Expression and promoter characterization of \textit{BbPacC}, a pH response transcription factor gene of the entomopathogenic fungus \textit{Beauveria bassiana}

Yong-Hong Zhou, Lei Hou, Yong-Jun Zhang, Yan-Hua Fan, Zhi-Bing Luo, Dan Jin, Qiao-Sheng Zhou, Yu-Jie Li, You Wang and Yan Pei

Biotechnology Research Center, Southwest University, 215 Tiansheng Road, Beibei 400716, Chongqing, PR China

To survive, the entomopathogenic fungus \textit{Beauveria bassiana}, which shows promise as a biocontrol agent for a variety of pests, including agricultural and forestry pests and vectors of human pathogens, must tailor gene expression to the particular pH of its environment. The pH response transcription factor gene \textit{BbPacC} and its flanking sequence were cloned from this fungus. Quantitative reverse transcription (RT)-PCR revealed that it is highly induced by alkaline pH and salt stress, and the expression level achieved twice that of the housekeeping gene \textit{c}-actin. A microfluorometric assay indicated that the 1479 bp promoter region could activate the expression of enhanced green fluorescent protein (EGFP) under the same conditions. Truncation analysis showed that the 1479, 1274, 1040, 888 and 742 bp promoters have similar efficiencies in activating expression of \textit{\beta}-glucuronidase (GUS). The GUS activities of corresponding transformants reached approximately 50\% that of those containing the strong constitutive promoter \textit{PtrpC}. A truncation upstream at the –572 bp position (referenced to the translation start codon ATG), however, resulted in a significant loss of GUS activity. Both the upstream absences of the –502 and –387 bp positions caused almost complete loss of GUS activity. These results suggest that \textit{PPacC} is an efficient, alkaline, and salt-inducible promoter, the core \textit{cis}-elements are mainly located within the –742 to –502 bp region, and promoters equal to or longer than 742 bp may be feasible for regulating gene expression in response to an ambient pH or salt stress.

INTRODUCTION

Entomopathogenic fungi, important regulating factors of insect populations in nature (St Leger \textit{et al.}, 1996), can be developed into mycoinsecticides to control a variety of pests, including agricultural and forestry pests and vectors of human disease (Xiao \textit{et al.}, 2012). They are capable of living in environments with a wide pH range. In soil for saprophytism, the ambient pH is very variable since it is influenced by many environmental factors (Ali-Shtayeh \textit{et al.}, 2003; Bidochka \textit{et al.}, 2000; Quesada-Moraga \textit{et al.}, 2007). When they parasitize a pest, the ambient pH is determined by the species, organ, age and diet of the host (Govenor \textit{et al.}, 1997; Schultz & Lechowicz, 1986; St Leger \textit{et al.}, 1998). While living in symbiosis with plants, the rhizospheric environment is mostly neutral or acidic (Behie \textit{et al.}, 2012; Blossfeld \textit{et al.}, 2010, 2011; Gao \textit{et al.}, 2011; Pava-Ripoll \textit{et al.}, 2011). In fermentation, pH of the medium usually influences the mycoinsecticide production (Hallsworth & Magan, 1996; Issaly \textit{et al.}, 2005; Bhanu Prakash \textit{et al.}, 2008; Ying & Feng, 2006). Additionally, chemicals, such as organic acids, alkaloids and ammonia, produced during the metabolism of the fungi, will lead to a wide range of pH alteration (Espeso \textit{et al.}, 1997; Fomina \textit{et al.}, 2005; St Leger \textit{et al.}, 1998, 1999; Tamerler \textit{et al.}, 1998). To survive, entomopathogenic fungi must be able to tailor gene expression to the particular pH of their growth environment. However, studies on the responses of fungal insect pathogens to alterations in the ambient pH are very limited, and the molecular mechanism of pH adaptability remains to be elucidated.

