Genetic stability of in vitro propagated transgenic \textit{Acacia mangium} expressing xyloglucanase

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\textbf{Abstract.} In 2011, a gene transformation experiment in \textit{A. mangium} was carried out to yield transgenic \textit{A. mangium} that overexpresses the xyloglucanase gene, enabling plants to grow faster. The transgenic \textit{A. mangium} has been maintained and continuously subcultured since. Here, we tested the stability of the xyloglucanase gene in transgenic \textit{A. mangium} following in vitro propagation. Molecular and morphological approaches were used. For all specimens tested, the wild-type \textit{A. mangium} (K) and two lines of transgenic \textit{A. mangium} named X11 and X21 were positive for the xyloglucanase gene after recurring subculture. Morphological observations did not reveal any alterations, and the cultures had undergone notable physiological deterioration.

\textbf{Keywords:} \textit{Acacia mangium}, in vitro, tissue culture, transgenic

\section{1. Introduction}
Wood is a forest product with many uses, including household applications, construction materials, pulp, paper, and fuel. The global demand for timber is increasing, especially for paper and pulp production. To fulfill this demand, the Indonesian government has developed a program called the Industry Plantation Forest (HTI) to develop crops that can grow rapidly for harvest. One forestry crop developed at the HTI is mangium (\textit{Acacia mangium}), which has several advantageous traits, including the ability to grow in the tropics, a rapid growth rate, tolerance to acidic and low nutrient soil, and the ability to compete with other plants (e.g., grasses) [1, 2].

Xyloglucan is a form of hemicellulose found in the central lamella of plant cell primary walls and the layers of gelatin walls in higher plants [3]. The 1,4-\(\beta\)-glucan chains act as a binder between cellulosic microfibrils. Xyloglucan contributes to the rigidity of cell walls when crossed with other microfibrils and it loosens cell walls during cell elongation [4]. Separation of microfibrils during elongation requires an enzyme that can break the bonds between microfibrils and hemicellulose. The overexpression of xyloglucanase in poplar causes the separation of xyloglucan and cellulose microfibrils. The degradation of xyloglucan by xyloglucanase enables stretching, thus promoting cell elongation [5].

A study of xyloglucanase (\textit{XEG}) gene transformation in transgenic \textit{A. mangium} mediated by \textit{Agrobacterium} was previously performed by Hartati et al. [6]. Overexpression of \textit{XEG} in transgenic \textit{A. mangium} resulted in xyloglucan levels in stems and leaf cell walls lower than those in wild-type
**A. mangium** and accelerated the process of cell elongation. Morphological observations of transgenic **A. mangium** showed faster growth in both stem length and diameter compared to wild-type **A. mangium** grown in soil media. This suggests that the transgenic **A. mangium** developed by Hartati et al. [6], can be harvested faster than wild-type **A. mangium**, helping to fulfill the demand for timber.

The transgenic **A. mangium** plants have been continually nurtured and subcultured at the Molecular Genetics Laboratory and Plant Biosynthesis Path Modification, Biotechnology Research Center, Indonesian Institute of Sciences (LIPI), Cibinong, for five years. After this duration of time, the stability of **XEG** genes in the transgenic plants propagated by tissue culture should be tested molecularly. Thus, the objective of this study was to examine the genetic stability of transgenic **A. mangium** subcultures. Gene stability tests included isolation of genomic **A. mangium** DNA, DNA amplification, and polymerase chain reaction (PCR) product analysis via electrophoresis gel. The gene expression tests included total RNA isolation, cDNA synthesis, and cDNA amplification with analysis via electrophoresis gel.

### 2. Materials and method

#### 2.1. Plant materials

Flower buds from potted one-year-old **A. mangium** mother plants were used for transformation with the **XEG** gene. The flowers were transformed with the **XEG** gene by spraying with an *Agrobacterium* solution after about 25 to 30 weeks. The flower will then produce seeds. The seeds were cultured in vitro in MS medium to select the transformed plants, which were then sub-cultured.

