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**RAP-PCR fingerprinting reveals time-dependent expression
of development-related genes following differentiation
process of *Bacillus thuringiensis***

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3 RAP-PCR fingerprinting reveals time-dependent expression of development-related
4 genes following differentiation process of *Bacillus thuringiensis*

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Abstract

Gene expression profiles are important data to reveal gene functions putatively involved in crucial biological processes. RNA arbitrarily primed-polymerase chain reaction (RAP-PCR) and specifically primed reverse transcription PCR (RT-PCR) were combined to screen differentially expressed genes following development of a commercial *Bacillus thuringiensis* (Bt) subsp. *kurstaki* strain 8010 (serotype 3a3b). Six differentially expressed transcripts (RAP1 to RAP6) were obtained. The RAP1 encoded a putative triple helix repeat-containing collagen or an exosporium protein H related to spore pathogenicity. The RAP2 was homologous to a ClpX protease and an ATP-dependent protease La (LonB) likely acted as virulence factors. The RAP3 was homologous to a beta subunit of propionyl-CoA carboxylase (PCC) enzyme (PCCB) required for the development of *Myxococcus xanthus*. The RAP4 had homology to a quinone oxidoreductase involved in electron transport and ATP formation. The RAP5 showed significant homology to a uridine kinase (UDK) mediated phosphorylation of uridine, azauridine. The RAP6 shared high sequence identity with 3-methyl-2-oxobutanoate-hydroxymethyltransferase (MOHMT) (EC 2.1.2.11) [also known as ketopantoate hydroxymethyltransferase (KPHMT) or PanB] involved in the operation of the tricarboxylic acid cycle. The findings described here would help to elucidate the molecular mechanisms underlying the differentiation process of Bt and unravel novel pathogenic genes.

Key words: *Bacillus thuringiensis*, development, RNA-arbitrarily primed PCR, differentially expressed gene, life cycle

Introduction

Bacillus thuringiensis (Bt) is a gram-positive, spore-forming bacterium capable of producing a number of toxins including insecticidal endotoxins (delta-endotoxins, ICPs), exotoxins, haemolysins, enterotoxins, chitinase and vegetative insecticidal proteins (VIPs) with toxicity to several insect orders, nematodes, mites and protozoa (Schnepf et al. 1998, Yu et al. 2007). Since the first report of resistance developing against Bt delta-endotoxins in the field in 1985, insect resistances to Bt had been discovered both in the laboratory for several insects and in a field population of *Plutella xylostella* (McGaughey 1985, Liu and Tabashnik 1997). A thorough understanding of Bt pathogenesis at the molecular level is important to develop new strategies for the resistance management.

The life cycle of Bt includes vegetative cell division, spore development (sporulation cycle) and lysis. Its vegetative cell is rod-shaped (2–5 µm long and about 1.0 µm wide) and divides into 2 uniform daughter cells by a division septum. Then the sporulation occurs and a fully sporulated cell of Bt contains ICPs lying along side the endospore. After that, the lysis of the sporulated cell happens, which might be due to nutrition limitation. Herein, ICPs and endospore release from the cell (Ibrahim 2010). The insecticidal activity of Bt was mostly due to ICPs produced during the process of sporulation. Comparative proteomic analysis revealed the metabolic changes of Bt during growth shift, indicating that the cells acquired energy, amino acid precursors and translation-related factors for the ICP synthesis (Gong et al. 2012). Our hypothesis is that Bt genes, whose expression was differentially regulated at different phases (vegetative growth, sporulation and lysis) of the life cycle, could be of special relevance for its pathogenesis.