The pH response transcription factor (PacC) is a key regulator for fungi in adjusting gene expression to adapt to ambient pH changes (Peñalva \textit{et al.}, 2008). To study
the function of genes involved in pH adaptation, a PacC coding gene, \textit{BbPacC}, and its flanking sequence were cloned from the entomopathogenic fungus \textit{Beauveria bassiana}. Quantitative RT-PCR revealed that the gene was highly induced by alkaline conditions and salt stress. To acquire an effective inducible promoter, the upstream sequence of \textit{BbPacC} was cloned and evaluated.

**METHODS**

**Strains and culture conditions.** \textit{Escherichia coli} DH5\textsubscript{a}, used for gene cloning and vector construction, was purchased from TaKaRa Biotechnology (Dalian, China). \textit{Agrobacterium tumefaciens} AGL1 was kindly donated by Dr Mark Guiltinan from Pennsylvania State University, University Park, PA, USA, and used to mediate fungal transformation. \textit{B. bassiana} CGMCC7.34 (China General Microbiological Culture Collection Center) was isolated from infected \textit{Pieris rapae} larva, and its culturing and conservation were performed as previously described (Zhou et al., 2012).

**Plasmid and nucleic acid extraction.** Plasmid DNA was isolated using the Plasmid Mini kit I (Omega). Extraction of \textit{B. bassiana} genomic DNA was performed according to the method of Fang et al. (2002). Total RNA was isolated using the method of Wan & Wilkins (1994).

**Sample preparations for RNA extraction.** To prepare samples at different fermentation stages, \textit{B. bassiana} conidia were inoculated into SDY broth (Sabouraud dextrose plus yeast extract; 40 g glucose l\textsuperscript{-1}, 20 g yeast extract l\textsuperscript{-1}, and 10 g tryptone l\textsuperscript{-1}, pH 7.0) at final concentrations of 1 x 10\textsuperscript{6} ml\textsuperscript{-1} and incubated in a rotary shaker (26 °C, 180 r.p.m.). The cultures were collected by centrifugation [12,000 r.p.m. (equal to 16,454 g), 5 min] at 2, 3, 4, 6, 8, 10, 12, 14 and 16 days for RNA extraction. In addition, the pH of the broth was measured at each time point.

For pH treatments, SDY-cultured (26 °C, 180 r.p.m. for 48 h) \textit{B. bassiana} hyphae were collected by centrifugation [6,000 r.p.m. (equal to 4,113 g), 5 min] and transferred into CZM (Czapek medium; 30 g sucrose l\textsuperscript{-1}, 2 g NaNO\textsubscript{3} l\textsuperscript{-1}, 0.5 g KCl l\textsuperscript{-1}, 1 g K\textsubscript{2}HPO\textsubscript{4} l\textsuperscript{-1}, 0.5 g MgSO\textsubscript{4}·7H\textsubscript{2}O l\textsuperscript{-1}, 0.01 g FeSO\textsubscript{4}·7H\textsubscript{2}O l\textsuperscript{-1}, pH 7.0), CZM-3MES (pH 3.0, buffered with 80 mM MES), CZM-4MES (pH 4.0, buffered with 80 mM MES) and CZM-8Tris (pH 8.0, buffered with 80 mM Tris). After incubation in a rotary shaker (26 °C, 180 r.p.m.) for 6 h, the hyphae were recovered by centrifugation [12,000 r.p.m. (equal to 16,454 g), 5 min] for total RNA extraction. For salt stress, the SDY-cultured (26 °C, 180 r.p.m., 48 h) \textit{B. bassiana} hyphae were transferred into CZM + 0.8 M NaCl. After incubation in a rotary shaker (26 °C, 180 r.p.m.) for 6 h, the hyphae were recovered by centrifugation [12,000 r.p.m. (equal to 16,454 g), 5 min] for total RNA extraction. Additionally, CZM-3MES (pH 5.0, buffered with 80 mM MES) and CZM-8Tris (pH 8.0, buffered with 80 mM Tris) were used as controls.

**Sample preparation for microfluorometric assay.** For the microfluorometric assay, conidia from 14-day-old colonies of 48 randomly select transformants were combined in 0.05 % Tween 80. The conidial suspension was diluted 1 : 10 with double-distilled water and stored at −20 °C for subsequent quantitative reverse transcription (RT)-PCR analysis.

