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Constitutive and Secretory Expression of the AiiA in *Pichia pastoris* Inhibits *Amorphophallus konjac* Soft Rot Disease

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Abstract

Amorphophallus konjac **is an important economic crop widely cultivated in Southeast Asia and Africa. However, *A. konjac* is seriously infected by soft rot pathogen. The endocellular acyl homoserine lactonase (AiiA) which is generated by *Bacillus* species has inhibitory effect on soft rot pathogen through disrupting the signal molecules (N-acylhomoserine lactones, AHL) of their Quorum Sensing system. The aim of our study is to obtain recombinant yeast which produces AiiA protein. The recombinant yeast *Pichia pastoris* GS115 was constructed to constitutive expression of the *AiiA* gene. The results of reverse transcript PCR analysis showed that the *AiiA* gene was expressed successfully in the yeast. Proteins extracted from YPDS showed the highest inhibition efficacy to *E. carotovora* compared with the other two mediums (YPD and LB) under tested conditions.**

Keywords

***Amorphophallus konjac*, Acyl Homoserine Lactonase, *Pichia pastoris*, Fusion-Expression**

1. Introduction

The genus *Amorphophallus* has been used as food, medicine, fodder and wine production [1]. *Amorphophallus konjac* which is one of the most widely cultivated species has been grown in China for more than 2000 years [2] [3]. *A. konjac* is a perennial plant with a huge commercial value to produce Glucomannan, which is a polysaccharide consisting of glucose and mannose residues at a molar ratio of 2:3 with β-1, 4 linkages [4]. Several clinical trials show that Glucomannan is responsible for lowering systolic blood pressure, total cholesterol and gly-

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cemia [5] [6]. Unfortunately, bacterial soft rot disease has a large impact on the yield of *A. konjac*. The soft rot *Erwiniae* which is the main soft rot disease bacterial on *A. konjac*, usually exits in soli, ground water and plant surface. Once inside the plant they reside in the vascular tissue and intercellular spaces of suberized or thin-walled parenchymatous tissues and will develop when environmental conditions become suitable, including free water, oxygen availability and temperature [7] [9]. During the *Erwiniae*-plant interaction, multiple cell wall degrading enzymes (exoenzymes) are secreted by *Erwiniae*, including pectinases, cellulases and proteases, which break down plant cell walls and release nutrients for bacterial growth. With successful release of nutrients during infection, other non-pectolytic bacteria will co-grow on the plant and, indeed, other pectolytic and non-pectolytic bacteria are often isolated from diseased plant tissues [8]. The facultative anaerobic pathogen *Erwiniae* causes maceration and rotting of parenchymatous tissues of all organs, eventually resulting in plant death [10]. For a long time, this disease has been a huge problem to *A. konjac* production.

Quorum signaling (QS) is an intercellular communication mechanism and widely employed by bacteria to coordinate behaviors such as bioluminescence, antibiotic synthesis, biofilm formation, adhesion, swarming, competence, sporulation, virulence, and others [11]. Using chemical signals organisms can detect their local population and change their gene expression so as to competitively optimize their behavior in their local environment. In the case of *Erwiniae*, N-acylhomoserine lactones (AHLs) are chemical signal molecules that responsible for regulating the production of plant cell wall degrading exoenzymes [9] and the antibiotic carbapen-3-em carboxylic acid which function in competing with other bacteria [12]-[14]. Degradation of AHLs has been proven as the efficient control of bacterial infections in transgenic plants [15].

The first AHLs degradation enzyme was identified from a soil bacterial isolate *Bacillus* species belonging to Gram positive bacteria, encoded by *aiiA* gene [16]. The enzyme has been subjected to study and is of interest as its resultant effect to disrupt bacteria's ability to communicate. The *Bacillus* aiiA enzyme is a metalloprotease containing two zinc ions in close proximity. AiiA degrades AHLs in a tail length independent manner but the tail is required for activity [17].

