









Improvement of biomass production in transgenic *Melia azedarach* L. plants by the expression of a *GA20-oxidase* gene

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Abstract: *Melia azedarach* L. is a multipurpose timber tree that is important in forestry plantations. Genetic modification to improve the growth rate and wood quality is important to produce new cultivars of *M. azedarach*. In this study, the effects of *AtGA20ox* gene expression on plant growth, wood formation, and biomass production in transgenic *M. azedarach* plants were investigated. *AtGA20ox* was inserted into the binary vector pBI101 under the control of the xylem specific promoter CAD4 and transferred to *M. azedarach* via *Agrobacterium*-mediated method. The integration and expression of the transgenes were confirmed using PCR, RT-PCR, and Southern blotting. The specificity of CAD4 in the xylem was indicated by the higher expression of the *AtGA20ox* gene in the stem tissues compared to the leaves. The larger stem diameter resulted from higher xylem cell numbers and a wider xylem zone in all the transgenic plants. In addition, the transgenic plants also grew more quickly. Consequently, transgenic *M. azedarach* increased its biomass production 2- to 4-fold compared to the wild-type plants at 90 days under greenhouse conditions. Our results suggest that the expression of ectopic *AtGA20ox* under the control of a xylem-specific promoter is an appropriate method to improve plant growth, biomass production, and wood formation in *M. azedarach* and other forestry plants.

Key words: Biomass production, CAD4, *GA20-oxidase* gene, *Melia azedarach* L., transformation, xylem

1. Introduction

Currently, the demand for wood products is increasing at a rapid rate, while the area of land devoted to forests is dwindling due to human activities. Therefore, the selection of wood species yielding both high quality and quantity is especially important for forest plantations. *Melia azedarach* L. is a multipurpose woody species and one of the most important species of the family Meliaceae. It is considered to be a fast-growing, drought-resistant plant and has been cultivated across ecological regions of Vietnam from the north to the south. It is widely used for construction, furniture, interior decoration, and sculptures due to its durable and termite-resistant wood. In addition, the leaves are used for green manure and as an antiseptic, grains for oil, and charcoal for high heat (Sharma and Sharma, 1995). Limonoids extracted from this plant have pharmacological activity to inhibit some types of cancer cells (Huang et al., 1996). Therefore, *M. azedarach* has been considered to be an important plant species in the strategy of forestry development in many countries, including Indonesia,

India, China, Australia, Vietnam, and the Philippines (Rubae, 2009). Increases in the plant growth rate, plant biomass, and wood quality are targets to improve the utilization of *M. azedarach* in forestry development and the wood industry. Genetic engineering approaches, such as plant transformation, could accelerate the process of *M. azedarach* cultivar improvement. Several previous reports showed the successes in *Melia azedarach* transformation using *Agrobacterium* mediated methods (Nirsatmanto and Gyokusen, 2007; Ngo et al., 2010; Dong et al., 2011).

An improvement in plant growth and biomass production through the manipulation of bioactive gibberellins (GAs) has been successfully applied in both monocot and dicot plants (Coles et al., 1999; Carrera et al., 2000; Biemelt et al., 2004; Fagoaga et al., 2007; García-Hurtado et al., 2012). GAs are tetracyclic diterpenoid phytohormones, which play an important role in regulating plant growth and affecting various developmental processes, including seed germination, stem elongation, flowering (Xu et al., 1995; Hedden and Kamiya, 1997;

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Olszewski et al., 2002; Yamaguchi, 2008; Hedden and Thomas, 2012; Dayan, 2016), and wood formation (Ragni et al., 2011). Although 136 natural GA structures have been identified, only a few are biologically active, including GA1 and GA4 (Xu et al., 1995; Yamaguchi, 2008). More highly bioactive GA concentrations are usually found in the growing parts, such as the young leaves and internodes, where they exhibit the high expression of GA biosynthetic-related genes (Hedden and Kamiya, 1997).

Therefore, the regulation of the bioactive GA biosynthetic pathway is important for plant development and response to environmental conditions (Hedden and Kamiya, 1997). In higher plants, the final steps of the GA biosynthetic pathway are completed in the cytoplasm and are catalyzed by GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) to generate various GA intermediates and mature bioactive GAs. GA20-oxidase catalyzes the metabolism of GA12/GA53 to inactive gibberellins (GA9/GA20) through three successive redox reactions. The 3 β -hydroxyl group is added to the GA9/GA20 to form the mature products (GA1/GA4) under catalysis by GA3-oxidase (Feuillet et al., 1995; Yamaguchi, 2008). The ectopic expression of the genes encoding GA20ox has improved the growth and biomass of different plant species, including the stem growth and xylem quality of woody trees such as hybrid aspen and citrus (Carrera et al., 2000; Eriksson et al., 2000; Biemelt et al., 2004; Fagoaga et al., 2007).