**Plasmid DNA was isolated using the Plasmid Mini kit I (Omega).** Extraction of \textit{B. bassiana} genomic DNA was performed according to the method of Fang et al. (2002). Total RNA was isolated using the method of Wan & Wilkins (1994).
inoculated into SDY-8.5Tris (pH 8.5, buffered with 80 mM Tris) or
SDY + 1.0 M NaCl broth to a final concentration of 10^6 ml^-1. After
incubation in a rotary shaker (26 °C, 180 r.p.m.) for 3 days, the
cultures were recovered by vacuum filtration through Whatman 54
paper and subjected to the GUS assay. Wild-type
B. bassiana
and a
PtrpC (a strong constitutive promoter from
Aspergillus nidulans
) -regulated GUS transformant were used as negative and positive
controls, respectively. The GUS assay on the crude hyphal protein was
performed according to the method of Jefferson
et al. (1987). The
GUS activity unit was defined as the quantity of enzyme required for
hydrolysing 4-MUG (4-methyl umbelliferyl glucuronide) to generate
1 nmol 4-MU min^-1 at 37 °C. The crude hyphal protein was
quantified by the Bradford method using BSA as the standard.

RESULTS

Gene cloning

Employing degenerate PCR and YADE walking, the coding
and flanking sequences of a pH response transcription
factor, BbPacC, a homologous gene in B. bassiana, were
cloned. Sequence analysis revealed that the 1773 bp ORF
encodes a protein consisting of 590 aa, and that the protein
is 71.6 %, 69.8 %, 68.5 % and 43.7 % homologous to PacC
proteins from Metarhizium anisopliae, Nectria haematococca,
Gibberella zeae and Aspergillus nidulans, respectively (Fig.
S1). Thus, the gene was designated BbPacC.

BbPacC is highly induced by alkaline pH and salt
stress

The pH response transcription factor is known to be a
central regulator of gene expression for fungal adaptation
to ambient pH changes (Peñalva
et al., 2008). In this study,
we found that during fermentation in SDY broth, the
extracellular pH of B. bassiana dropped from 7.0 (initial)
to 4.17 (lowest) in the first 3 days, then gradually rose to
8.31 at 16 days. The trend of BbPacC expression was
similar to the alteration in extracellular pH. When the
extracellular pH was lower than 7.0, BbPacC expressed
generally at a background level, but when the pH rose
higher than 7.0, the expression of BbPacC rose accordingly.
The highest expression level of BbPacC achieved was more
than twice that of the housekeeping gene c -actin, which
was used as the internal reference (Fig. 1a). These results