As one of the anti-quorum signaling strategies, degradation of AHL-signaling molecules using aiiA enzyme could have potential applications in attenuating plant disease. In this paper, a safely and constitutive expressional recombinant yeast was reported that efficiently produces aiiA protein using for attenuating plant disease. The recombinant yeast *Pichia pastoris* GS115 was constructed to constitutive expression of the *aiiA* gene. The *aiiA* gene expression was confirmed by reverse transcript PCR analysis in the yeast. AiiA enzyme products were extracted from yeast fermentation broth and effectually inhibited the biassay of *Erwinia carotovora.*

2. Materials and Methods

2.1. Bacteria, Medium, and Culture Conditions

Escherichia coli DH5*α* and BL21 were grown in Luria-Bertani broth (LB) (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, pH 7.0) at 37˚C for propagation of plasmids and protein expression, respectively. The strain of *P. carotovora* subsp. *carotovora* (P.c.c) was isolated from an infected corm of konjac from Hubei province in China (Registry number: FJ463871). *Bacillus thuringiensis* strain 4Q7 was cultured in LB medium at 28˚C. Host strain *P. pastoris* GS115 was purchased from Invitrogen (USA). YPD (1% yeast extract, 2% peptone, 2% glucose) and YPDS (YPD, 1 M sorbitol) medium were prepared as described in the manual of the *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, USA).

2.2. Extraction of Total DNA, RNA, PCR and Sequencing

Based on the sequence of *aiiA* gene from the NCBI (NC_018877.1), gene specific primers were designed for *aiiA* gene cloning (P1: 5'GTCGGATCCATGACAGTAA AGAAGCTTTA3', P2: 5'GTCGCGGCCGCCTATATATACTCAGGGAACA3'). DNA was isolated from *B. thuringiensis* or *P. pastoris* by the modified CTAB method [18]-[20]. Total RNA from *P. pastoris* was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To eliminate genomic DNA contamination, purified RNA was treated with RNase-free DNase I (Takara, Dalian, China) before final ethanol precipitation. Next, 3 µg of total RNA was reversely transcribed into cDNA by reverse-transcription with Superscript II (Invitrogen) and an oligo-dT20 primer. The final cDNA was stored at −20˚C before use.

Polymerase chain reactions (PCRs) were carried out in a PTC-100 Thermal Cycler (MJ Research, Massachu-

setts, USA) with gene-specific PCR primers following the procedure: initial denaturation at 94˚C for 3 min, 35 amplification cycles of 94˚C for 20 s, 54˚C for 30 s, 72˚C for 1 min and final polymerization step of 72˚C for 7 min. Each PCR mixture (25 µl) contained 0.1 µM primers of P1 and P2, 20 mM dNTP (Dingguo Ltd., China) (10 mMol of each), 40 mM $MgCl_2$, Taq polymerase buffer, 10 ng template-DNA, 1U Taq polymerase (Promega Co., China) and 15 µl mineral oil were added to each of the reaction solutions. The final PCR product was resolved in 1% agarose gel and purified using the AxyPrep gel purification kit (Axygen, Union City, CA, USA). PCR products sequencing was performed by Sangon biotech (Shanghai, China). The protein O-glycosylation site was identified by NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) [21].

2.3. Plasmid Construction

For *aiiA* gene expression in *E. coli*, the full length of *aiiA* gene without stop codon was amplified using gene-specific PCR primers as described above. After restricted by *BamH*I and *Not*I, the 762 bp fragment was cloned into the expressional vector pET22b between *BamH*I and *Not*I restriction enzyme sites to form pET22b-aiiA (**Figure S1**). Similarly, *aiiA* gene without stop codon was cloned into the expressional vector pGAPZ*α*-A between *EcoR*I and *Not*I restriction enzyme sites for expression in *P. pastoris* to form pGAPZ*α*-A-aiiA. The primers using for vector construction were list in **Table 1**.

2.4. Expression, Purification and Detection of Recombinant AiiA in *E. coli*

E. coli BL21 cells (10 µL) containing a phagemid pET22b-aiiA was inoculated into 20 mL of LB liquid medium plus 100 µg/mL ampicillin and incubated overnight at 37˚C on a shaker (200 rpm). The next day, 2 mL of the cultured cells was transferred to 100 mL of LB medium with the antibiotics and grown to an OD 600 value of 0.5. After addition of IPTG to a final concentration of 0.4 mM, the culture was incubated at 16˚C for 12 h with shaking. The cells were collected by centrifugation (10 min, 3000 g, 4˚C). The resulting pellets were resuspended in 1 mL of buffer containing 30 mM Tris/HCl and 1 mM EDTA and stored for 15 min on ice, followed by ultrasonication. After centrifuging (10,000 g, 15 min, 4˚C), the supernatant was collected and dialyzed by Ni-NTA-agarose (Qiagen, Chatsworth, CA) and the pellet was resuspended in 8 M-urea.