However, the overexpression of *GA20ox* under the control of a constitutive promoter, such as 35S, may cause adverse effects such as a reduced stem diameter, small leaves, and poor rooting (Eriksson et al., 2000; Mauriat et al., 2014). Jeon et al. (2015) also observed undesirable phenotypes in transgenic poplar, including poor root growth and leaf development, when *PdGA20ox* was expressed under the regulation of 35S. When the 35S promoter was replaced by DX15, a xylem-specific promoter isolated from a hybrid poplar (*Populus maximowiczii* \times *Populus nigra* clone NM7) (Ko et al., 2012), no such negative phenotypes were detected. In this study, a construct containing *AtGA20ox* under the control of a xylem-specific promoter CAD4 (cinnamyl alcohol dehydrogenase) was transferred into *M. azedarach* using an *Agrobacterium*-mediated method with the aim of improving plant growth, biomass production, and wood formation in this species.

2. Materials and methods

2.1. Materials

M. azedarach L. seeds of dominant trees provided by the Institute of Forestry Biotechnology, Vietnam National University of Forestry, were used as the initial plant material. The *AtGA20ox* gene and xylem-specific promoter CAD4 were isolated from *Arabidopsis* and *Populus trichocarpa*, respectively. The plant transformation binary vector pBI101 containing the *AtGA20ox* gene under the regulation of the CAD4 promoter was constructed from our previous research (Nhung et al., 2017) (Figure 1) and transformed into *A. tumefaciens* strain C58. The list of primers designed and used for PCR, RT-PCR, and Southern blot to confirm the insertion and expression of transgenes is shown in Table 1.

2.2. Plant transformation, selection, and regeneration

M. azedarach L. seeds were surface sterilized using EtOH 70% for 3 min, washed 3 times with 100% commercial bleach solution (10 min/time), and washed 5 times with sterile water. The sterilized seeds were germinated in 250-mL Erlenmeyer flasks, and 14-day-old hypocotyl segments were used for the *Agrobacterium*-mediated transformation method as described by Dong et al. (2011). Briefly, 1-cm hypocotyl segments were inoculated with bacterial suspension for 30 min and transferred to cocultivation medium for 2 days in the dark at 25 °C. Subsequently, the explants were washed with sterilized distilled water and cultured on the first selective medium (SM1) containing Murashige and Skoog (MS) medium, 2 mg/L BAP, 30 g/L sucrose, 500 mg/L cefotaxime (Zeiss Pharmaceuticals Pvt. Ltd., Baddi, India), and 50 mg/L kanamycin (Bio Basic Inc., Markham, Canada). After 14 days, young shoots regenerated from explants were subcultured into the second selective medium (SM2) supplemented with 200 mg/L kanamycin for 2–3 weeks. Healthy shoots (approximately 3 cm with 4 or 5 leaves) were transferred to rooting medium (RM) containing MS medium, 15 g/L sucrose, 0.3 mg/L IBA, 250 mg/L cefotaxime, and 50 mg/L kanamycin. Shoots with well-established roots were transferred to the soil in an acclimation room after 3 weeks.

2.3. Genomic DNA extraction and PCR analysis

To confirm the T-DNA insertion, genomic DNA was extracted from the leaves of 30-day-old putative transgenic

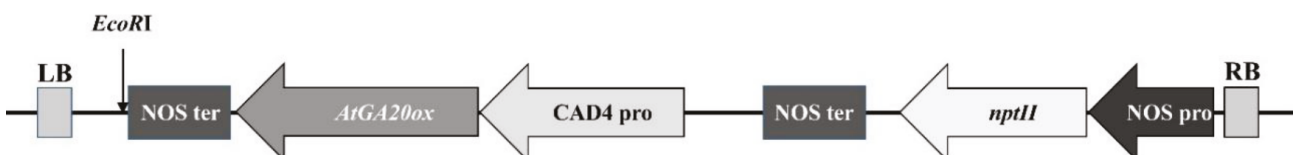


Figure 1. Diagram of pBI101/CAD4::*AtGA20ox* transformation vector. NOS ter: Nopaline synthase terminator; *AtGA20ox*: *A. thaliana* gibberellin (GA) 20-oxidase; CAD4 pro: cinnamyl alcohol dehydrogenase 4 promoter; *nptII*: neomycin phosphotransferase II gene; NOS pro: nopaline synthase promoter; LB: left border; RB: right border.

Table 1. List of primers used in this study.