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Purpose and name of primer</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification of BbPacC</td>
<td></td>
</tr>
<tr>
<td>PacC-F</td>
<td>CCGAAGTTCTCTATGAACAC</td>
</tr>
<tr>
<td>PacC-R</td>
<td>GTGCTTGACGTCTTTC</td>
</tr>
<tr>
<td>Amplification of the internal reference γ-actin</td>
<td></td>
</tr>
<tr>
<td>Actin-F</td>
<td>ATGGAGGAAGAAGTTGCTGC</td>
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<tr>
<td>Actin-R</td>
<td>ACACGGAGCTGTTGAGAA</td>
</tr>
<tr>
<td>Promoter deletion analysis</td>
<td></td>
</tr>
<tr>
<td>EcoRI-P1479-F</td>
<td>CTGGaaaaccTGCGTGACTGACAAGCCTGA</td>
</tr>
<tr>
<td>EcoRI-P1247-F</td>
<td>CAGGaaaaccATGAGGGGATGGAGGTT</td>
</tr>
<tr>
<td>EcoRI-P1040-F</td>
<td>GTGCGaaaaccGGCGAGTTCTAGTCAAGC</td>
</tr>
<tr>
<td>EcoRI-P888-F</td>
<td>TGATgaaaccGCAACGCCCTCCACTACTAC</td>
</tr>
<tr>
<td>EcoRI-P742-F</td>
<td>CTCGgaaaccCCTGAAAATGGCAAAAGCA</td>
</tr>
<tr>
<td>EcoRI-P572-F</td>
<td>GAGGgaaaccGCTACAAAGCGAATGCTG</td>
</tr>
<tr>
<td>EcoRI-P502-F</td>
<td>GTGTTgaaaccCTTATGGGTCTTCCCGCAAG</td>
</tr>
<tr>
<td>EcoRI-P387-F</td>
<td>TCTAgaaaccCCTCCTGACGAAATGATTTC</td>
</tr>
<tr>
<td>NotI-PgagggccgTGCAAGCGGTTGAGATAGCT</td>
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<tr>
<td>Promoter mutation analysis</td>
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<tr>
<td>Generation of Pmut1</td>
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<tr>
<td>Pmut1-F</td>
<td>AAAGCAGCGACCAGCGCTGGTTTTGAGGTTGTGCT</td>
</tr>
<tr>
<td>Pmut1-R</td>
<td>GCAACACCCTCAAAACCGCGCTCGGCTCGTCTT</td>
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<tr>
<td>Generation of Pmut2</td>
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<tr>
<td>Pmut2-F</td>
<td>AAAGCAGCGACCAGCTGGTTTTGAGGTTGTC</td>
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<tr>
<td>Pmut2-R</td>
<td>GCAACACCCTCAAAACCTGGCTCGGCTGTTT</td>
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<tr>
<td>Generation of Pmut3</td>
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<tr>
<td>Pmut3-F</td>
<td>TACGGGTGTTGCCCAAGGCTCGACGCTGCTAATC</td>
</tr>
<tr>
<td>Pmut3-R</td>
<td>AGTTAGCGCGTGCGCCGCTTGGGCAACACGGA</td>
</tr>
<tr>
<td>Generation of Pmut4</td>
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<tr>
<td>Pmut4-F</td>
<td>AGACGAGCGGCGGCTGGTTGATACAGCGG</td>
</tr>
<tr>
<td>Pmut4-R</td>
<td>CCCTTGTACCCCAAGCGCAGCGCTCAGCTGCTT</td>
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</table>

The nucleotides in lower case are recognition sites for EcoRI or NotI; the four nucleotides in front of the recognition sites are protective bases.
Fig. 1. Expression pattern of the pH response transcription factor BbPacC response to ambient pH changes and salt stress. At 2, 3, 4, 6, 8, 10, 12, 14 and 16 days, the transcripts of BbPacC in SDY-fermented cultures of B. bassiana were detected by quantitative RT-PCR. The pH value of the cultures was determined at each time point (a). Forty-eight-hour-old B. bassiana hyphae were transferred into CZM of different pH values. After induction (26 °C, 180 r.p.m.) for 4 h, the induced hyphae were recovered and subjected to quantitative RT-PCR analysis (b). Forty-eight-hour-old B. bassiana hyphae were transferred into CZM-5MES (pH 5.0), CZM, CZM + 0.8 M NaCl (0.8 M NaCl) and CZM-8Tris (pH 8.0). After induction (26 °C, 180 r.p.m.) for 6 h, the hyphae were recovered and subjected to quantitative RT-PCR (c). Forty-eight-hour-old B. bassiana hyphae were transferred into SDY, SDY-5MES (pH 5.0), SDY-8Tris (pH 8.0), SDY-0.8 M NaCl, SDY-0.8 M sorbitol, CZM, CZM-5MES (pH 5.0), CZM-8Tris (pH 8.0), CZM + 0.8 M NaCl (0.8 M NaCl), CZM + 0.8 M sorbitol (0.8 M sorbitol), BS, BS-5MES (pH 5.0), BS-8Tris (pH 8.0), BS + 0.8 M NaCl (0.8 M NaCl) and BS + 0.8 M sorbitol (0.8 M sorbitol). After incubation on a rotary shaker (26 °C, 180 r.p.m.) for 4 h, the hyphae were recovered and subjected to quantitative RT-PCR (d). γ-actin was used as the internal control. Three replicates were performed for each sample, bars indicate SD.
indicated that expression of \( BbPacC \) was highly induced by an alkaline pH but suppressed by acidic conditions. When induced with media of different pH, the expression of \( BbPacC \) was still positively correlated with the value of the ambient pH (Fig. 1b). In \textit{Fusarium oxysporum}, PacC was found to be necessary for salt resistance (Caracuel \textit{et al.}, 2003). This prompted us to investigate the response of \( BbPacC \) to salt stress, and the results show that in addition to an alkaline pH, \( BbPacC \) was also induced by salt stress (Fig. 1c).

