in a transformation system. The promoters that belong to a particular fungus can better cooperate with its own trans-acting elements, and so we selected the promoter of *M. brunnea* beta-tubulin as a housekeeping gene with great stability and high promotion efficiency.

As a the first step of promoter cloning, the sequence of the beta-tubulin gene was obtained By comparison of

the beta-tubulin gene sequences of three fungal species, two conserved regions were found and selected as candidates for primer design. A method called CODEHOP (Cheng *et al*., 2010) was used to design degenerate primers consisting of a core region and a clamp region. The core region of the nucleotide (nt) sequence of the primer encodes 3-4 conserved amino acids (aa) and determines the specificity of the primer while the clamp region plays a role in stabilizing annealing with the template. Thus, a degenerate PCR can succeed as long as the core region amino acids are correct (Rose *et al*., 1998). This method greatly improves the success of homologous cloning. The full-length cDNA fragment of the beta-tubulin gene was then cloned and sequenced using RACE (GenBank accession No. JN967643). The *M. brunnea* beta-tubulin gene is 1598 bp long and comprises a 1341 bp open reading frame that encodes a protein of 447 aa with a theoretical molecular mass of 49.2 kDa. The DNA sequence of this gene was obtained by PCR using *M. brunnea* DNA as a template. As a result, five introns were found (Fig. S2), and the primers designed for promoter amplification were in the upstream of the *Eco*RI and *Hind*III restriction sites. Adapter ligation-mediated PCR were introduced here for promoter cloning as it was used to identify T-DNA insertional mutants in the Arabidopsis genome (O'Malley *et al*., 2007). Finally, the beta-tubulin promoter, a 780 bp fragment, designated *pTubulin*, was cloned by genome walking method (Fig. S3). Promoter analysis showed that it had many transcription factor-binding sequences.

***A. tumefaciens*-mediated transformation of *M. brunnea*.** The beta-tubulin promoter and the hygromycin B-resistance gene were fused by overlapping PCR. Overlapping PCR has been used for site-directed mutagenesis and gene splicing (Heckman and Pease, 2007). Here we ligated the promoter and hygromycin B-resistance gene using overlapping PCR instead of restriction ligation. The overlapping fragment was inserted into in the *Ase*I site of the pMDC83 vector. Thus, the vector with a hygromycin B-resistance gene promoted by a native beta-tubulin promoter was constructed (Fig. 1a).

M. brunnea has a very low tolerance to hygromycin B, in this it differing from most fungi, thus a relatively lower concentration of hygromycin B should be used. It is worth noting that conidia that have just been produced can hardly to grow in any medium containing hygromycin B. Thus, *M. brunnea* conidia were incubated on PDA at concentrations of 1, 5, 10, 15, and 25 μg hygromycin B ml^{-1}, for determining the minimum inhibitory concentration.

