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A simple method of plant sectioning using the agarose embedding technique for screening intracellular green fluorescent protein

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ABSTRACT It is difficult to observe plant tissue sections transformed using the agroinfiltration method under a fluorescent microscope. This is due to the softness of the post-transformation plant. This research was conducted to optimize the sectioning of tobacco stems transformed through the agarose embedding technique. Optimization was conducted at various agarose concentrations: 2%, 4%, and 6%, followed by five minutes of incubation at various temperatures: –80 °C, 4 °C, and 25 °C. The stems were then cut using a scalpel and examined under a fluorescence microscope. The results showed that the embedding method using 6% agarose was more effective at producing a tobacco stem section than 2% or 4% agarose. Meanwhile, incubation at 25 °C was better suited to the transformed tobacco stems than at 4 °C or –80 °C. Green fluorescent protein (GFP) could be determined under a fluorescent microscope when using the optimum method. Thus, the optimum method for creating sections of transformed tobacco stems by embedding was to use 6% agarose followed by incubation at 25 °C for 5 min. The optimum result can be applied to obtain a slight section of tobacco stem in order to observe a recombinant protein or other anatomical structures.

KEYWORDS Agarose; Embedding; Green fluorescent protein; Plant section; Transformation

1. Introduction

Plant transformation using the agroinfiltration method is widely used in genetic engineering. However, observing the transformed plants is challenging, particularly for plants transformed by the agroinfiltration method. The plant has to be immersed in the infiltration medium to facilitate plant infection by *Agrobacterium tumefaciens* (Suhandono et al. 2014). It causes transformed plant tissue to become softened and hard to be sectioned into a single layer. Thus, the plant internal anatomy is not clearly observed under the microscope. In molecular biology study, detection of the precise location of protein recombinant in transformed plant tissue is also important to be observed due to the unclearness of the internal anatomy of the plant section.

Paraffin-embedded sectioning is a common technique for plant anatomical observation (Pace 2019). However, obtaining a thin layer of plant section requires many steps. The aldehyde-based fixatives used in this technique can also affect the native and recombinant protein (Li et al. 2015; Hobro and Smith 2017). Therefore, it is unsuitable for transformed plant sectioning to detect recombinant protein or biomolecular markers such as green fluorescence protein (GFP), particularly in plant organs. Moreover, the fluorescence microscope as a tool for observing the fluorescence of GFP has a limitation of observation because of the low resolution (Wang and Lai 2021). On the other hand, Wahyuningsih et al. (2016) reported that the fluorescence of GFP in transgenic tobacco could not be seen as a clear figure under UV light.

Cryohistological is an alternative sectioning method that can maintain the integrity of the plant tissue. It also permits the detection of the fluorescence signals of GFP in plants (Knapp et al. 2012). In certain methods, a low temperature, approximately from –25 °C to –5 °C, must be maintained at the cutting blade (Liyanage et al. 2017). There are a lot of cryohistological methods using different chemicals and techniques, such as paraformaldehyde, glutaraldehyde, sucrose, dimethyl sulfoxide (DMSO), ethylene glycol (EG), sucrose, trehalose (Lestari et al. 2018), propanediol, PIPES, EGTA, manganese sulfate, triton X-100, glycine (Celler et al. 2016), n isopentane, liquid nitrogen, and cryotome (Vidot et al. 2018). However, those methods are laborious and time-consuming. It is also suitable for thick samples due to curling and shattering instead of thin and soft plant tissue (Liyanage et al. 2017). Thus, a simple method must be elaborated to obtain a single thin

and soft transformed plant layer.

Agarose is a suitable candidate to embed thin and soft transformed plants to generate an easy sectioning process. It is quickly changed into a gell state since the gelling process of this polysaccharide is under 35 °C. It is also reported that agarose could encapsulate mammalian cells due to the temperature-sensitive water solubility property (Moo-Young 2019). The utilization of agarose can be combined by using appropriate temperature incubation. Thus, this research optimized plant sectioning after being transformed by the agroinfiltration method using different concentrations of agarose and temperature incubation variations to screen intracellular GFP. The optimum result will be helpful in determining a recombinant protein of the transformed plant sections.