To study the effects of nutrients and hyperosmotic stress on the expression characterization of \( BbPacC \), we investigated the response of \( BbPacC \) to different nutritional conditions and hyperosmotic stresses. The results showed that the expression of \( BbPacC \) varied under different nutritional conditions (BS, basic CZM medium and rich SDY medium), but was not induced by these conditions, whereas \( BbPacC \) expression was induced by alkaline pH and salt stress under all three nutrient conditions (Fig. 1d). The results also showed that \( BbPacC \) can be induced by 0.8 M NaCl, but cannot be induced by 0.8 M sorbitol (Fig. 1d) and even 1.5 M sorbitol (Fig. S4), which is also able to cause hyperosmotic stress and reduced growth rate but does not generate salt stress (data not shown), indicating that the induction of \( BbPacC \) by NaCl is actually by salt stress, but not by increased osmolarity or reduced growth rate.

**Sequence characterization of \( PPacC \)**

Sequence analysis revealed that the 1500 bp upstream sequence of \( BbPacC \) has no typical TATA box, but contains a transcription start site (−95 bp), an initiator of transcription (Inr), three CT-rich regions and four CAAT boxes. In addition to these typical \( cis \)-acting elements of a eukaryotic promoter, as many as 14 PacC-binding motifs (GCCARG) were found within the 1500 bp promoter region (Fig. 2). These may explain why \( BbPacC \) was highly induced by alkaline pH.

**Microfluorometric assay**

To confirm the specificity of \( PPacC \), a 1479 bp fragment of \( PPacC \) (P1479) was cloned and fused to upstream of EGFP.

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**Fig. 2.** Sequence characteristics of the pH response transcription factor promoter \( PPacC \). Nucleotides are numbered with reference to the translational start codon (ATG). The promoter sequence is shown in lower case, the upper case nucleotide in the black shadow is the predicted transcription start site (tsp), the lower case nucleotides in the black shadow form the potential transcription initiator (Inr), the underlined nucleotides are putative CT-rich regions, the boxed nucleotides are CAAT boxes, and the PacC-binding motifs (GCCARG) are in grey-shadowed boxes. The arrows indicate the positions of the primers designed for promoter truncation.
to construct the expression vector pK2-P1479–EGFP. Mediated by Agrobacterium tumefaciens AGL1, the vector was transferred into B. bassiana. Microscopic fluorescence testing showed that the randomly selected EGFP transformant generated strong green fluorescence under alkaline condition (Fig. 3c) and salt stress (Fig. 3d).

**Promoter truncation and mutation analysis**

To determine the core region and efficiency of PPacC, eight promoter truncation constructs were generated using GUS as the reporter gene (Fig. S2). The resulting vectors, pK2-Promoters–GUS, were transferred into B. bassiana through the Agrobacterium tumefaciens-mediated method. Southern blot analysis showed that the occurrence of multi-copy integration in the transformants is low (see Fig. S3). The GUS activity assay showed that promoters P1479, P1274, P1040, P888, and P742 displayed similar high efficiency. The GUS activity [62.99–123.91 U (mg protein)^{-1}] was approximately 50% of that from the strong constitutive promoter PtrpC. For promoter P752, the GUS activity of the corresponding transformants significantly declined to 16.19 to 27.06 U (mg protein)^{-1}. For P502 and P387, the GUS activities were similar to wild-type, which only displayed background levels [8.21–17.20 U (mg protein)^{-1}].