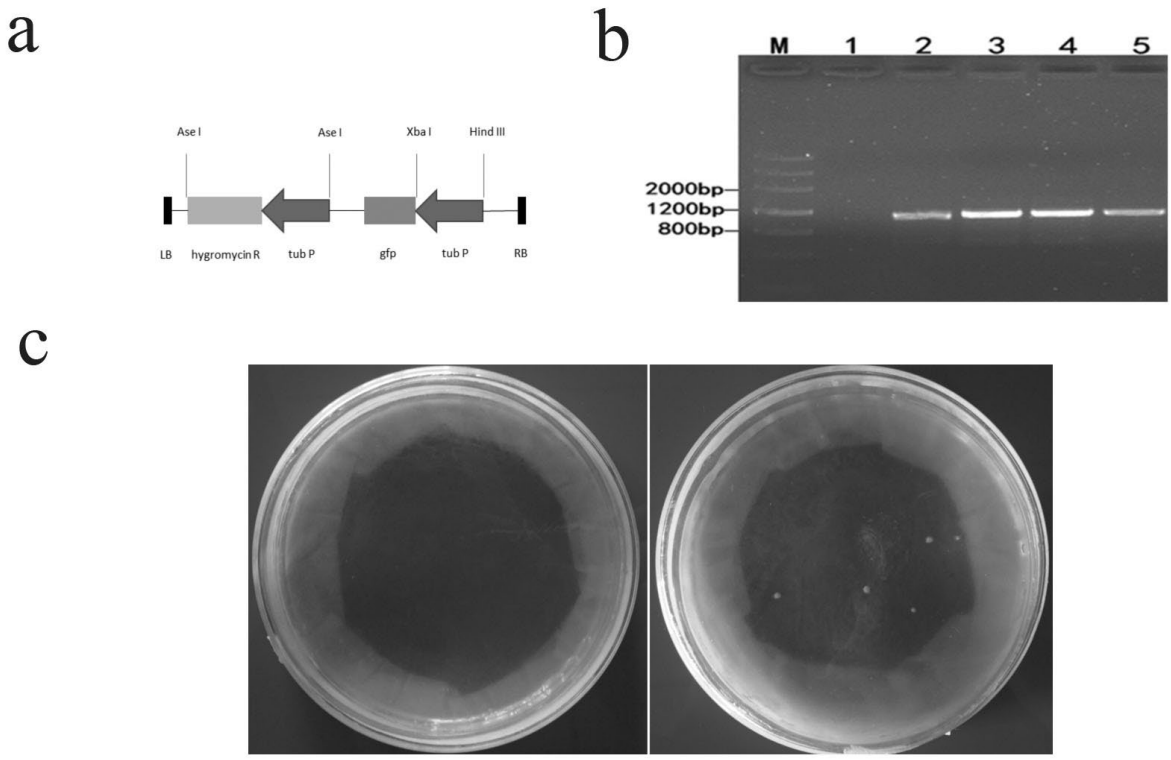

Fig. 1. Vector construction, *Agrobacterium tumefaciens* mediated transformation of *M. brunnea*. a. The re-constructed vector. b. Detection of *M. brunnea* transformants. PCR products amplified using primers specific for the hygromycin B resistance gene using DNA as templates which isolated from wide-tpye *M. brunnea* and four of the *M. brunnea* transformants, 1050 bp is the expected PCR product size. Lane 1: Tiangen Marker III. Lane 2: wide-tpye *M. brunnea* DNA as template. Lanes 3-7: *M. brunnea* transformants DNA as templates. c. Transformants of wild-tpye (left) and transgenic *M. brunnea* (right) on selection medium.

We selected the concentration of 10 µg hygromycin B ml^{-1} because when the hygromycin B concentration was increased to 10 µg ml^{-1} , the conidial growth was completely suppressed. Of the three *A. tumefaciens* strains (GV3101, EHA105, LBA1100) used for transformation only LBA1100 yielded positive transformants.

M. brunnea is a slow-growing fungus and controlling growth of *A. tumefaciens* was a challenge. Cefotaxim and timentin are two antibiotics of common usage and are used separately in most experiments (Rolland *et al.*, 2003; Weld *et al.*, 2006). As we found each of these two antibiotics failed to suppress the growth of *A. tumefaciens*, they were mixed and added to the growth medium thus obtaining complete suppression of bacterial growth. T-DNA containing the hygromycin B-resistance gene in the pMDC83-hyg-gfp binary plasmid was transferred to cells of *M. brunnea* which allowed them grow on selective medium, whereas the negative control was unable to grow. Totally, 27 hygromycin B-resistant colonies were obtained from three independent transformation. The transformation frequency of *M. brunnea* (approximately 10 transformants/10^6 conidia) was consistent with the experiments carried out with other fungal species. *B. cinerea* and *S. sclerotiorum*, for example, are close to *M. brunnea* in DNA sequence, and their transformation frequencies were similar to that of *M. brunnea* (Rolland *et al.*, 2003; Weld *et al.*, 2006).

In the course of the present study several efficient modifications were developed to improve the transformation efficiency of *M. brunnea*. First, the addition of a pre-germination step and the extension of the co-cultivation period had a positive impacs on the transformation of *M. brunnea* which grows slowly. This is consistent with previous studies that have proposed that a two-day or longer co-cultivation period was beneficial for maximizing the transformation efficiency of slow-growing fungi (Mullins *et al.*, 2001). Moreover, it was found that acetosyringone, a compound that induces the expression of virulence genes, was necessary for successful transformation (Lohrke *et al.*, 2001), as confirmed by the great reduction of the transformation efficiency of the control in which acetosyringone was not used. Acetosyringone is required for transformation in both fungi and plants, indicating that they share a similar transformation mechanism (De Groot *et al.*, 1998). The significance of acetosyringone in fungal transformation has also been discussed by others (Mullins *et al.*, 2001; Meyer *et al.*, 2003).