With the aim of identifying such genes, the RNA arbitrarily primed PCR (RAP-PCR) technique (Welsh et al. 1992), a method closely related to the differential display technique, was employed in this study. The techniques of differential display can be applied to evaluate modifications in transcriptomes according to various conditions in

prokaryotes (Dachet et al. 2010). RAP-PCR technique used random oligonucleotide primers to create a unique cDNA fingerprint for a given microorganism in a particular physiological state, thus providing a powerful tool for assessing differential gene expression in prokaryotes (Benson et al. 2000, Bidle 2003, Bidle and Bartlett 2001, Cabanes et al. 2000, Chia 2001, Dũ and Kolenbrander 2000, Holmstrøm and Gram 2003, Shepard and Gilmore 1999, Smalley et al. 2002, Steger et al. 2002, Fung et al. 2009, Schedel et al. 2011). Real-time or reverse transcription PCR was further used to make sure the cDNA were derived from real differentially expressed genes. Then the cDNA were cloned, sequenced and analyzed. According to the published papers, it is not necessary to do repetition in RAP-PCR. Unlike RAP-PCR, RAPD was often used to obtain the evolution relationship of two or more close species. Thus, the RAPD pattern was usually followed with the construction of phylogenetic trees. To obtain reliable trees, it is necessary to do the repetition of RAPD. RAP-PCR had been successfully used to investigate bacterial development, bacterial virulence genes, microbial responses to environment, and host infection (Benson et al. 2000, Bidle 2003, Bidle and Bartlett 2001, Cabanes et al. 2000, Chia 2001, Dũ and Kolenbrander 2000, Holmstrøm and Gram 2003, Shepard and Gilmore 1999, Smalley et al. 2002, Steger et al. 2002, Fung et al. 2009, Schedel et al. 2011). We also have successfully applied RAP-PCR to obtain differentially displayed mRNA related to resistance to Bt subsp. *israelensis* of *Aedes albopictus* selected *in vitro*-activated Cyt1Aa6 (Zhang et al. 2012). Bt subsp. *kurstaki* 8010 (serotype 3a3b) is a commercial strain in China (Guan 1997). In this study, we compared the RNA fingerprints of the bacterial cells during different growth phases and were then able to reveal differentially expressed genes following Bt 8010 development. Our results showed that RAP-PCR is a promising method for obtaining a better understanding of gene expression profiles of Bt during development.

Materials and Methods

Bacteria strains and plasmids

The Bt strain used in this study was Bt subsp. *kurstaki* 8010 (serotype 3a3b), a commercial strain isolated by our lab (Guan 1997). *Escherichia coli* JM109 and pMD18-T vector (TaKaRa, Japan) were used for molecular cloning experiments. Luria-Bertani (LB) medium (1% bactotryptone, 0.5% yeast extract, 1% sodium chloride) was used for the growth of bacteria. Bt strain 8010 was cultivated at 30 °C while *E. coli* strain was grown at 37 °C.

RNA preparation

The overnight cultures of Bt strain 8010 were sub-cultured in a 200 ml fresh LB broth with a 1:100 dilution. The growth of cells was monitored by measurements of optical density at 600 nm periodically with a spectrophotometer (Pharmacia, Sweden) and by observations made under an electron microscope (Olympus U-SPT, Japan). The sub-cultured cells obtained at 5 different time points (8 h, 30 h, 39 h, 42 h and 46 h of sub-culturing) were recovered for RNA extraction, respectively, using the SV Total RNA Isolation System according to the manufacturer's instructions (Promega, USA).

The integrity of the RNA was assessed by electrophoresis of 5 µl of each sample through a 1% agarose-0.66 M formaldehyde gel in MOPS running buffer [20 mM MOPS (morpholinepropanesulfonic acid, pH 7.0), 8 mM sodium acetate, 1 mM EDTA (pH 8.0)] at 3 to 4 V/cm [23]. RNA concentration was determined spectrophotometrically by measuring the OD₂₃₀, OD₂₆₀ and OD₂₈₀ of a 1:500 dilution in DEPC-treated water.