2. Materials and Methods

2.1. Plant Transformation

The cloning plasmid, pART Test7 plasmid, was obtained from the Queensland University of Technology. It contained the *GFP* gene controlled by a constitutive promoter, CaMV 35S. The plasmids were transferred to *A. tumefaciens* AGL1 using heat shock transformation following the method of Hofgen and Willmitzer (1988). The confirmation of positive transformed bacteria was assessed by polymerase chain reaction (PCR) technique using a pair of primers for CaMV 35S promoter amplification (5′-CCT AAC AGA ACT CGC CGT AAA GA-3′ for forward primer and 5′-CCC GTG TTC TCT CCA AAT GAA ATG-3′ for reverse primer). Positive bacteria-harbouring GFP-inserted plasmids were used for plant transformation.

Plant transformation followed the method of Park (2006), Suhandono et al. (2014), and Leckie and Stewart (2011), with modification of the optical density (OD) of bacteria, the duration of vacuum, and the percentage of surfactant Silwet-408. *A. tumefaciens* containing pART 121 were cultured until the absorbance of optical density (OD) at 600 nm reached approximately 0.8. The transformation was carried out by soaking bacteria and tobacco stems in the infiltration medium (2.22 g/L Murashige-Skoog (MS) Salt, 2% sucrose, 200 mM acetosyringone, and 0.01% surfactant Silwet-408). Then, it was placed in a desiccator, and the vacuum was applied at 21.5 mmHg for 30 min. Afterward, bacteria and tobacco were cocultivated at 25 °C for three days in dark conditions. Tobacco stems were disinfected using 400 ppm cefotaxime in ½ MS Salt media.

2.2. Optimization of Transformed Plant Sections

Liquid agarose was made by boiling agarose in various concentrations: 2%, 4%, and 6%. Then, tobacco stems were cut and embedded in different concentrations of agarose: 2%, 4%, and 6% at 25 °C. The scalpel was used to make a cross-cut section of the tobacco stems. All tobacco stems were cut about 0.3 cm thick after the gel solidified. Then, the thin layer was formed and placed on the object glass. A drop of water was added to it and closed by cover glass. Explants were examined under a fluorescent microscope at 450–490 nm. The optimum result of agarose concentration was used for the temperature optimization. The tobacco stems were embedded at –80 °C, 40 °C, and 25 °C for 5 min. Then, all tobacco stems were cut and examined under a fluorescent microscope at 450–490 nm (Figure 1).

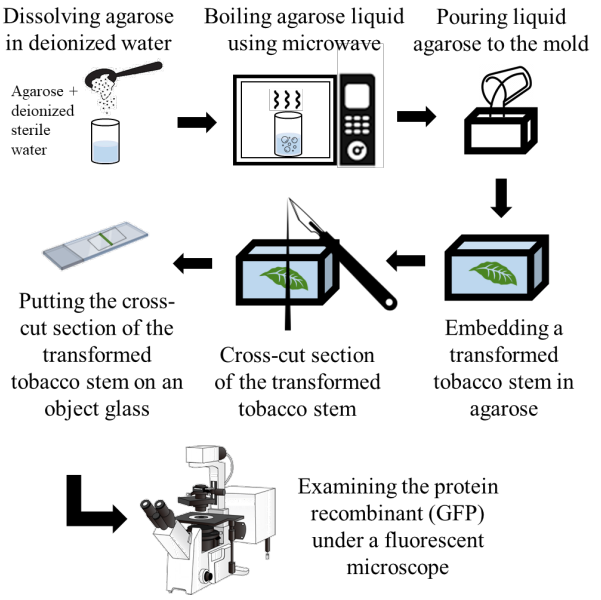

FIGURE 1 The embedding process of the transformed tobacco plant in agarose liquid.

3. Results and Discussion

Positive transformed bacteria were confirmed by PCR technique using primers for CaMV 35S promoter, which is controlled the gfp gene expression. The size of the amplicon product was about 500 bp (Figure 2C), confirming the existence of CaMV 35S which had a precise length of 507 bp. Thus, the bacteria could be used for plant transformation. The result of the plant transformation could be determined by detecting GFP appearance in the trans-