Primers	Nucleotide sequence (5'-3')	PCR products (bp)	Note
<i>GA20ox-F</i>	CCCGGGATGGCCGTAAGTTTCGTAACAA	1176	For PCR, RT-PCR
<i>GA20ox-R</i>	GAGCTCTCACAGATCCTCTTCTGAGATGA		
<i>Actin-F</i>	TCCTTAAAGATGGGCGATGC	186	For RT-PCR
<i>Actin-R</i>	CCATTCCAACCATAACACCAG		
<i>sGA20ox-F</i>	GGCATCAGCGAGGAGCTTAT	698	For Southern blots
<i>sGA20ox-R</i>	GGTGGCGTCACTACTCTGTC		

plants cultivated under greenhouse condition and analyzed using polymerase chain reaction (PCR). The control leaves from untransformed plants were sampled using the same procedure. Genomic DNA was extracted using the CTAB procedure modified from Dellaporta et al. (1983). PCR was performed using specific primers for the *AtGA20ox* gene (*GA20ox-F/R*), and their sequences are shown in Table 1. The amplification program for this study was performed at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 50 s and a final extension of 72 °C for 10 min. The PCR products were separated using a 0.8% agarose gel and visualized on a UV transilluminator.

2.4. Southern blot analysis

Southern blot analysis was used to confirm the transgenic status of the PCR positive plants for the *AtGA20ox* gene. Total genomic DNA isolated from the leaf material was digested with *EcoRI*, which cuts only once in T-DNA, fractionated using 2.0% agarose gel electrophoresis, and transferred to a Zeta-Probe GT nylon membrane (Bio-Rad, Hercules, CA, USA). The *GA20ox* probe was generated by PCR using the *sGA20ox-F/R* primers. The probe was labelled with ³²P using a Prime-It RmT Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA). Hybridization and membrane washing were conducted based on the Zeta-Probe GT manufacturer's instructions at 65 °C.

2.5. Gene expression assessment using reverse transcription PCR (RT-PCR)

Total RNA was extracted from the leaves and young stems of 90-day-old nontransformed and *AtGA20ox* PCR-positive transgenic plants using total RNA extraction with the TRIzol reagent (Life Technologies). First-strand cDNAs were synthesized from total RNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). Semiquantitative RT-PCR was performed using a pair of gene-specific primers. Reaction conditions comprised a step at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 50 s and ending with a cycle of 72 °C for 7 min. The housekeeping gene *Actin* was used as an internal control.

2.6. Phenotypic analysis of transgenic plants

Transgenic plants harboring *AtGA20ox* under the control of the CAD4 promoter and nontransformed plants were propagated in vitro. After the roots formed, the plants were transferred to plastic pots containing mixed soil and cultured in the greenhouse. The stem length was measured every 15 days, while the number of internodes, internode length, and petiole length, as well as plant biomass, were recorded at the 90th day. To examine the internode and petiole lengths, only the 3rd, 4th, and 5th internodes and petioles of the 3rd, 4th, and 5th leaf from the top were measured. Data analyses were performed using Microsoft Excel and SPSS 20.

2.7. Histological analysis

M. azedarach stem cross-sections were prepared from internode number 9 and stained with 0.05% toluidine blue O for 1 min. Images were recorded using a Meiji Techno MA151/MT05 C-mount 0.5× microscope (at 10× and 40×). Xylem width and cell pile number were identified as described by Jeon et al. (2015) using ImageJ software (NIH).

3. Results

3.1. Generation of transgenic *M. azedarach*, confirmation of the presence of transgene, and shoot propagation

3.1.1. Generation of transgenic *M. azedarach* plants

M. azedarach was genetically transformed using *A. tumefaciens* C58 harboring a CAD4::*AtGA20ox* cassette. More than 30 samples that formed shoots were produced from approximately 300 hypocotyl segments on medium containing 200 mg/L kanamycin. Healthy shoots that were 3 cm in length and produced 4 or 5 leaves were transferred to RM supplemented with 50 mg/L kanamycin (Figure 2A). Fifteen potential lines transformed with a pBI101/CAD4::*AtGA20ox* construct were successfully rooted, transferred to the soil in the acclimation room, and moved to a greenhouse for additional analysis.

3.1.2. Transgene confirmation and shoot propagation

After 4 weeks under greenhouse conditions, new leaves were collected from putative transgenic lines (plants

rooted well on RM with 50 mg/L kanamycin) and used to isolate DNA. Specific *AtGA20ox* gene primers were designed and used for PCR to confirm the presence of the transgenes. The expected 1.2-kb fragment containing the *AtGA20ox* coding sequence was observed in nine out of the 15 putative transgenic plants. Due to the long life cycle of *M. azedarach*, we used in vitro micropropagation to establish a large transgenic population for phenotypic and further studies. In this study, stem segments with adventitious buds were cut from putative lines and grown on shoot induction medium to multiply transgenic lines. Transgenic lines 4, 64, and 82 were randomly selected from nine putative lines and used three times for vegetative propagation. We confirmed the presence of the transgenes after each vegetative propagation cycle using PCR with specific primers (GA20ox-F/R) (Figure 2B). Plants propagated from three transgenic events were transferred to the soil under greenhouse condition for phenotyping.