RAP-PCR

RAP-PCR was performed essentially as previous described (Welsh et al. 1992). Arbitrary 10-mer primers with 50% of G+C content were obtained from Operon

Technologies (USA). First strand synthesis was performed using AMV First Strand cDNA Synthesis Kits (BBI, Canada) according to the manufacturer's instructions. Second strand synthesis was performed in 50 µl of reaction mixtures containing 0.7 µl of first strand reaction mixtures, 0.5 µl (2.5 U) of *Taq* polymerase (BBI, Canada), 3 mM MgCl₂, 1×PCR Buffer, 0.5 mM each of the four dNTPs and 0.8 µM arbitrary primer. RAP-PCR cycling profile was 40 cycles of 94 °C for 1 min, 37 °C for 2 min and 72 °C for 1 min 30 sec, with a final extension step at extension at 72 °C for 10 min. Products were resolved on polyacrylamide gels prepared in TBE buffer and were electrophoresed until the xylene cyanol band reached the bottom of the gel. RAP-PCR fingerprints were visualized by silver staining as previously described (Dachet et al. 2010, Sambrook et al. 1989).

Isolation and cloning of RAP-PCR fragments

After silver staining, individual bands representing differentially expressed products were cut and placed into a microcentrifuge tube containing 50 µl of distilled water for overnight elution at 70 °C. A portion of the eluted fragment was applied for the subsequent PCR with the original primer used in RAP-PCR amplification. The PCR products were electrophoresed on a 2% agarose gel and the bands of the correct size were extracted using the UltraCleanTM 15 DNA Purification kit (MO BIO, Canada). Each purified PCR product was ligated into pMD18-T vector (TaKaRa, Japan) by TA cloning, and transformed into *E. coli* JM109. Transformants were identified by PCR and sequencing.

DNA sequencing

Cloned cDNAs were sequenced on both strands using *BcaBEST*TM Sequencing Primer RV-M (5'-GAGCGGATAACAATTTACACAGG-3') and *BcaBEST*TM Sequencing Primer M13-47 (5'-CGCCAGGGTTTCCCAGTCACGAC-3') by ABI

PRISM Model 377 DNA system and sequences obtained were compared to those in the GenBank database by using the BLAST search algorithm to identify similarities to known sequences.

Confirmation of differential gene expression

Once a candidate differentially expressed fragment was sequenced, a pair of specific primers with 20-23 nucleotides in length (Table 1) were designed based on the sequencing results and used for specifically primed reverse transcription PCR (RT-PCR). RT-PCR was performed under the following conditions-30 cycles of 1 min at 95 °C, 1 min at 58 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Aliquots of each PCR product were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Synthesis of a PCR product of the correct size and expression pattern was considered verified for differential expression of the original RAP-PCR result. 16S rRNA was used as a control gene not affected by the growth kinetics by using the general bacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-ACGGCTACCTTGTTACGACTT-3').

Results

Growth process of Bt strain 8010

The growth of Bt strain 8010 experienced three typical phases— phase of vegetative growth, sporulation and lysis (data not shown). Briefly, cells grew exponentially during the vegetative phase. At the onset of the stationary phase (at 30 h of sub-culturing), cells began to sporulate with the endospores forming at one end of the cells and intracellular crystallized inclusions deposited at the other end. Upon mother cell lysis (at 46 h of sub-culturing), the spores and inclusions were released.

RAP-PCR analysis

The five total RNA extracts from the cells of 8010 at 8 h, 30 h, 39 h, 42 h and 46 h of sub-culturing were used for RAP-PCR with several arbitrary primers. By RAP-PCR, 19 putative differentially expressed fragments were obtained. However, due to the inability to re-amplify 4 excised fragments, a total of 15 putative differentially expressed gene fragments, designated RAP1 to RAP15, were obtained finally. One example of RNA fingerprinting of RAP-PCR with arbitrary primer A-07 (Operon, USA) was shown in Fig. 1.

The putative differentially expressed fragments, which responded to different growth phases, as represented by changes in bands appearance or disappearance or by differences in bands brightness, could be identified. When a putative differentially expressed fragment from RAP-PCR products was found, it was purified from the gel, re-amplified with the same arbitrary primer and electrophoresed on 2% agarose gel. The corresponding correct single band, compared to the original RAP-PCR products, was purified and used for the further cloning and sequencing. If several bands of various sizes appeared in the re-amplified product, it suggested that the putative differentially expressed fragment was purified with the nearby fragments in the original RAP-PCR products. In this case, target PCR fragments were subject to several rounds of gel purifications before cloning.