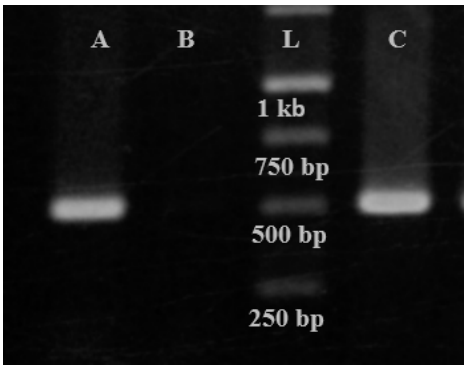

FIGURE 2 CaMV 35S promoter confirmation. Positive control (A). Negative control (B). Ladder 1 kb (L). The amplicon product of positive transformed bacteria sample (C).

formed tobacco stems under a fluorescence microscope. However, the tobacco stems could be observed clearly if only the tissue had been sectioned into a thin layer. The embedding and sectioning process using agarose brought to the process of GFP observation in tobacco stems.

The embedding method with 2% and 4% agarose could not produce a single layer of transformed tobacco stems. The explants were visible as tangential sections instead of a single cross-section layer (Figures 3b and 3c). However, the green fluorescence of GFP in the tobacco stems could be detected under a fluorescence microscope. The negative control showed black and red colors instead of green (Figure 3a). The chlorophyll appeared red under a fluorescence microscope at 450–490 nm.

The embedding method using 6% agarose produced a thin layer of the cross-section of the transformed tobacco stem (Figure 3d). It could confine the tobacco stem without affecting its structure, and the plant tissues could be determined under the fluorescent microscope. In Figure 3d, the result showed that the green fluorescence of GFP was found in the three layers of the tobacco stem: dermal, ground, and vascular tissue.

The optimization of the transformed tobacco stem sectioning method using different temperatures was shown in Figure 4. It was shown that the most apparent transformed tobacco stem sectioning was at 25 °C (Figure 4d). There was some disruption of the ground tissues in the explants at 4 °C (Figure 4c) and –80 °C (Figure 4b).

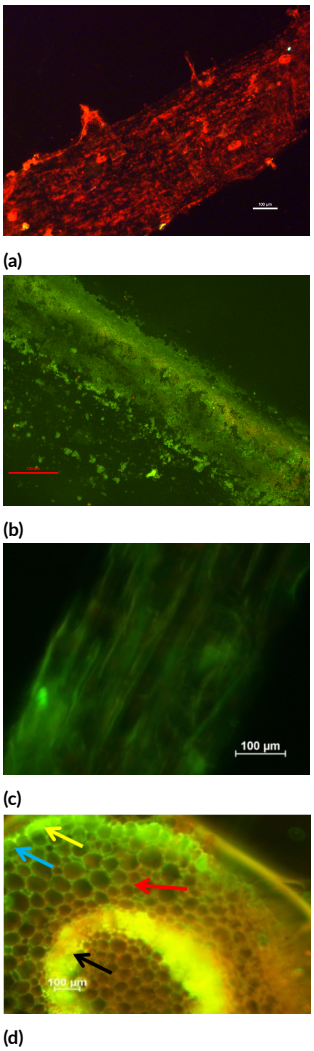

FIGURE 3 The transformed tobacco stem sections under a fluorescent microscope using the embedding method in various agarose concentrations. The tobacco stem was transformed by *A. tumefaciens* without pART Test7 as a negative control (a). Tobacco stems were transformed by *A. tumefaciens* harboring pART Test7 and sectioned using 2% agarose (b), 4% agarose (c), and 6% agarose (d). A blue arrow pointed to the dermal tissue. A red arrow pointed to the ground tissue. A black arrow pointed to the vascular tissue. A yellow arrow pointed to the green fluorescence from GFP.