3.2. Transgene insertion and expression analysis

3.2.1. Detection of the copy number of *AtGA20ox* in transgenic lines using Southern blotting

To confirm the stable integration of the T-DNA, transgenic plants were investigated using Southern blot analysis. The genomic DNA of transgenic *M. azedarach* was digested with *EcoRI* restriction enzyme, which cut once within the T-DNA region, enabling an estimate of the copy number of the transgenes, as well as the identification of different events. The digested DNA revealed one single or two hybridization bands when hybridized to the 700-bp GA20ox probe (Figure 2C), which indicates the integration of only one copy of T-DNA into the genome of transgenic lines 64 and 82 and two copies in transgenic line 4. DNA from a nontransformant did not show any hybridization signals. Different insertion patterns of the transgenic lines indicated that the three lines tested are independent events. The low copies of the transgene (1–2 copies) demonstrate the potential of the *Agrobacterium*-mediated method for *M. azedarach* transformation.

3.2.2. Analysis of the expression level of transgenes using semiquantitative RT-PCR

The transcription levels of the *AtGA20ox* gene under the regulation of the CAD4 promoter were examined in the leaves and young stems of the transgenic plants at 90 days under greenhouse conditions. cDNA was synthesized from total RNA and used for RT-PCR using GA20ox-F/R specific primers (Table 1). The binary vector pBI101/CAD4::*AtGA20ox* and DNA of the wild-type plants were used as the positive and negative control, respectively, and the *Actin* gene was used as the internal control. The RT-PCR results showed that the *AtGA20ox* gene was expressed in all tested transgenic lines, while no expression was observed in both leaf and stem tissue samples of nontransformed plants (Figure 2D). Importantly, the expression level

of *AtGA20ox* in the stem tissue was obviously higher compared to that in the leaves. This result clearly indicated that the CAD4 promoter was more specifically regulated in the xylem tissue compared to the leaves. No difference in the gene expression levels was noted between the transgenic plants and one and two copies of *AtGA20ox* using RT-PCR. The quantitative expression level of *AtGA20ox* in different tissues, especially in the stem, will be examined in the future.

3.3. Phenotyping transgenic *M. azedarach* plants

Young plants of the three lines 4, 64, and 82 and nontransformed plants micropropagated in vitro were grown in the greenhouse. The growth rate and other morphological characteristics were evaluated. After 3 months, all the transgenic plants grew faster than the nontransformed control plants (Figure 3A). The stem length of the transgenic events was increased 5-fold compared to the wild-type for line 4 and approximately 3-fold for lines 64 and 82 (Figure 3B). The development of the wild-type plants increased gradually from day 1 to day 60, while the growth of the transgenic plants increased dramatically after 30 days, especially in line 4 (Figure 3C). On average, the stem elongation of the transgenic plants varied from 0.6 cm to 0.97 cm per day compared to only 0.19 cm per day for the wild-type plants.

We found that the overgrowth of the transgenic plants was the result of the rapid elongation of the stem between the nodes rather than the number of internodes (Table 2). The number of internodes was 18 or 19 for both the transgenic and nontransgenic lines, while the stem length was 6.1, 6.7, and 7.9 cm for transgenic lines 82, 64, and 4, respectively, compared to 1.1 cm for the nontransgenic lines. In addition, longer leaf petioles were observed in all the transgenic lines compared to the control plants, and its length averaged from 5.7 to 6.3 cm for the transgenic lines and only 4.9 cm for the nontransgenic ones. More importantly, the stem diameter of the transgenic lines had also improved. It was found to be significantly larger than that of the nontransgenic control plants in transgenic line 4 and to slightly increase in lines 64 and 82 (Figure 4A).

Together with the longer internodes, the fresh weight of all three transgenic lines was significantly higher than that of the wild-type plants. The highest weight was obtained in the transgenic line 4 (9.8 g), followed by line 64 (6 g) and line 82 (5.1 g). However, the fresh weight of the wild-type plants was only 2.5 g under the same greenhouse condition (Figure 4B). All these data indicated that the expression of *AtGA20ox* increased the growth and biomass production of transgenic *M. azedarach*.

3.4. Wood formation analysis of transgenic *M. azedarach* plants

The ectopic expression of *AtGA20ox* under the control of the CAD4 promoter obviously improved the development