#### 2.2. Stability of xyloglucanase on transgene Acacia mangium

##### 2.2.1. DNA isolation

For the isolation of **A. mangium** genomic DNA, samples were extracted from leaf and stem tissue from laboratory cultures of GMMJBT with X_{11} and X_{21} strains and wild-type **A. mangium** (K) from in vitro cultures. Field samples were used as negative controls. DNA isolation was performed by the CTAB method [7]. Briefly, a 0.5 mg sample of **A. mangium** was treated with liquid nitrogen, and then 600 µL buffer of CTAB extract containing 0.2 % mercaptoethanol solution mixture was added to loosen the cell walls. The samples were then incubated at 65 °C for 1 hour. Debris including proteins, lipids and other cell wall components were removed by adding 600 µL of chloroform:octanol (24:1) solution, followed by centrifugation at 8,000 rpm for 15 min at 4 °C. This process was repeated several times. The supernatant was then mixed with 100 % ethanol and allowed to stand for 12 hours to precipitate the DNA. Next, the samples were centrifuged at 10,000 rpm for 25 min at 4 °C. The supernatant was removed and the pellet was washed by adding 400 µL of 70 % ethanol and centrifuging at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was dried. The pellet was rehydrated with ddH₂O and stored at -20 °C until used as template DNA for amplification.

##### 2.2.2. PCR amplification

DNA amplification was performed using a Thermo Scientific kit. Approximately 2 µL of templates of **A. mangium** DNA was inserted into a tube with 1 µL buffer 10X, 0.6 µL MgCl₂, 1 µL dNTP mix, 0.2 µL DNA polymerase, 0.25 µL forward primer, and 0.25 µL reverse primer. The mixture was homogenized by vortexing for 10 sec, before inserted into the thermal cycler for the amplification process. The PCR conditions are given in table 1. The primer sequences were specific for **XEG** genes. The primers were constructed from *Aspergillus aculeatus* (AaXEG, accession number AY160774). The forward primer sequence used was 5’-GCTGCCAGTCTAGAGCGCCGACG-3’ and the reverse primer sequence was 3’-CAACGCACTGGCCGCCGACTGCCCCCTC-5’. The PCR product was then analyzed by gel electrophoresis with agarose gel concentration of 1 % in TAE 1x buffer solution.
Electrophoresis was carried out for 60 min at 70 volts with the. The agarose gel was then soaked in 0.07 % ethidium bromide solution for 15 min. Results were visualized using a UV transilluminator and documented using a digital camera.

2.3. Expression of xyloglucanase by transgenic Acacia mangium

2.3.1. RNA isolation. The gene expression test was performed by a two-step method. First, reverse transcription PCR (RT-PCR) was started by isolating total RNA, followed by cDNA synthesis and amplification. RNA isolation was then performed using a Total Geneaid RNA Mini Kit (Plant).

2.3.2. cDNAs synthesis and amplification. The results of total RNA isolation of transgenic A. mangium were synthesized to cDNA using a RevertAid cDNAs Synthesis Kit. About 1 μL RNA template was added to a PCR tube with 1 μL of oligo (dT), 4 μL 5x reaction buffer, 1 μL RiboLock RNase Inhibitor, 2 μL 10 mM dNTP mix, and 1 μL RevertAid M-Mulv RT. The sample was then homogenized by vortexing. Next, the tube was inserted into the thermal cycler. The initial incubation temperature was set at 25 °C for 10 min, the final incubation temperature was 42 °C for 60 min, and the termination temperature was 70 °C for 5 min. The cDNA was then amplified by the PCR method using the same primers described in section 2.2.2.

2.4. Plantlet observation

Morphological observations of transgenic A. mangium were performed on a sub-cultured plantlet in MS medium containing IAA 0.5 mg/L. The parameters included stem height, number of leaves, and number of roots. Observations were made daily for eight weeks.

3. Results and discussion

3.1. Stability of XEG in transgenic A. mangium

The results of DNA isolation had a ratio range at A260/A280 of 1.489–2.056. DNA is considered pure if it has a ratio range at A260 / A280 of 1.800–2.000. This suggests that some of the DNA isolates used in this study still contained protein or phenol [8]. However, they can still be used as a template in the amplification process [9]. The results of gel electrophoresis showed that the XEG gene has a band size of 709 bp (figure 1).

