A CHEMICAL TRANSFORMATION OF GREEN FLUORESCENT PROTEIN (GFP) GENE INTO GRAM-POSITIVE ENDOPHYTIC BACTERIUM, *Bacillus cereus* EB2

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The efficacy of biological control agents (BCAs) such as bacteria, fungi, actinomycetes and mycorrhiza, to control *Ganoderma* disease in oil palm has shown potential positive effect (Angel *et al*., 2016; Ramli *et al*., 2018; Shariffah-Muzaimah *et al*., 2018; Sundram *et al*., 2015). Reports of Gram-negative endophytic bacteria (EB) from genera *Pseudomonas* and *Burkholderia* as BCAs for controlling various plant diseases including *Ganoderma* disease in oil palm (Maizatul-Suriza *et al*., 2012; Nasyaruddin *et al*., 2011; Ramli *et al*., 2016) have been published. Currently, the use of Gram-positive bacteria as BCAs in agriculture shows advantageous potential because Gram-positive bacteria are generally safer than Gram-negative bacteria, and the spore forming character of *Bacillus* species lends a longevity advantage to its use as an agricultural agent (Olubajo and Bacon, 2008). This transformation protocol was initiated to investigate and verify the colonisation nature of EB as an endophyte in oil palm roots. However, Gram-positive endophytic bacteria are difficult to transform since the composition of Gram-positive's cell wall hinders the uptake of foreign DNA (Rattanachaikunsopon and Phumkhachorn, 2009). In the present days, electroporation is the most commonly used transformation method for Gram-positive bacteria (Pyne *et al*., 2013; Zhang *et al*., 2015). However, electroporation requires a substantial investment in specialised equipment. Induce competency though chemical treatment is the least used method compared to other protocols, a few studies demonstrated the effectiveness of this procedure to introduce exogenous DNA into the cells (Ren *et al*., 2017; Vojcic *et al*., 2012).

Therefore, this study was initiated in parallel along with the *in vivo* assessment of the endophytic bacteria in oil palm. The broad host range of plasmid containing constitutively expressed green fluorescent protein (GFP) gene is useful for tracking bacterial colonisation inside the plants (Sun *et al*., 2014). GFP is preferred for observing live cells, because the use of GFP does not require fixation of plant tissues and no substrate or cofactor is necessary (Kandel *et al*., 2017). As a control, the Gram-negative endophyte, *Pseudomonas aeruginosa* EB35 was used. We have described here the adapted chemically prepared competent cells and transformation protocol for pBBRGFP-45 plasmid, which harbours the kanamycin resistance and GFP genes, into *Bacillus cereus* EB2 and optimised the concentration of antibiotic for both selected endophytes. The transformation procedure was crucial since the verification of the endophytic nature of the *B. cereus* EB2 was verified via confocal microscopy imaging.

WHY DOES MPOB PROVIDE THE SERVICE?

Researchers interested in pursuing any genetic transformation studies need to be familiar with genetic transformation protocol, involving setting up electroporation system, conduct verification and visualisation of endophytes characteristic for internal colonisers and biological control agents using GFP gene. These activities are time consuming and costly. Therefore, MPOB offers you this GFP tagged *Bacillus cereus* endophyte service to reduce time and avoid the cost taken to set up the facilities for genetic transformation.

PROCEDURES

The cells were made competent using respective chemical treatment to *Bacillus cereus* EB2 and *Pseudomonas aeruginosa* EB35 (Sirajuddin and Sundram, 2020), followed by transformation protocol reported for *Pseudomonas aeruginosa* (Chuanchuen *et al*., 2002). In this study, kanamycin concentration in the selective medium was also optimised. Preliminary findings using qualitative analysis of colony polymerase chain reaction (PCR)-GFP demonstrated the presence of putative

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positive transformants for *B. cereus* EB2 and *P. aeruginosa* EB35. The positive transformants were then verified using molecular techniques such as observation of putative colonies on specific media under UV light, plasmid extraction and validation analyses, followed by fluorescence microscopy. Therefore, this finding demonstrated the potential of chemically prepared competent cells and the crucial step of heat-shock in foreign DNA transformation process of Gram-positive bacterium, namely *B. cereus*, was required for successful transformation.

NOVELTY/HIGHLIGHTS OF THE TECHNOLOGY

- Demonstrates the co-existing between naturally occurring/wild and introduces pBBRGFP-45 plasmid DNAs in the *B. cereus* EB2 and *P. aeruginosa* EB35 cells.
- Confocal visualisation of GFP gene exhibits the endophytic nature of *B. cereus* EB2 and *P. aeruginosa* EB35 cells by colonising the internal tissues of oil palm ramets.