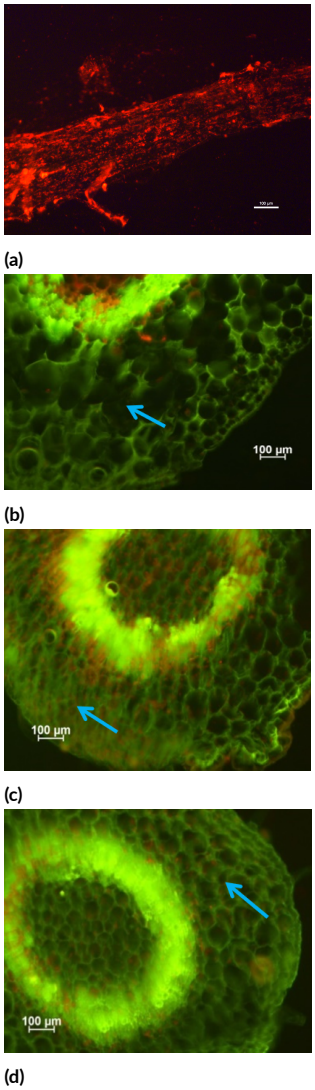

FIGURE 4 The transformed tobacco stem sections under a fluorescent microscope using the embedding method at different temperatures. The tobacco stem was transformed by *A. tumefaciens* without pART as a negative control (a). Tobacco stems were transformed by *A. tumefaciens* harboring pART and sectioned by the embedding process at –80 °C (b), 4 °C (c), and 25 °C (d). A blue arrow pointed to the ground tissue. The redness was unfiltered chlorophyll that fluorescence under a fluorescent microscope.

3.1. Discussion

Kunkel and Asuri (2014) reported that horseradish peroxidase enzymes were stable in 0.5% and 2% agarose under denaturing environment. It was assumed that the pore size of agarose could also maintain the structure of GFP in transformed tobacco stems. However, the sectioning method using 2% and 4% agarose could not produce a single layer of the cross section. It was due to the less dense agarose gel that made the gel too soft to obtain a good section. Thus, the plants could not be appropriately restrained in agarose gel during the sectioning process. In this research, the ability of agarose to encapsulate explants was up to 6% concentration. However, the concentration of agarose above 6% is not suitable for the embedding process of the transformed plant. It requires more than 60 °C to turn the agarose into a liquid state. On the other hand, the fluorescence of GFP tends to gradually lose after 60 °C and lost entirely at 80 °C due to the state of the protein that becomes unstable under high temperatures (Sokalingam et al. 2012; Cai et al. 2020). The native protein can also denature at the high temperature of the embedding process. It will disrupt the structure of the transformed plant tissue.

The optimization of transformed plant sectioning at different temperatures showed that the low temperatures at 4 °C and –80 °C caused the transformed tobacco stem to be softened. The frozen gel thawed during the sectioning process, and then it diffused into the plant tissue, causing tissue rupture. It was suggested that the embedding process required a cryoprotectant to induce tolerance to dehydration (Lestari et al. 2018; Kitin et al. 2020). Cryoprotectants can decrease cell water at low temperatures to reduce the damaging effect of the concentrated solutes in the cells. It is due to the interaction between cryoprotectants and water through hydrogen bonding (Whaley et al. 2021). Many cryoprotectants have been used for cryohistological: glycerol (Kitin et al. 2020), sucrose (Lestari et al. 2018), dimethyl sulfocide (DMSO) (Bekheet et al. 2020), polyvinylpyrrolidone (PVP) (Soonthornkalump et al. 2020), and polyethylene glycol (PEG) (Roque borda et al. 2021). However, Kaur et al. (2022) reported that the steady state fluorescence anisotropy of GFP is about 10–70 °C.

In this study, the fluorescence of GFP from the transformed tobacco stem was detected in various agarose concentrations at all temperature conditions. Therefore, this method is suitable for screening intracellular proteins of the transformed plant tissue. However, this method is unsuitable for a tiny cross-section of the transformed plant, for instance, tobacco root, maize root, and tobacco leaf. It requires an additional compound to harden the explant. This method is preferable for a large cross-section of the transformed plant, including tobacco stem, banana stem, maize root, and banana leaves. The small cross-section of the plant tends to lose its attachment to the gel. Hence, it is difficult to cut using a scalpel even though it is still embedded in the gel.

The transformed plant was prepared just before the screening of the intracellular recombinant protein due to the half-life time of the recombinant protein of GFP wild-type was 26 hours. Adibzadeh et al. (2019) Thus, this simple agarose embedding method becomes a rapid method for preparing the transformed plant section.

4. Conclusions

The optimum method for making sections of the transformed plants by embedding was using 6% agarose and then incubating at 25 °C for 5 min. The cross-section of transformed plant tissues and the GFP fluorescence could be examined using the fluorescence microscope.

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Authors' contributions

SS, NI, and FMD designed the study. NI carried out the laboratory work. NI, FMD, and SS analyzed the data. NI, FMD, and SS wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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