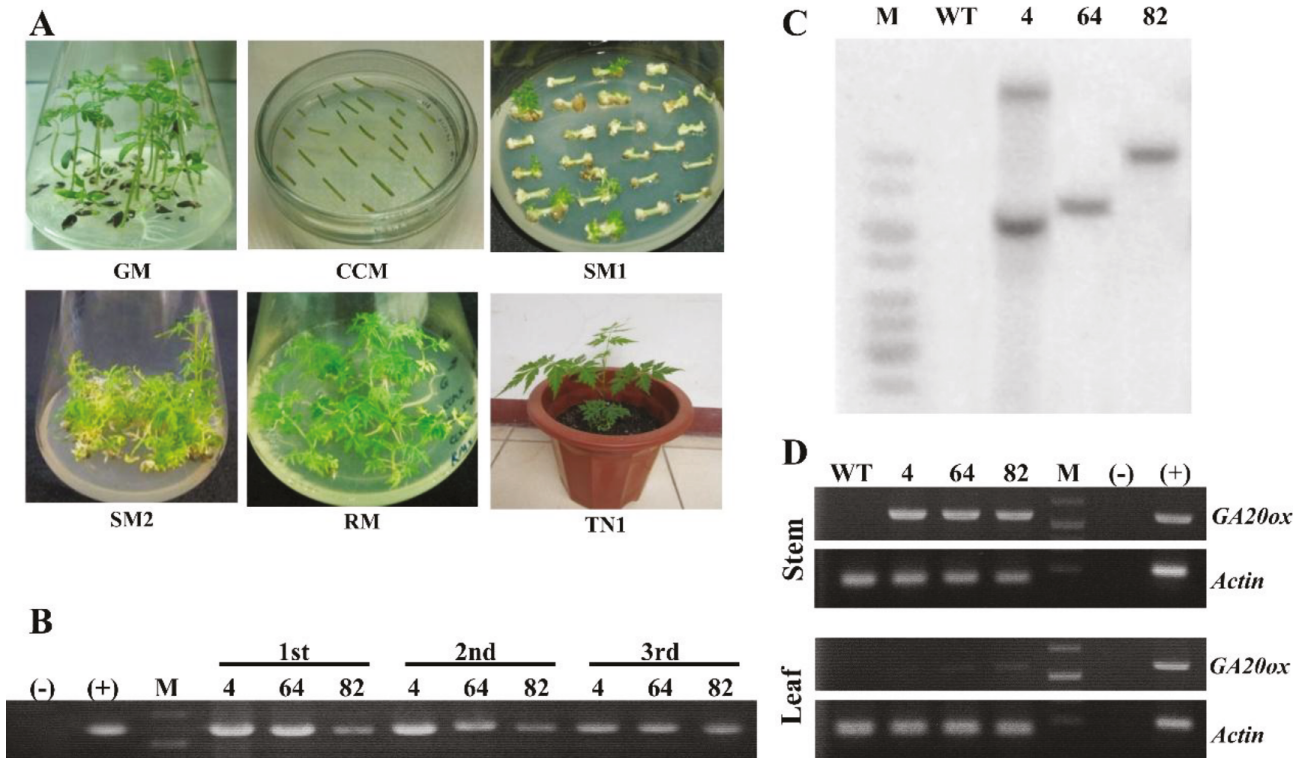


Figure 2. Production and molecular analysis of transgenic *M. azedarach* plants expressing the *AtGA20ox* gene under the regulation of the xylem-specific promoter CAD4. A) Transformation and selection procedure of *M. azedarach*. Seedlings established from seeds on MS medium (GM); 1-cm-length hypocotyl segments on coculture medium (CCM); shoot formation on the first selective medium containing 50 mg/L kanamycin (SM1); shoot development on the second selective medium containing 200 mg/L kanamycin (SM2); shoots rooted on rooting medium containing 50 mg/L kanamycin (RM); putative transgenic lines in soil (TN1). B) PCR analysis to determine the presence of transgenes in transgenic lines (4, 64, and 82) 3 times (1st, 2nd, and 3rd) with in vitro propagation. C) Southern blot analysis using an *AtGA20ox* probe. M: DNA ladder, 1 kb. D) Transcript expression of *AtGA20ox* in the leaf and stem of transgenic *M. azedarach* lines using semi-quantitative RT-PCR. M: DNA ladder, 1 kb; (-): negative control used DNA of wild-type plant; (+): positive control (pBI101/CAD4::AtGA20ox vector).

of the plant stem and may affect the formation of wood in transgenic *M. azedarach*. Cross sections of the 9th internodes were prepared for histological analysis to examine that hypothesis. A remarkable increase in xylem differentiation in all the transgenic lines was observed (Figure 5A). The xylem width increased from 628 μm in the nontransgenic plants to 737.0 μm and 1000.7 μm in the transgenic lines (Figure 5B). In the transgenic plants, the enlargement in xylem width also correlated with increased xylem cell numbers. The number of xylem cells increased from 41.3 in the nontransformed plant to 44.3–60.3 in the transgenic lines (Figure 5C). Line 4 showed the highest number of xylem cells, as well as xylem width. Therefore, internode elongation and the longer xylem width of the transgenic lines could be a result of the increase in cell division in both the vertical and horizontal directions.