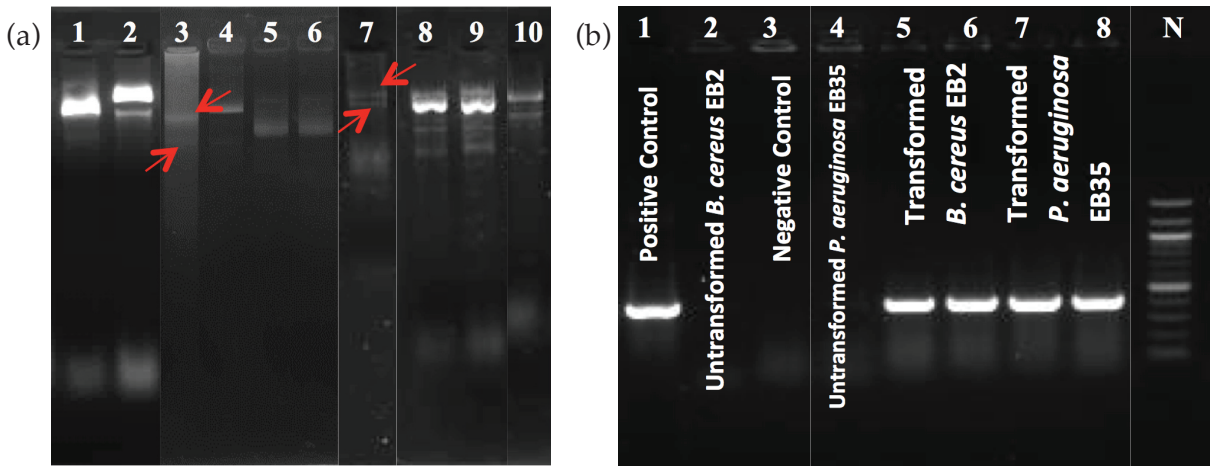

Figure 1. Presence of naturally occurring and/or introduced plasmid in the bacterial cells. (a) Detection of plasmid DNAs isolated from untransformed B. cereus *EB2 (lane 3) and* P. aeruginosa *EB35 (lane 7) and transformed cells of* B. cereus *EB2 (lanes 4-6) and* P. aeruginosa *EB35 (lanes 8-10) using agarose gel electrophoresis while E. coli DH5α harbouring pBBRGFP-45 plasmid (lanes 1-2) as control. The arrowhead indicates plasmid DNA native to* B. cereus *EB2 and* P. aeruginosa *EB35 cells, respectively. (b) Plasmid PCR-GFP using the introduced (lanes 5-8) or naturally occurring (lane 2 and 4) plasmid DNAs that were isolated from their respective cells as templates in the amplification assay and positive (lane 1: extracted pBBRGFP-45 plasmid from* E. coli *DH5α) and negative (lane 3: sterile distilled water) controls; N: 100 bp ladder (NEB, UK).*