Verification of time-dependent expression of differential expressed genes following Bt development by specifically primed RT-PCR

Based on sequencing results, 15 specific primer pairs were designed and used for the RT-PCR to confirm differentially expressed genes. Of the 15 candidates from the RAP-PCR, 9 were shown to be false positives by repeating the RT-PCR with respective specific primers and were eliminated from further analysis. The remaining 6 fragments

(RAP1 to RAP6) were confirmed by RT-PCR to be derived from differentially expressed genes (Fig. 2). For example, the level of the RAP1 transcription was significantly increased during later growth phases, with maximal expression detected at 39 h. Higher RAP2 expressions were seen at 39, 42 and 46 h compared those at 8 and 30 h. The transcription levels of the RAP3 were higher at 39 and 42 h compared with those at 30 and 46 h, while there was a dramatic decrease in the expression of the RAP3 at 8 h. Furthermore, the expressions of the RAP4 were stronger at 30, 39 and 42 h compared with those at 8 and 46 h. The expressions of RAP5 and RAP6 were gradually increased in a time-dependent manner at 30 h, 39 h, 42 h and 46 h compared with that at 8 h. The GenBank accession numbers of RAP 1 to RAP6 were AY646412, AY646414, AY646409, AY646407, AY646406 and AY646410, respectively. We proposed that time-dependent increase of the expressions of the RAP1 to RAP6 may play roles in the development or pathogenesis of Bt.

Characterization of the differentially expressed genes following Bt development

The differentially expressed genes (RAP1 to RAP6) from Bt 8010 showed high degrees of similarities to Bt proteins possibly involved in sporulation, stress response and pathogenesis (Table 2 and Fig. 3). Of the 6 differentially expressed genes, the RAP1, including BclB C-terminal domain in a number of proteins that have extensive collagen-like triple helix repeat regions, showed 93.6% identity (294/314 bp) with triple helix repeat-containing collagen / exosporium protein H from Bt BMB171. The RAP2 showed 97.6% identity (577/591 bp) with ClpX protease and ATP-dependent protease La (LonB) from the *B. cereus* group and the close proximity of these two genes indicated they might be co-transcribed from the same operon. Genome sequence search revealed that second ATP- dependent protease La was located adjacent to the above La gene. These 3 genes were highly conserved among the *B. cereus* group, indicating the existence of a putative operon. The others (RAP3 to RAP6) encoded gene products that displayed

varying degrees of similarities to a diverse range of proteins (Table 2), mostly involved in metabolism and energy formation. More specially, RAP3, RAP4, RAP5 and RAP6 showed 97.0% (230/237 bp), 91.4% (265/290 bp), 99.7% (295/296 bp) and 98.9% (554/560 bp) identity with propionyl-CoA carboxylase beta chain (PCCB), quinone oxidoreductase, uridine kinase (UDK) and 3-methyl-2-oxobutanoate-hydroxymethyltransferase (also called ketopantoate hydroxymethyltransferase or PanB), respectively, from the *B. cereus* group.

Discussion

Changes in gene expression can be evaluated by differential display methods, so that effects on bacterial metabolic activity can be estimated by variation in transcription profiles (Dachet et al. 2010). In this study, the RAP-PCR technique has been applied to elucidate the molecular mechanism(s) underlying the differentiation process of Bt and seek for differentially expressed genes.

cDNA amplified fragment length polymorphism (cDNA-AFLP) and RAP-PCR were the most frequently used methods for evaluating modifications in transcriptomes. However, cDNA-AFLP requires restriction sites so transcripts without correct sites will not be revealed at all, while RAP-PCR uses acrylamide electrophoresis that lacks the resolution necessary to efficiently separate amplimers (Dachet et al. 2010). Another drawback of both techniques is the high number of false positives generated (Nagel et al. 2001). This explained why 9 of the 15 candidates from the RAP-PCR were shown to be false positives.