4. Discussion

M. azedarach L. is a multipurpose timber species and an important plant for forestry development strategy in

Vietnam and other Asian countries. Several attempts have been made to improve the plant growth and wood quality of forestry trees, including this species (FAO, 2004). However, there are certain limitations using conventional breeding methods due to the long life-cycle of forestry plants. Therefore, genetic engineering combined with vegetative propagation is a more suitable method to genetically improve forestry species. The overexpression of the *GA20ox* gene has been demonstrated to improve plant growth rate and biomass production in a number of plant species, including both monocots and dicots (Carrera et al., 2000; Eriksson et al., 2000; Vidal et al., 2001; Biemelt et al., 2004; Dayan et al., 2010; García-Hurtado et al., 2012; Ayano et al., 2014; Voorend et al., 2015; Do et al., 2016). However, undesirable phenotypes were reported when constitutive promoters, such as 35S and ubiquitin, were utilized to direct the *GA20ox* gene (Eriksson et al., 2000; Mauriat et al., 2014; Jeon et al., 2015). For example, longer leaves, internodes, and tillers but narrow leaves and slender stems were observed in monocot transgenic

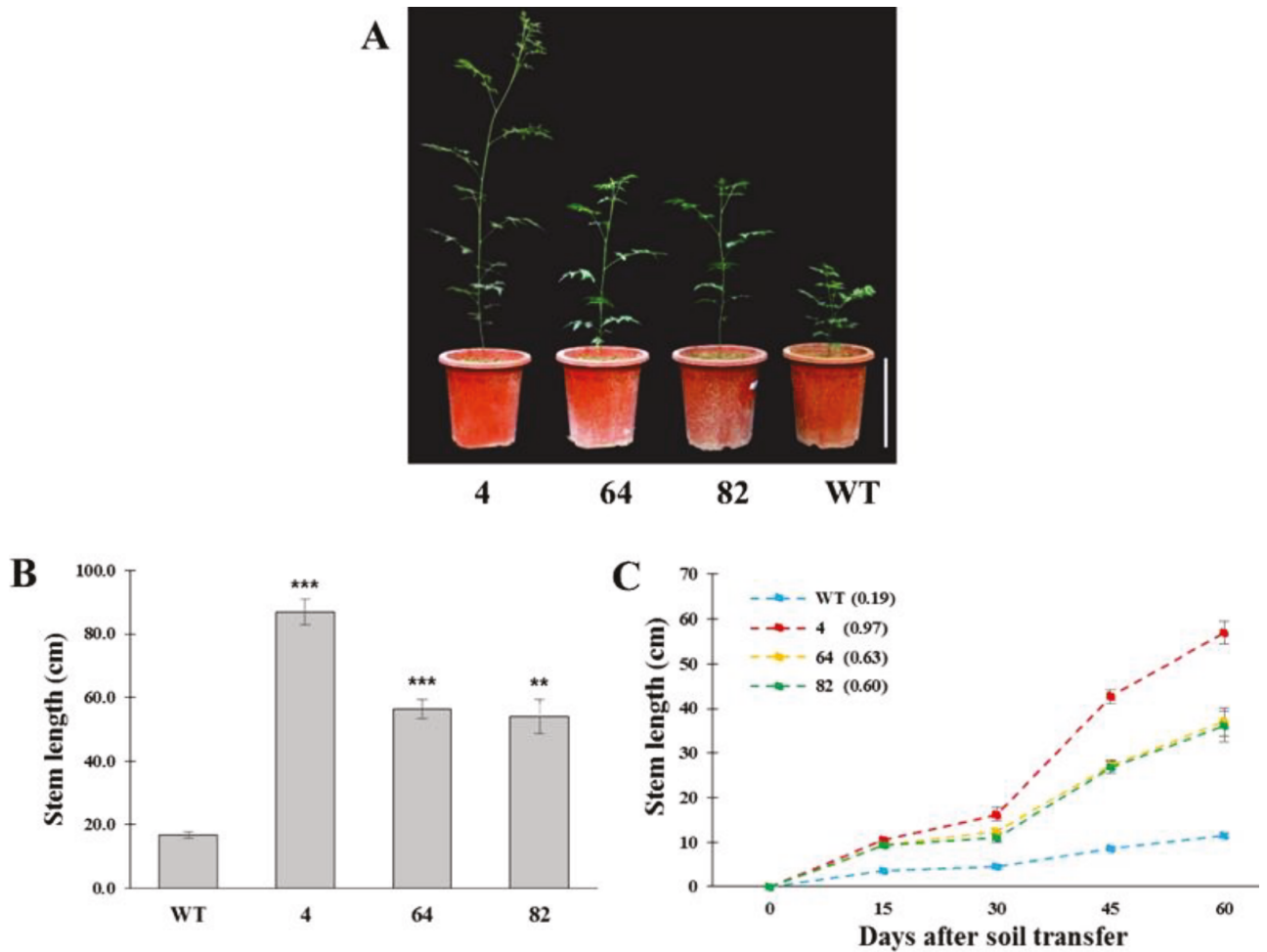


Figure 3. Characterization of the morphology of transgenic *M. azedarach*. A) Transgenic *M. azedarach* lines (4, 64, and 82) and the wild-type at 90 days under greenhouse conditions. Scale bar is 30 cm. B) Average stem length (n = 3) at 90 days grown in the soil. The significant difference was determined using Student's t-test (***: P < 0.001, **: P < 0.01). C) Stem elongation. Stem length was recorded at different time points. Elongation rate (cm/day) is shown with each line. Error bars indicate the SE (n = 3).