Proteins were separated by 12% (w/v) SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot was developed using a 1:5000 diluted monoclonal anti-His antibody and AP-conjugated goat anti-mouse IgG antibody. The colorimetric reaction was carried out by using the BCIP/NBT Color Development Kit (Boster). The immunoblot membranes were scanned with Epson perfection V500 Photo.

2.5. Pichia Pastoris Transformation

The recombinant plasmid pGAPZ*α*-A-aiiA was linearized by digestion with *Bgl*II and then transformed into *P. pastoris* GS115 by electroporation at 1.5 kV with a 2 mm cuvette. Then, 800 µL ice-cold sorbitol was immediately added to the cuvette, and the mixture was spread on YPDS plates containing 100 µg/mL Zeocin. Then the plates were incubated at 30˚C for 3 days until colonies form. The selected transformants were inoculated into new YPDS plates containing 100 µg/mL Zeocin. Ten of Zeocin-resistant *P. pastoris* transformants were chosen for the presence of insert detection.

Table 1. Sequences of primers used for this study.

Primer	Sequence (5’-3’)
aiiAF	CTGCGGATCCGACAGTAAAGAAGCTTTATTTCATCC
aiiAR	GTCGCGGCCGCTATATATTCTGGGAACACT
aiiA-His-F	AGTAGCGTATGGATATCGGAATTAAT
aiiA-His-R	CAAAAAACCCCTCAAGACCCG
pGAP-F	GTCCCTATTTCAATCAATTGAA
AOX1	GCAAATGGCATTCTGACATCC

2.6. Fermentation of Recombinant AiiA in Shake-Flasks

Two positive transformants were selected and inoculated into 300 mL YPD, YPDS or LB medium. Flasks were cultured at 16˚C and 30˚C, for one to three days at 200 rpm. The supernatant were collected by centrifugation at 5000 g, 4˚C for 10 min. Crystalline ammonium sulfate is slowly added to the supernatant layer to a final concentration of 450 g/L (70% saturation), and the mixture is stirred for 2 h at 4˚C. Proteins were collected by centrifugation (13000 g, 15 min, 4˚C) and resolved by PBS.

2.7. *In Vitro* Bioassay of Purified Proteins on P.c.c

The CPA media was used to assay the growth of P.c.c instead of *A. konjac* leave or shoot basal discs. The CPA media contained 1% hydroxypropyl methylcellulose, 1% pectin, 5.6 g/L NH_4NO_3, 0.5 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4 \cdot 7H_2O$ and 1% Agarose (Low melting point). The antibacteria activity of aiiA protein was studied by agar diffusion test using CPA media. Overnight cultures of P.c.c were diluted 1:10,000 into fresh, prewarmed CPA media (35˚C) and 20 mL mixture was added into a 9 cm petri dish. Wells were made in each plate using a 5 mm sterile puncher and 50 μL aqueous solutions of protein or control buffer were placed in respective wells. The plates were incubated at 28˚C for 3 d and then photographed.

3. Results

3.1. Gene Cloning and Sequence Analysis

The full length without stop codon (750 bp) was amplified from *B. thuringiensis* strain 4Q7 using the primers aiiAF/aiiAR or aiiA-His-F/aiiA-His-R to clone into pET22b expression vector (expressed in *E.coli*) or pGAPZ*α*-A expression vector (expressed in *P. pastoris*). The recombination *aiiA* gene encoded a 269 (in *E.coli*) or 369 (in *P. pastoris*) amino acid polypeptide with N-terminal secretion peptides and C-terminal His-tag. After splicing, the fusion protein was secreted with a calculated molecular mass of 29.2 kDa and a pI of 4.87. No potential O-glycosylation site was identified by NetOGlyc 4.0 Server.