To test the mitotic stability of transformed fungi, transformants obtained from selective PDA medium were serially transferred onto ordinary PDA, then transferred onto selective PDA with 10 µg hygromycin B ml^{-1}. This was done three times. All the five transformants tested were able to grow on the selective medium.

Transformants were verified by PCR assays and fluorescence detection. To confirm the truthfulness of transformation, PCR amplification of the hygromycin B-resistance gene was performed on genomic DNA extracted from 15-day-old cultures of the transformants and the wild type strain (Fig. 1b, c). Four selected putative transformants yielded the predicted amplicon, and sequencing results confirmed the presence of the hygromycin B-resistance gene. This gene was not amplified from the negative control.

Fluorescence was observed in the GFP-transformed *M. brunnea* isolates providing convincing proof of successful transformation (Eckert *et al.*, 2005; Figueiredo *et al.*, 2010; Vieira and Camilo, 2011). After the GFP gene was cloned into the plasmid used for transformation, its expression was observed and analyzed. The mycelia of both wild type and transformed colonies were examined using epifluorescence microscopy. GFP-associated green fluorescence was detected in transgenic mycelia, but not in wild-type *M. brunnea*. Bright-field image of wild-type *M. brunnea* is also shown (Fig. 2c). Up to now, the infection processs of *M. brunnea* remains unclear. The GFP transformants may help us to observe invasive growth and cell to cell movement in infected tissues (Horowitz *et al.*, 2002; Linsell *et al.*, 2011; Lakshman *et al.*, 2012).

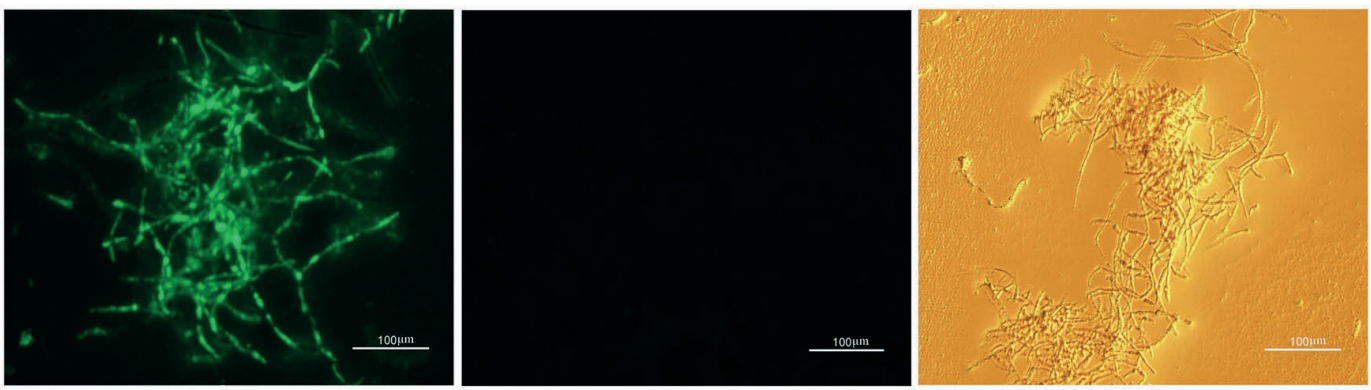

Fig. 2. Green fluorescent protein fluorescence from *M. brunnea* transformants. The fluorescence caused by GFP protein was observed in transformed *M. brunnea* mycelia and wide-type *M. brunnea* mycelia under green light excitation (a, b). The bright-field image of *M. brunnea* mycelia (c). Bar=100 µm.