Table 2. Morphology of transgenic *M. azedarach*.

Lines	Number of internodes	Internode length (cm)	Petiole length (cm)
WT	18.3 ± 0.6	1.1 ± 0.2	4.9 ± 0.7
4	19.7 ± 1.5	7.9 ± 0.6*	6.3 ± 0.7*
64	18.3 ± 1.5	6.7 ± 0.7*	5.8 ± 0.5*
82	18.7 ± 0.6	6.1 ± 0.5*	5.9 ± 0.3*

Data were collected from plants at 90 days under greenhouse condition. Mean values are shown with SE (n = 3). *: Significant difference from wild-type (WT) plants at P < 0.01.

plants, such as switchgrass (Do et al., 2016) or maize (Voorend et al., 2015). These phenomena were also observed in different dicots, such as *Populus* (Eriksson et al., 2000), potato (Carrera et al., 2000), tobacco (Biemelt et

al., 2004), and citrus (Fagoaga et al., 2007). In this result, the xylem-specific promoter CAD4, which was identified in the study of Barakat et al. (2009), was utilized to drive the expression of the *AtGA20ox* gene. Our expression

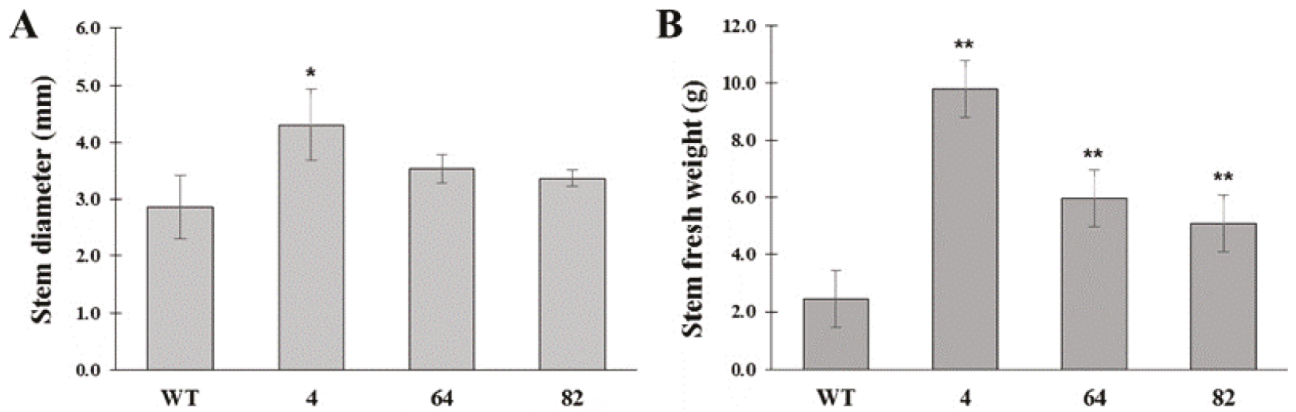


Figure 4. Stem diameter and fresh weight of the transgenic and wild-type *M. azedarach*. A) Stem diameters were measured 10 cm above the ground. B) Fresh weight of the primary stems at 90 days in the greenhouse. Error bars indicate the SE (n = 3). The statistical significance of differences was determined using Student's t-test (*: P < 0.05, **: P < 0.01).