Of the 6 putative pathogenic genes identified from this study, RAP1 encoded a protein showing high sequence identity with triple helix repeat-containing collagen and exosporium protein H from the *B. cereus* group. The function of *Bacillus* helix repeat-containing collagen was mostly unknown while the spores of the *B. cereus* group harbored a loose balloon-like exosporium. The particular adherence and hydrophobic

properties conferred by the exosporium suggested that it might possibly be of significance to spore pathogenicity (Todd et al. 2003). Typically, the ICPs of Bt were deposited against the forespore, developed outside the exosporium, and were separated from spores after sporulation (Zhu et al. 2011). However, in a few strains, such as in Bt subsp. *finitimus* strains and Bt subsp. *oyamensis* strain LBIT-113, the ICPs were located between the exosporium and the spore coat and continue to adhere to the spore after mother cell lysis. This phenotype has been previously described as spore-crystal association (SCA) (Zhu et al. 2011). The protein composition of the exosporium was complex. Some may act as structural elements playing a protective role while others may be strongly absorbed, moderating the spore's germination rate (Nagel et al. 2001, Terry et al. 2011). To date, their role in determining spore architecture and spore function is still poorly understood (Terry et al. 2011).

The RAP2 contained part of two genes encoding ATP-dependent clp protease ATP-binding subunit ClpX and ATP-dependent protease La (LonB), respectively. Clp protease was originally identified *in vitro* as an ATP-dependent protease consisting of two components, the ATPase subunit and the protease (ClpP) subunit (Hwang et al. 1988). ATP-dependent proteins were believed to play roles in differentiation processes and might be virulence factors for bacterial infection in mice (de Crécy-Lagard et al. 1999, Coleman et al. 2009). ClpA and ClpX ATPases are members of a large family of closely related ATPases found in both prokaryotic and eukaryotic cells. They can function as molecular chaperones independent of ClpP. The organization of the Clp protease is complex with its functions and targets varying from one organism to another. In *Lactococci lactis*, ClpX is involved in proteolysis of heat damaged proteins and cryoprotection (Skinner and Trempey 2001). *Escherichia coli* ClpX was an ATP-dependent machine for protein unfolding and translocation and functioned with the ClpP peptidase to degrade target substrates (Stinson et al. 2015). In *B. subtilis*, Clp is involved in the regulation of competence and sporulation (de Crécy-Lagard et al. 1999,

Pan and Losick 2003)., *B. subtilis* DegS-DegU two-component regulatory system was regulated by the ClpXP-Spx regulated proteolysis system (Shiwa et al. 2015). ClpXP protease was responsible for Epsilon antitoxin degradation (Brzozowska and Zielenkiewicz 2014). The canonical *E. coli* protease La (Lon-Ec), a conserved and much studied protease important for homeostasis by targeting abnormal proteins and unstable regulatory proteins, played a role in host infection (Coleman et al. 2009). Lon protease was conserved from bacteria to humans and regulated cellular processes by degrading different classes of proteins including antitoxins, transcriptional activators, unfolded proteins, and free ribosomal proteins (Osbourne et al. 2014). Sequence analysis revealed that another gene, which also codes for ATP-dependent protease La, was located adjacent to the above protease La gene. These 3 genes were highly conserved among the *B. cereus* group, indicating the existence of a putative operon. Thus, further examination of the existence of such an operon and how it regulates the *clp* gene expression in Bt will be helpful.