Our establishment of a transformation system can be used for producing mutants in *M. brunnea* through large-scale insertional mutagenesis which may contribute to uncover new pathogenicity loci (Combier *et al*., 2003; Jeon *et al*., 2007). Meanwhile, it will also be helpful in the study of gene function, especially of virulence genes, for the transformation system could be used for gene over-expression and gene disruption after the corresponding plasmids are constructed.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table 1. Oligonucleotide primers used in this study.

Primers	Sequences
Long strand of adapter	GTAATACGACTCACTTAGGGCACGCGTGGTCGACGGCCCGGGCTGC
Short strand of adapter Hind III	AGCTGCAGCCCG
Short strand of adapter EcoR I	AATTGCAGCCCG
AP1	GTAATACGACTCACTATAGGGC
AP2	TGGTCGACGGCCCGGGCTGC
Beta-tub ORF forward	ATGCGCGAGATTGTTCAC
Beta-tub ORF reverse	CTCCTCTTGCTCTAGAGGTG
Beta-tub reverse inner	GGACGGCAACAGCACGGAAGGAGTGAG
Beta-tub reverse outer	AAGATAGCGGAGCAGGTCAAG
Beta-tub forward outer	TCCCAGATCGAatgatggcnac
Beta-tub forward inner	TTTCGCTCCACTGACCTCCmgnggngcnca
Beta-tub- forward(Ase I)	ATTAATCTGATTCCATGACGTCTG
Beta-tub-hyg- reverse	CAGGCTTTTTCATTGTGAAGGTTTTAAGG
Beta-tub-hyg- forward	CCTTAAAACCTTCACAATGAAAAAGCCTG
Hyg reverse(Ase I)	ATTAATCTATTTCTTTGCCCTCGGACG
Beta-tub promoter F(Hind III)	CCCAAGCTTCTGATTCCATGACGT
Beta-tub promoter R(Xba I)	GCTCTAGATGTGAAGGTTTTAAGGT
Hyg forward	ATGAAAAAGCCTGAACTCACCG
Hyg reverse	TTTCTTTGCCCTCGGACGAGTGC

Fig. S1. Multiple sequences alignment of beta-tubulin proteins from *Aspergillus clavatus*, *Botrytis cinerea* and *Sclerotinia sclerotiorum*.