Since the difference between the efficiencies of the 742 bp and 572 bp fragments was striking, it is interesting to determine whether the sole PacC-binding motif and CAAT box are the functional elements in the −742 bp to −572 bp region. Thus, we mutated A4 of the sole PacC site to T (Pmut1), and deleted the PacC site GCCAGG (Pmut2), the CAAT box (Pmut3), and the whole −742 bp to −572 bp region (Pmut4), respectively. These four mutated fragments were fused upstream of the reporter gene GUS to construct expression vectors. The vectors were transferred into B. bassiana by Agrobacterium tumefaciens-mediated transformation. GUS assay showed that Pmut1–GUS, Pmut2–GUS, and Pmut3–GUS transformants had similar GUS activities with that of P1479–GUS, but the activity in Pmut4–GUS transformants was significantly lower than that of P1479–GUS transformants. These data suggest that the sole PacC-binding motif and CAAT box are not the functional elements in −742 bp to −572 bp region.

**DISCUSSION**

Using constitutive or inducible promoters to regulate target genes in engineered strains is important for gene function analysis and strain improvement. In entomopathogenic fungi, the most frequently used constitutive promoters include the glyceraldehyde-3-phosphate dehydrogenase promoter (PgpdA) and the tryptophan C gene promoter (PtrpC) from Aspergillus nidulans (Fang et al., 2004; Ma et al., 2009). Recently, some endogenous constitutive promoters have also been successfully used in fungal pathogens (Cao et al., 2012; Liao et al., 2008). Although constitutive promoters can activate gene expression under all circumstances in the cell, constant expression of some genes may generate negative effects in transgenic tissues, especially inappropriate expression of genes that are either deleterious or lethal. Inducible promoters can regulate transcription of target genes under specific conditions, thus avoiding lethal or growth inhibitory effects that are usually associated with constitutive promoters (Liao et al., 2009; Roslan et al., 2001). In entomopathogenic fungi, some inducible promoters have been successfully used. For instance, using the inducible promoter PMcCl to regulate transcription of a scorpion toxin gene AaIT in hemolymph by Metarhizium anisopliae increased fungal toxicity 22-fold against tobacco hornworm (Manduca sexta) caterpillars and ninefold against adult yellow fever mosquitoes (Aedes aegypti) (Wang & St Leger, 2007). Liao et al. have established the ethanol inducible alc system in B. bassiana, which regulates gene expression through ethanol induction (Liao et al., 2009). However, these inducible promoters are not sufficient for the diverse research on fungal insect pathogens. Thus, more inducible promoters are required for specific experimental conditions.

Many fungi grow over a wide pH range and their gene expression is tailored to the environmental pH. The pH response transcription factor (PacC) is a key regulator of gene transcription when fungi undergo an ambient pH.
alteration (Peñalva et al., 2008). Under alkaline conditions, PacC upregulates some alkaline pH inducible genes but prevents expression of acid-expressed genes (Then Bergh et al., 1998; Cupertino et al., 2012; Espeso & Arst, 2000; Kanda et al., 2008; Merhej et al., 2011; Tilburn et al., 1995). The transcription of the PacC gene is self-regulated in a feedback manner. Thus, it is also upregulated in response to alkaline conditions (Tilburn et al., 1995). In addition, PacC in some filamentous fungi was found to be involved in salt resistance (Caracuel et al., 2003). BbPacC was induced by both alkaline conditions and salt stress (Fig. 1). Microscopic fluorescence testing showed that its promoter can efficiently activate the expression of reporter gene EGFP under the same conditions. Additionally, we found that the expression level of BbPacC can reach twice that of the housekeeping gene G-actin (Fig. 1a). The promoting efficiency of PPacC on the reporter gene GUS was approximately 50% that of the strong constitutive promoter PtrpC (Fig. 4). These results suggest that PPacC can be used for efficient activation of gene transcription under alkaline conditions or salt stress.