The percentage of XEG gene stability is shown in table 2. XEG genes appeared in all of the samples tested. Our findings agreed with Yin et al. (2004), who stated that transgenic plants still carry a gene insertion to the next derivative through mitotic cell division [10].

Table 1. PCR cycle conditionsa.

| Process       | Time  | Temperature | Repetition |
|---------------|-------|-------------|------------|
| Pre-Denaturation | 1 min | 95 °C       | 1 x        |
| Denaturation   | 30 sec| 95 °C       |            |
| Amplification  |       |             |            |
| Annealing      | 45 sec| 58 °C       | 30 x       |
| Extension      | 1 min | 72 °C       |            |
| Final extension| 7 min | 72 °C       | 1 x        |
| Hold          | unlimited | 4 °C   |            |

aThermoScientific (2016), modified
Figure 1. DNA amplification of transgenic and wild-type A. mangium using specific primers for the XEG gene (AaXEG). A fragment of 709 bp can be seen in lane 1, which is positive control (AaXEG plasmid DNA). Fragment bands can be seen from transgenic A. mangium X21 (lanes 2–7) and X11 (lanes 8–13) and from wild-type A. mangium (lanes 14–19). Lane 20 is a negative control in which DNA templates were replaced with ddH2O.

Table 2. Percentage of XEG genes.

| Type of plant | Positive XEG (%) |
|---------------|------------------|
| K             | 100              |
| X11           | 100              |
| X21           | 100              |

Rooke et al. (2003) also suggested that the inheritance of inserted genes in plants would increase if the plants were kept in tissue culture [11]. However, the negative control of wild-type A. mangium, which was not transformed by the XEG gene, showed amplification of DNA bands the same size as XEG genes. This result may have been caused by errors during the subculture process, such as bacterial contamination, or by human error [12].

For further verification, wild-type A. mangium from the field was used as a negative control. The tip of the leaf was used to isolate genomic DNA. Visualization of the PCR electrophoresis gel showed that wild-type A. mangium from the field did not have an XEG gene band, as shown in figure 2. This demonstrates that the presence of XEG genes in wild-type plants in vitro was caused by contamination during maintenance.

3.2. Expression of XEG in transgenic A. mangium

Reverse transcription PCR (RT-PCR) of some samples used in the gene stability test was performed to determine the expression of the xyloglucanase gene using a total RNA template of A. mangium. This procedure was done to ascertain whether wild-type A. mangium positively expresses the XEG gene or not. The RT-PCR results are shown in figure 3. As shown, wild-type A. mangium from the field did not express the XEG gene. This confirms the assumption that in vitro wild-type A. mangium was contaminated during the maintenance process.
Figure 3. Results of RT-PCR of A. mangium with the XEG gene. Lane 1 is positive control of AaXEG plasmids, lanes 2-4 are wild-type A. mangium field collection samples, lanes 5-7 are in vitro wild-type A. mangium, lanes 8-10 are transgenic A. mangium X11, and lanes 11-13 are transgenic A. mangium X21.

3.3. Plantlet observation
The subcultured A. mangium in MS medium containing IAA 0.5 mg/L showed no growth. Thus, data on growth could not be obtained. The subculture of A. mangium showed physiological deterioration such as fallen leaves, broken stems, and dead plants (browning). Physiological deterioration of the A. mangium subculture may be due to several factors such as stress hormones, plantlet age, and recurrent subculture [13, 14].

4. Conclusion
The xyloglucanase genes in transgenic A. mangium, X11 and X21 strains remained stable after being subculturated several times on MS medium with various concentrations of growth regulators, that characterized by the presence of a 709 bp band PCR product on the electrophoresis gel. However, the culture of A. mangium from XEG gene transformation did not grow well, had physiological deterioration that marked by lack of stem growth, reduced leaf number, and abnormal root appearance.

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