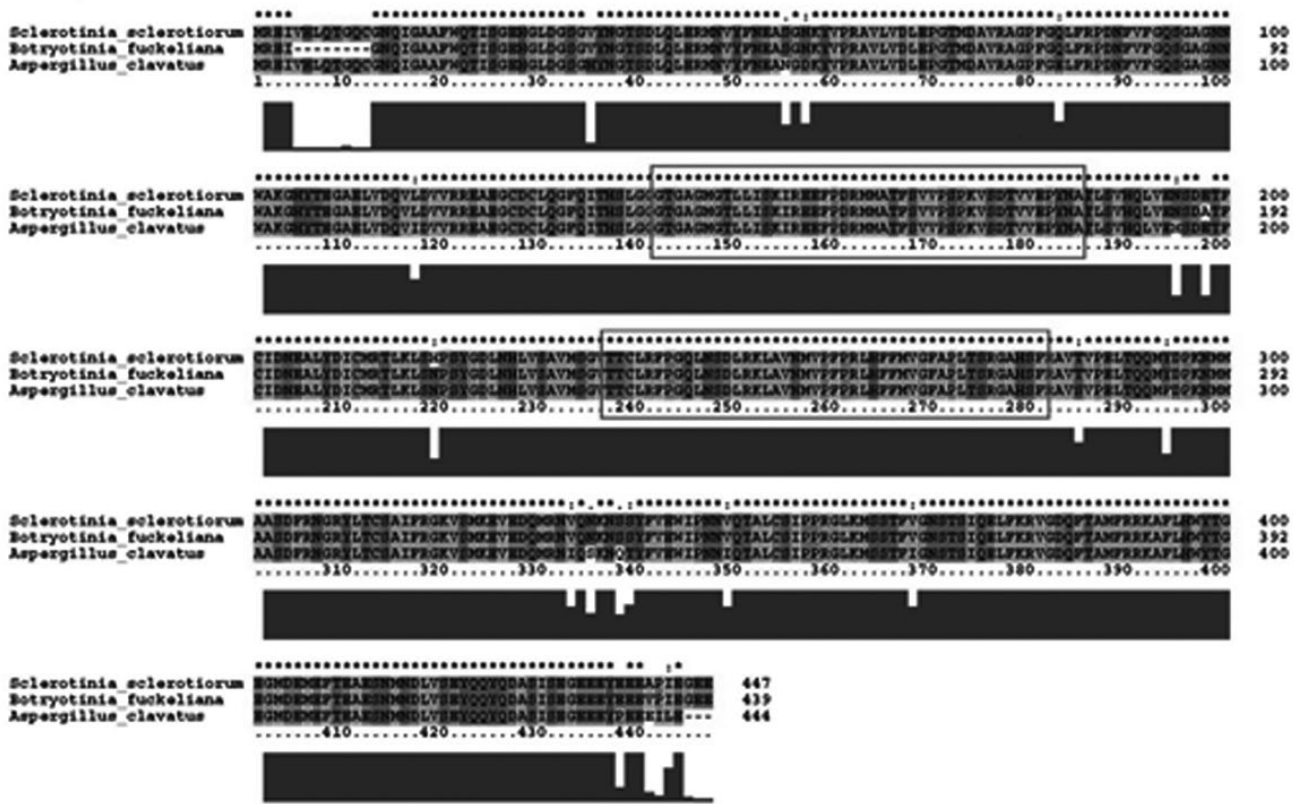

Fig. S2. The exon-intron structure of *M. brunnea* beta-tubulin gene.

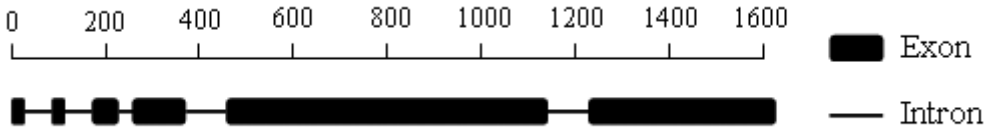

Fig. S3. The promoter analysis of beta-tubulin gene. The sequence of the beta-tubulin promoter was analysis to identify the possible promoter elements. Putative *cis*-acting elements are underlined.

AGCTTCCTCGTGTTTGCTGACTCCAAATCAACACCCGT
GCR1 AP-1
AGTCGAGATGCCTAAGGGAGATGACATGTTTCTCTGAG
GATA-1
CTCCCACTTGAATAGCCTGGTCCAATCTCTTTGCAGCGG
AAGACAGCTGGGTACGTTGGCCACTGGCCGCCTGAGA
AP-4
GGCGGTGAACTTGTCCACCCATTTCTGGACTATCTGACT
CdxA NIT2
GATTTTGATCTGGTTCTCAGAGGAAGGATCACCAGAAG
HSF
CCGAGAGCAGCAGCTCGGTAATGGATGTAGCTGCTTCG
GCCAGAGGAGACGGCCCTGTGCTCTCTACTGCATCTGG
AACATATGCATTTGATGCATGGGTTAAGTAGCCGTTGGC
HSF
ATATTAATGTGAGGATCGGGTCGATGCCCGGGATAAAG
CdxA GATA-X
CCCTTGATTTCTGATTCCATGACGTCTGGGTATGGCGAG
CdxA CREB
TTGTCGAACGCAATATCTGGAATTGCTTCCGAGCGACA
NIT2
ATTGCAAGTCGAGATTCAATAAGTCACATTTCGATTCTT
GCCTTGATTTTCAGGCCTCAAGTCGGTACTAGGGCTAG
CGCGGTGCACGGCCAGTAAACAGAGCTAGGGCAGGGG
SRY ADR1
TGGGCGCGATCGCGACTCCTTTTCCTTTACCGCTCCTTT
GATA-1 StuAp
CTCCTGCTCAACATCATCTCGACGACCTCAATTCCTCGA
Skn1
CAGCTCGCTCCATCACCTTCGGAAAACATCATCTCGAC
ZID
GACCTCAATTCCTCGACAGCTCGCTCCATCACCTTCGC
ZID
AACCCAACCCAACTCATTACCATCTCCAGTCCCGAAAC
cap HSF
CTTAAAACCTTCACA