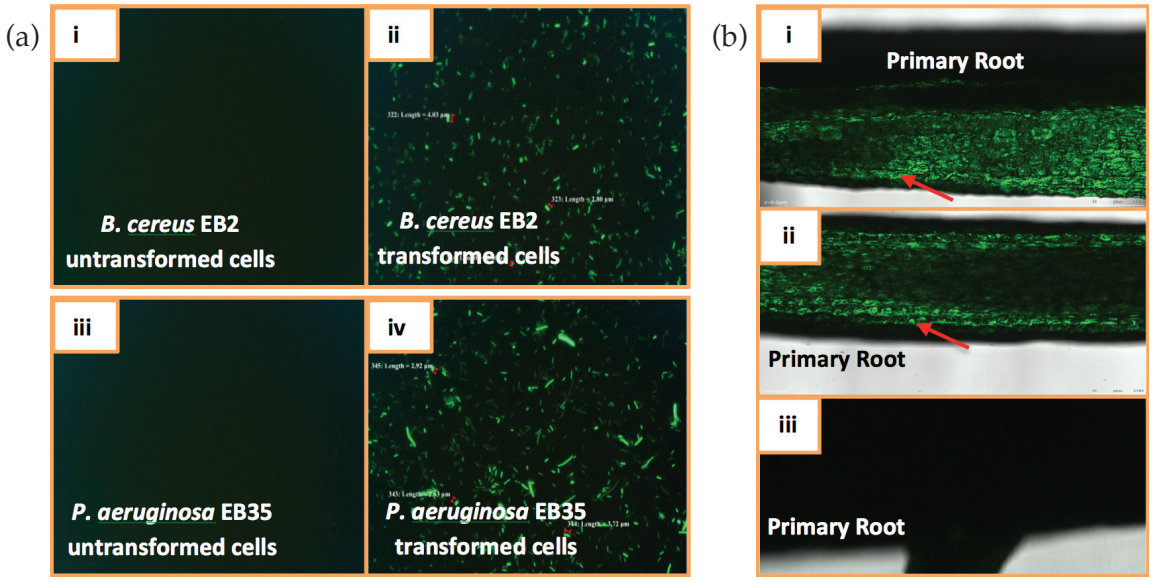

Figure 2. Appearance of GFP signals as green bright spots/lines in bacterial cells or oil palm ramet roots, respectively. (a) Bacterial cells harbouring pBBRGFP-45 plasmid were displayed as bright green rods (ii and iv) that are not observed in untransformed cells (i and iii) under fluorescence microscopy (X40) observation. (b) Oil palm ramet roots colonised by B. cereus *EB2-gfp (i) and* P. aeruginosa *EB35-gfp (ii), respectively, were visualised under 10X magnification. Arrows indicate the GFP signals in specified roots. No GFP spots were observed for the control primary root (iii).*

The presence of both wild and foreign plasmid DNA in the cells (*Figure 1a*, lanes 5-6 and 8-9) signifies that a single cell may have multiple co-existing plasmids, and in this study, these natural plasmids do not interfere with the transformation result but to a certain limit of co-existing. Following this, the visualisation of expected amplified products (~442 bp) indicates the existence of the introduced plasmid DNAs in the transformed bacterial strains (lanes 5-8) while in the untransformed cells (lanes 2 and 4), only the naturally occurring plasmid DNAs are present, thus no amplified product is detected (*Figure 1b*). In addition, fluorescence microscopy observation also displayed the positive introduction of pBBRGFP-45 plasmid into *B. cereus* EB2 and *P. aeruginosa* EB35 cells (*Figure 2a*). GFP signals appeared as green bright spots/lines in the specified root (i and ii) whereas no GFP spot was observed in control primary root (iii) as via confocal visualisation (*Figure 2b*).

SERVICE OFFERED

The procedure outlines the transformation for *B. cereus* EB2 and *P. aeruginosa* EB35 endophytes which involve the following steps (*Figure 3*) as follows:

The cost of Gram-positive bacterium *B. cereus* transformation service per sample is estimated to be RM2900. The time required to complete the procedures is approximately eight weeks, excluding the optimisation period. Prices are subjected to change depending on customer's enquiry and cost of consumables.

THE CLIENTS

The service is available to all stakeholders in the agricultural industries, scientific communities from research organisations and universities.

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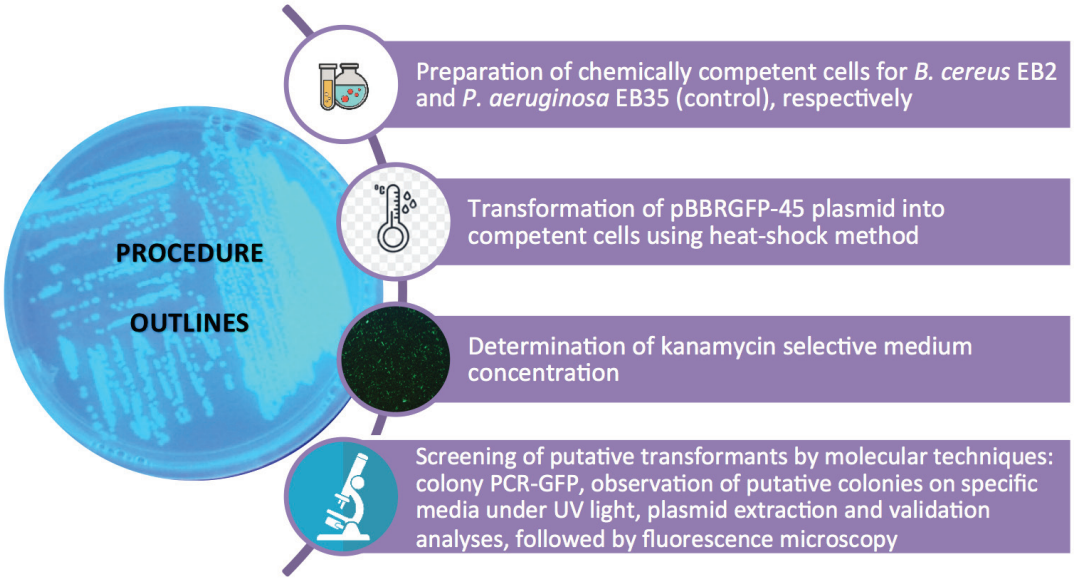

Figure 3. General workflow for endophytic bacteria transformation and subsequent qualitative analyses to verify the protocol's ability in introducing foreign DNA, pBBRGFP-45 plasmid harbouring kanamycin resistance and green fluorescent protein (GFP) genes into a Gram-positive bacterium, Bacillus cereus *EB2.*

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