In an acidic environment, the PacC precursor adopts a self-closed conformation, protecting itself from activation through intramolecular interactions involving the C-terminal domain. Meanwhile, under alkaline conditions, the self-closed conformation shifts to an ‘open’ conformation (protease-accessible), enabling the two steps of proteolysis. This results in the removal of the C-terminal negative repression domain (Diez et al., 2002). The remaining N-terminal activation domain goes into the nucleus (Mingot et al., 2001) where it activates transcription of genes preferentially expressed under alkaline conditions and represses those expressed under acidic conditions. In both cases, this is by binding to GCCARG

Fig. 4. Crude protein GUS activity of transgenic and wild-type B. bassiana. For each truncation, the transformants cultured in SDY-8.5Tris (pH 8.5) and SDY+1.0 M NaCl (1 M NaCl) broth for 3 days were subjected to a GUS assay. The strong constitutive promoter PtrpC was used as positive control. Wild-type B. bassiana (WT) was used as negative control. Three replicates were performed for each sample. Error bars indicate SD. All the values under the same stress were grouped and analysed by ANOVA, in which the values of PtrpC, the truncations and WT were compared with that of the full-length P1479 promoter, respectively (**P<0.01).

Fig. 5. Promoter mutation assay. Four promoter mutations, mutation A4 of the sole PacC site to T (Pmut1), deletion the PacC site GCCAGG (Pmut2), the CAAT box (Pmut3) and the whole −742 bp to −572 bp region (Pmut4), were generated by PCR-based site-directed mutagenesis methods (a). GUS transformants cultured in SDY-8.5Tris (pH 8.5) and SDY+1.0 M NaCl (1 M NaCl) broth for 3 days were subjected to a GUS assay. The full-length promoter P1479 was used as positive control. The wild-type B. bassiana (WT) was used as negative control. Three replicates were performed for each sample, error bars indicate SD. Values under the same stress were grouped and analysed by ANOVA analysis. Values of Pmut1, Pmut2, Pmut3, Pmut4 and WT were compared with that of P1479. (**P<0.01).
motifs in the promoter region of the target genes (Espeso & Peñalva, 1996; Espeso et al., 1997; Tilburn et al., 1995). Sequence analysis revealed that there are 14 GCCARG motifs within the 1500 bp upstream of BbPacC, and 13 of the motifs are within the −720 to −1 bp region. This information suggests that a PPacC promoter equal to or longer than 720 bp might be efficient and specific. This speculation was proven correct by the microscopic fluorescence testing (Fig. 3) and promoter truncation analysis (Fig. 4).

To analyse the functions of the genes involved in pH adaptation and ion homeostasis, it is necessary to seek efficient inducible promoters to regulate gene expression under corresponding experimental conditions. To this end, PPacC might be a suitable candidate since it is efficient in response to ambient pH changes and salt stress. Additionally, for engineered fungal strains in which PPacC is used to regulate the target gene, it might be practical to increase the yield of target product by adjusting the pH or salt concentration of the media.

For salt stress, the concentration of NaCl was nearly constant, and the stress resulted from salt on the fungal growth may be stronger than the basic condition which can be alleviated by the acidification of medium. That is why the response of BbPacC to alkaline pH was more pronounced than to salt stress in Fig. 1d.

In GUS assay, the results may be distorted by the occurrence of homologous recombination events restoring the endogenous context of the promoter fragment, multicopy transformations, chromosomal position effect (integration into transcriptionally active or inactive genomic regions) and other unknown factors. It would be very complicated and time consuming to completely eliminate these error-causing factors by molecular characterizations. To minimize the errors caused by these factors, we increased the biological replicates in GUS assay up to 48 randomly selected transformants (48 biological replicates) for each promoter truncation or mutation. In addition, since the occurrence of homologous recombination event and multicopy integration in B. bassiana using A. tumefaciens-mediated method is low (Fang et al., 2004; Ma et al., 2009), this transformation method was employed in the present study.

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