3.2. Expression and Purification of Recombinant AiiA in *E. coli*

To investigate whether the fusion aiiA protein would inhibit *E. carotovora in vitro*, we expressed the fusion aiiA protein by *E. coli* expression system. After addition of IPTG (0.4 mM) for 6 h, the fusion aiiA protein was significantly detected by SDS-PAGE compared with the no IPTG addition sample (**Figure 1(a)**). The aiiA fusion

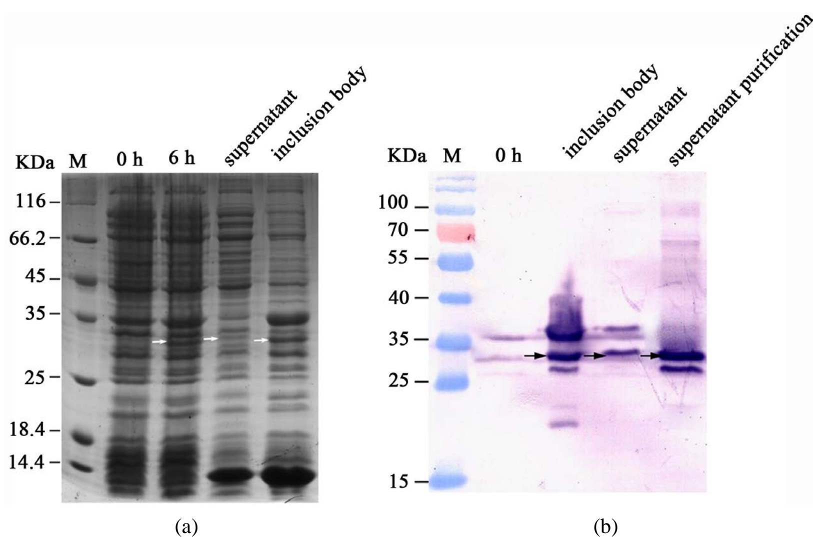

Figure 1. SDS-PAGE and Western blot analysis of proteins extracted from transgenic *E. coli*. (a) SDS-PAGE analysis of total proteins extracted from *E. coli* cultured for 0 h, 6 h and 12 h (supernatant and inclusion body) after adding IPTG; (b) Western blot analysis of proteins in supernatant and inclusion body extracted from *E. coli* cultured for 12 h after adding IPTG. M, protein molecular mass standards; White arrows labeled the aiiA protein band.

protein existed both in supernatant and precipitate (**Figure 1(a)**). This indicated the toxicity of aiiA fusion protein to *E. coli* cell. When adding IPTG to 1 mM or changing the expression condition to 28˚C, most of aiiA fusion proteins formed inclusion bodies (data not shown). This phenomenon supplementary verified our hypothesis. To increase the production of aiiA fusion protein, the concentration of 0.4 mM IPTG was chosen to induce protein expression. After culturing for 12 h at 16˚C, *E. coli* cells were collected for protein extraction and purification and the purification soluble protein concentration was 200 ng/μL (**Figure 1(b)**).

3.3. Effect of Purified aiiA Fusion Proteins on *E. carotovora*

Agar diffusion test was used to detect the inhibition of aiiA fusion proteins on *E. carotovora*. The *E. carotovora* cells were mixed with CPA media medium and perforated with sterile puncher. The well adding 200 ng/μL aiiA fusion protein remarkably inhibited *E. carotovora* growth. Howerer, the well adding PBS (control to aiiA fusion protein), the purified protein from *E. coli* and the purified protein from control bacteria (bacteria transformed with empty plasmid vector instead of expressing aiiA fusion protein) were fully covered with *E. carotovora* colonies (**Figure 2**). Our results indicated the aiiA fusion protein had the ability to inhibit *E. carotovora* growth and was valuable for further reproduction.

3.4. Expression and Fermentation of Recombinant AiiA in *P. pastoris*

With the expression vector pGAPZ*α*, the aiiA protein was transformed into *P. pastoris* GS115. The presence of insert expression cassette was detected using PCR and the 1100 bp fragment was amplified by primers pGAP-F/AOX1 (**Table 1**; **Figure 3(a)**). To verify the aiiA fusion gene was expressed in transcriptional level, RNA was extracted from two positive *P. pastoris* transformants and was reverse transcript into cDNA. The 770 bp aiiA transcriptional fragment was amplified using primers aiiAF/aiiAR (**Figure 3(b)**).

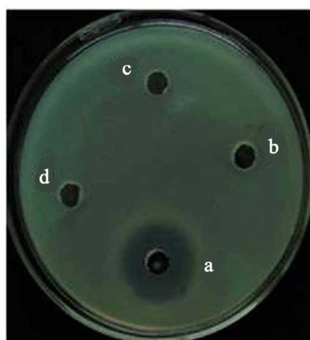

Figure 2. Bioassay of purified proteins from transgenic *E. coli* on *E. carotovora.* The activity of *aiiA* protein was studied by agar diffusion test using CPA media. 50 μL samples were placed in respective wells. The plates were incubated at 28˚C for 3 d and photographed. a: *aiiA* fusion protein; b: PBS; c: the purified protein from *E. coli*; d: the purified protein from control bacteria (bacteria transformed with empty plasmid vector instead of expressing aiiA fusion protein).