Other differentially expressed genes (RAP3, RAP4, RAP5 and RAP6) obtained were mostly involved in metabolism and energy formation. The RAP3 was homologous to PCCB, which was the beta subunit of propionyl-CoA carboxylase (PCC) enzyme (Ugarte et al. 1999). It was required for the development of *Myxococcus xanthus* and its deficiency can cause propionic academia (Ugarte et al. 1999, Kimura 1997). The PCC was involved in the catabolism of branched chain amino acids, odd-numbered fatty acids, cholesterol, and other metabolites while the PCC in *Streptomyces coelicolor* can carboxylate different short chain acyl-CoAs (Arabolaza et al. 2010; Chiu et al. 2014).

RAP4 had homology to quinone oxidoreductase of *B. cereus* ATCC 14579 and seemed to be involved in electron transport and ATP formation. Disulphide bond formation catalyzed by thiol-disulphide oxidoreductases (TDORs) was a universally conserved mechanism for stabilizing extracytoplasmic proteins. A TDOR enzyme (BdbD) of *B. subtilis* had been shown to require a quinone oxidoreductase for activity (Kouwen et

al. 2007).

RAP5 showed significant homology to the uridine kinase (UDK) of the *B. cereus* group, which was a phosphotransferase that mediated phosphorylation of uridine, azauridine and cytidine to produce uridine- azauridine- and cytidine-monophosphate (UMP / aza-UMP / CMP) (Gagyi et al. 2003). The ATP-driven conversion of uridylate monophosphate into uridylate diphosphate was an essential metabolic step in prokaryotes (Meier et al. 2008).

RAP6 shared high sequence identity with 3-methyl-2-oxobutanoate-hydroxymethyltransferase (MOHMT) (EC 2.1.2.11) [also known as ketopantoate hydroxymethyltransferase (KPHMT) or PanB] from the *B. cereus* group. This enzyme was indispensable for the synthesis of bacterial pantothenate (vitamin B₅), which was an essential precursor of coenzyme A (CoA), a key molecule in many metabolic reactions including the synthesis of phospholipids, synthesis and degradation of fatty acids, and the operation of the tricarboxylic acid cycle (Kleinkauf 2000, Villasenor et al. 2011).

If more primers were used, we certainly would obtain more candidate genes for Bt pathogenesis. However, we think it is more urgent to elucidate the precise functions of the 6 putative pathogenic genes (RAP1 to RAP6) by gene knockout and gene complementation. Herein, the genes are currently being disrupted through homologous recombination using pRN5101 thermosensitive vector as previously described (Tran et al. 2011). Based on the comparison of RAP1 gene knock-out mutant with the wild type strain, Exp protein conferred protection to the cells by avoiding premature germination of the cells (unpublished data). Herein, it plays a critical role in the development or pathogenesis of Bt. In conclusion, RAP-PCR was a reliable fingerprinting method to reveal differentially expressed genes following Bt development.

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Text to figures

Fig. 1 The products of RAP-PCR with arbitrary primer A-07 [Lane 1-5 = the products amplified from the total RNA of Bt 8010 at 8 h, 30 h, 39 h, 42 h, 46 h of sub-culturing, respectively; Lane M = 100 bp DNA Ladder Plus. Arrows indicated some differentially expressed fragments. The designation of the corresponding RAP-PCR products was indicated in Table 2]

Fig. 2 Reverse transcription PCR (RT-PCR) analysis of 6 differentially expressed gene in Bt 8010 [RT-PCR with respective specific primers was performed using the total RNA extracts from the sub-cultured cells at 8 h (lane 1), 30 h (lane 2), 39 h (lane 3), 42 h (lane 4) and 46 h (lane 5) as the templates. The RAP1 to RAP6 corresponded to those given in Table 2]

Fig. 3 Comparision of 6 RAP genes of Bt 8010 with genomic sequence of Bt BMB171 [(A) RAP1. (B) RAP2. (C) RAP3. (D) RAP4. (E) RAP5. (F) RAP6. Gene symbols and gene loci were given above and below each gene, respectively. The RAP gene coordinates were indicated with lines above the genes of Bt BMB171]

Fig. 1

Draft

Fig. 2

Fig. 3

Table 1 Specific primers used to confirm the differentially expressed genes

Designation ^a	Primers	Sequences
RAP1	RAP1F	5'-GCAACAGGTTTAACTGGTGCAAC-3'
	RAP1R	5'-GGAGATAATGCAGCTGTTGC-3'
RAP2	RAP2F	5'-CGGGTGCTCGTGGACTTCGTTC-3'
	RAP2R	5'-GTTGCGGATTCGGACCACAAAG-3'
RAP3	RAP3F	5'-TCTTCGGTACAGTTGCTTCACC-3'
	RAP3R	5'-GAAGGGTGGCGTATTATTCCAC-3'
RAP4	RAP4F	5'-GCCGCTGTGAAATCATCAATGG-3'
	RAP4R	5'-CTTAATTCCCATCACAGGAGCG-3'
RAP5	RAP5F	5'-GACCATTATCAAACGCAAGCGG-3'
	RAP5R	5'-ATGGGGACGAATAAGCCTGTTG-3'
RAP6	RAP6F	5'-TACGAAAGCTGTACGCCGCG-3'
	RAP6R	5'-GCGCCAATCCCAATTGTCGG-3'
RAP7	RAP7F	5'-CGGGTGCACCAAGTACAGAAG-3'
	RAP7R	5'-TGCAGCGCTATTAGCATTCGTC-3'
RAP8	RAP8F	5'-AAACGGGTGACCTCCTGTATTC-3'
	RAP8R	5'-TTGAGCAGCGAACGGTAAGTTC-3'
RAP9	RAP9F	5'-GTACCAACACATTTTAGGGC-3'
	RAP9R	5'-GGTGTGAACTCGGATTTGG-3'
RAP10	RAP10F	5'-TTGCGATAAAGTTGCCCGAAGC-3'
	RAP10R	5'-CGGATGTGCTGGATAATGACAC-3'
RAP11	RAP11F	5'-TAGTGCTTACTGATCGTCGCAC-3'
	RAP11R	5'-ATCTAACAAGTTATCCCCCCCC-3'
RAP12	RAP12F	5'-ACGGACTCGCATTCTATTCC-3'
	RAP12R	5'-TAGACCACCATAAGAACCCC-3'
RAP13	RAP13F	5'-GTTGTAGAGACCCATGACAG-3'
	RAP13R	5'-ACGGACTCCATGAGAGAAAC-3'
RAP14	RAP14F	5'-CTCGAACTATCTGACTGCAC-3'
	RAP14R	5'-TTGATTGCTGAGGGGATGTC-3'
RAP15	RAP15F	5'-GGGTGACGGAGGAGCGAGAC-3'
	RAP15R	5'-AGCGCCATATTTTCCCGGCC-3'

^a RAP1–RAP6 were the differentially expressed transcripts.

Table 2 Homology analysis of nucleotide sequences of DNA fragments derived from RAP-PCR

Designation ^a	Band size (bp)	Origin	Closest homology (accession no.); % Similarity ^b
RAP1	314	39 h	Bt BMB171 (NC_014171); 294/310 (95%); Triple helix repeat-containing collagen/ Exosporium protein H
RAP2	591	42 h	Bt BMB171 (NC_014171); 573/581 (99%); ATP-dependent Clp protease ATP-binding subunit ClpX, ATP-dependent protease La
RAP3	237	42 h	Bt BMB171 (NC_014171); 219/228 (96%); propionyl-CoA carboxylase beta chain
RAP4	290	42 h	Bt BMB171 (NC_014171); (262/281) 93%; Quinone oxidoreductase
RAP5	296	42 h	Bt BMB171 (NC_014171); 285/286 (99%); Uridine kinase
RAP6	560	46 h	Bt BMB171 (NC_014171); (554/560) 99%; 3-methyl-2-oxobutanoate- hydroxymethyltransferase

^a RAP1–RAP6 were the differentially expressed transcripts. The GenBank accession numbers were AY646412, AY646414, AY646409, AY646407, AY646406 and AY646410, respectively.

^b The percent similarity was showed at the nucleotide level.