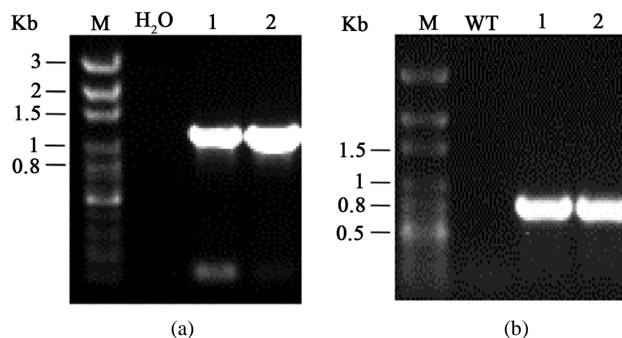

Figure 3. PCR and RT-PCR analysis of transgenic *P. pastoris.* Amplicons of aiiA gene from transgenic *P. pastoris* genome (a) and transcript (b). M; DNA molecular marker, 1; transgenic *P. pastoris*-1, 2; transgenic *P. pastoris*-2; H_2O and wild-type cDNA were used as negative control, respectively.

Three fermentation mediums including LB, YPD and YPDS were selected to research the efficacy of aiiA fusion protein production at the temperature of 16˚C and 30˚C. Proteins were extracted 3 d post inoculated from the supernatant of fermentation broth. The antibateria of extracting solution was measured by agar diffusion as describe above. With the same condition, proteins extracted from YPDS showed the highest inhibition efficacy to *E. carotovora* compared with the other two mediums (YPD and LB) (**Figure 4**). Protein expression at 30˚C was higher than at 16˚C (date not shown). Finally the YPDS was chosen to express the aiiA fusion protein at 30˚C for 3 d.

4. Discussion

The soft rot is a serious damage to *A. konjac* production which is mainly caused by *E. carotovora*. Current protective measures rely on chemical control, producing bactericide-resistant pathogens and other undesirable environmental consequences [22]. Alternative strategies must be found to protect *A. konjac* crops from *Erwiniae* pathogens. The use of biochemical tools is gaining great momentum in crop protection and these may be a supplement or an alternative to chemical pesticides control. The increased understanding of the quorum sensing has made possible using the enzyme aiiA as a diagnostic tool [23] [24]. AiiA have been expressed in several pathogens or transgenic plants [10] [16] [25]-[27]. However, these transgenic strains were not suitable for aiiA production due to the attenuated growth caused by aiiA or the bacterial security. To improve the production of aiiA, aiiA was constitutively and secretory expressed in an atoxic fungi *P. pastoris*.

E. carotovora secretes exoenzymes including pectinases, cellulases and proteases, that contribute to the pathogenesis of plant. AHLs regulate the production of plant cell wall degrading exoenzymes in *E. carotovora* [12]. *In vitro* bioassay of *E. carotovora* growth, the cell number was comparatively rare at 200 ng/μL aiiA proteins (extracted from *E.coli*). That indicated AHLs were degraded by aiiA fusion proteins and the transcription of the exoenzyme structural genes were not coordinated to high levels by AHLs in *E. carotovora.*

The promoter of the gene (GAP) encoding the GAPDH protein has recently been characterized and shown to express recombinant proteins to high levels in *P. pastoris*, depending on the carbon source used [28]. The level of expression seen with the GAP promoter can be slightly higher than that obtained with the AOX1 promoter (an inducible promoter usually used in *P. pastoris* expression) [29]. Moreover, the GAP promoter is a constitutive promoter and it is convenient for aiiA fusion protein production. After cloning the aiiA-His gene to GAP promoter expression cassette, the expression cassette was transformed into *P. pastoris* and inserted into the genome sequence. The transcription of aiiA-His was detected by RT-PCR. Three days post inoculation, high level transcription of the aiiA-His gene was detected in transgenic *P. pastoris*.

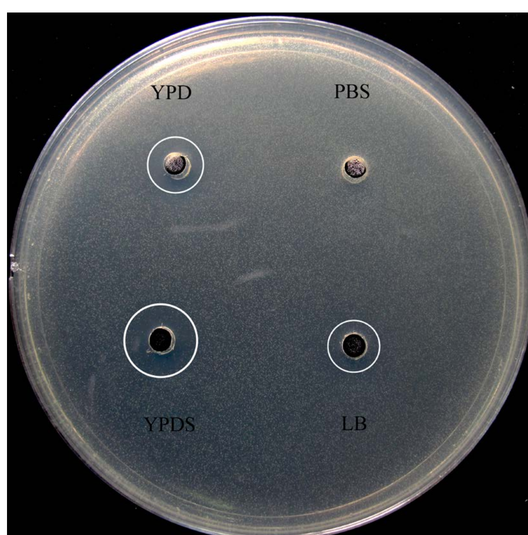

Figure 4. Bioassay of purified proteins on *E. carotovora* from different transgenic *P. pastoris* fermentation broth. AiiA proteins were extracted from *P. pastoris* fermentation broth (LB, YPD and YPDS) 3 d post inoculation. 50 μL aqueous solutions of protein were placed in respective wells. The plates were incubated at 28˚C for 3 d and photographed. PBS was used as negative control.

To improve the production of aiiA-His, three fermentation mediums (LB, YPD and YPDS) were selected to research the efficacy of aiiA fusion protein production under 16˚C and 30˚C. According to the result of agar diffusion test, highest antibacteria activity was observed in YPDS-grown cells, although aiiA-His was constitutively expression in all three fermentation mediums at 30˚C. That was consist with the Northern blot analysis of total RNA isolated from *P. pastoris* cells grown on glucose-carbon sources [28].

5. Conclusion

In conclusion, we constitutively expressed of the AiiA-His gene in *P. pastoris* and achieved high-yield fermentation of AiiA-His protein. The AiiA enzyme products extracted from yeast fermentation broth effectually inhibit the growth of *E. carotovora.* Our results indicated that direct application of AHL-lactonase to control *E. carotovora* infection might be an effective alternative of chemical control to avoid the emergence of bactericide-resistant strains.

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Supplement

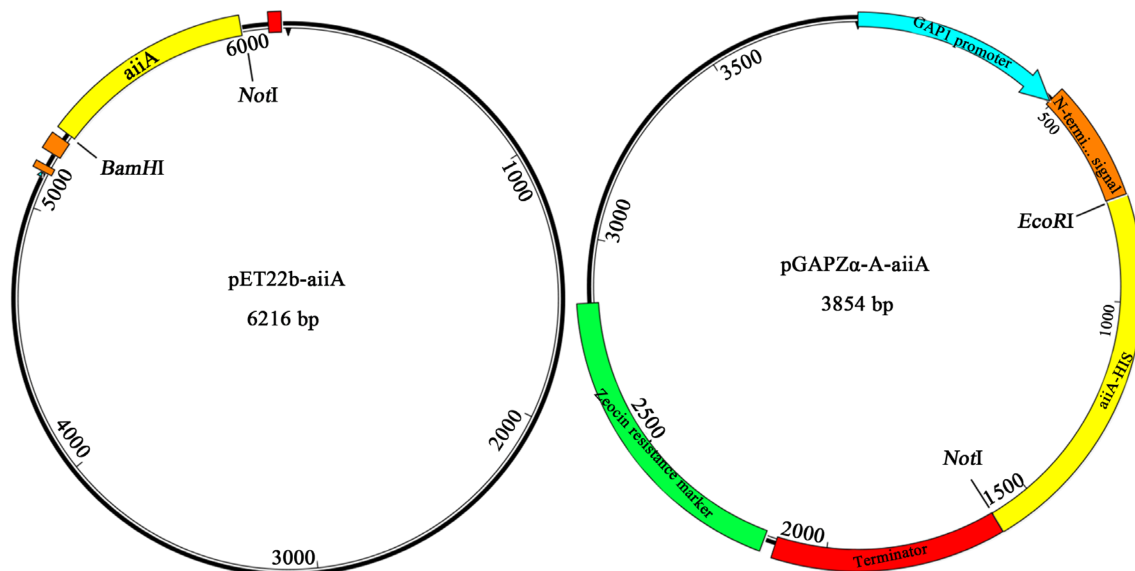

Figure S1. Plasmids used in this study.