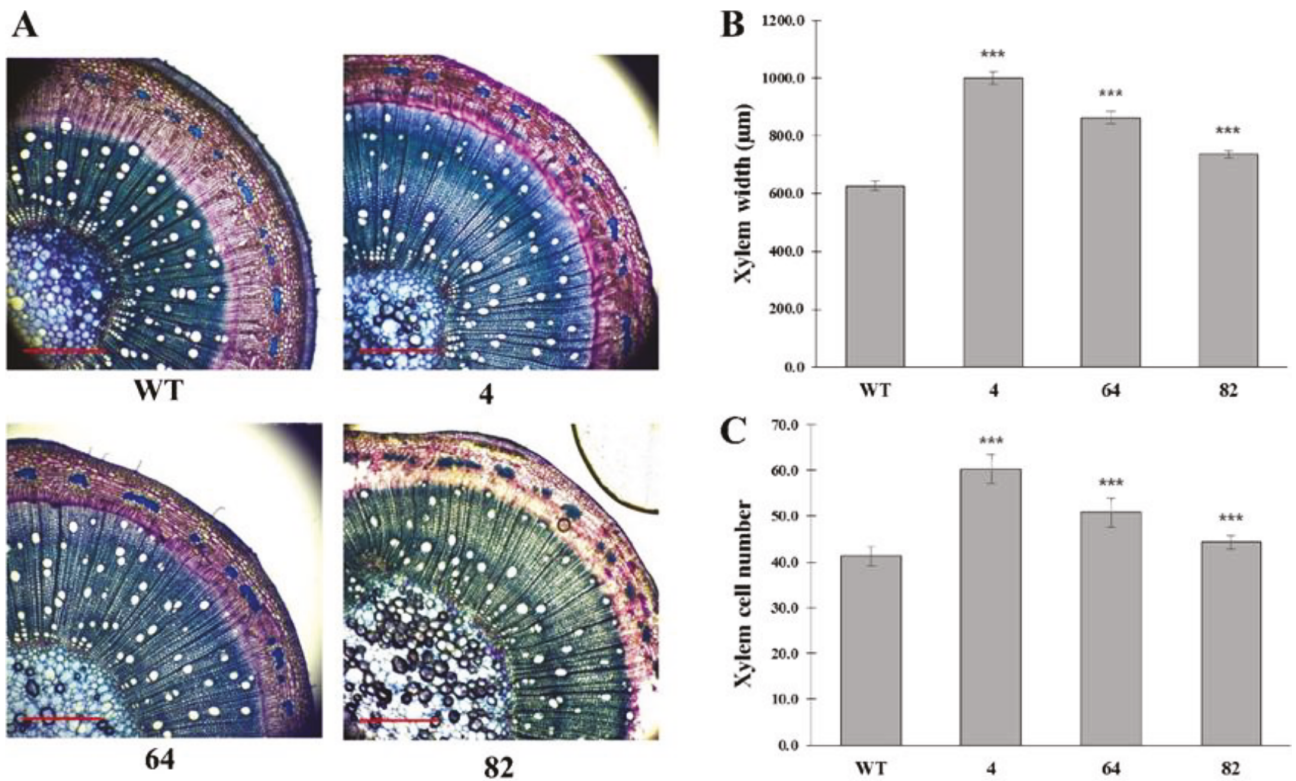


Figure 5. Wood formation of transgenic *M. azedarach* and the wild-type. The 9th internodes of the primary stem of 90-day-old soil-grown plants were used for histological analysis and measurement. A) Stem cross-sections of the transgenic plants and the wild-type were stained with 0.05% toluidine blue O. Scale bars indicate 500 µm. B) Xylem width. C) Xylem cell number. Error bars indicate SE (n = 3). Significant differences were determined using Student's t-test (***: P < 0.001).

analysis of *AtGA20ox* in the *M. azedarach* transgenic plants reconfirmed the specificity of CAD4 in xylem tissue. The expression of the *AtGA20ox* gene was found to focus on the stem rather than leaves. No negative effect on plant architecture was observed. However, internode and stem sizes, stem diameters, xylem cell numbers, and xylem zones increased compared to those in the wild-

type. Consequently, the specific expression of *AtGA20ox* in the xylem promoted the growth rate and biomass production of the transgenic plants. The expanded stem diameter and xylem zone were also reported in transgenic poplar, which was transformed with the *PdGA20ox* gene under the regulation of DX15, another xylem-specific promoter (Jeon et al., 2015). Therefore, it is obvious that

the regulation of the GAs using a xylem-specific promoter could be a potential approach to enhance the growth rate and improve biomass production, especially for woody plants.

The overexpression of the *GA20ox* genes is known to increase bioactive gibberellins such as GA1 and GA4, in addition to morphological modifications (Carrera et al., 2000; Eriksson et al., 2000; Fagoaga et al., 2007; García-Hurtado et al., 2012). Do et al. (2016) showed the correlation between the alteration of switchgrass phenotypes, the *GA20ox* gene copy number, and the bioactive GAs contents. No significant difference in the transcript abundances of the *GA20ox* gene was found between several switchgrass transgenic lines, but higher bioactive gibberellin contents and more morphological alterations were obtained in transgenic lines with more copies of the transgenes. In our study, the two independent lines 64 and 82, which carried a single copy of *AtGA20ox*, showed no difference in stem length, growth rate, and biomass production (Table 2; Figure 4). Faster growth rate and higher biomass production was obtained in the transgenic line 4 with two copies of *AtGA20ox*. This demonstrated that greater amounts of bioactive GAs could be obtained in transgenic *M. azedarach* with a higher copy number of transgenes. However, Fagoaga et al. (2007), Voorend et al. (2015), and Do et al. (2016) all observed weak stems or tillers in transgenic plants with higher bioactive levels. Therefore, additional research needs to be conducted to identify the optimal copies of *AtGA20ox* for *M. azedarach* growth and biomass improvement.

This study reports the development of transgenic *M. azedarach* plants expressing the *AtGA20ox* gene under the control of the CAD4 promoter using an *Agrobacterium*-mediated transformation method. The integration of

AtGA20ox was detected in nine transgenic lines out of 15 regenerated plants. Southern blot hybridization of three randomly selected transgenic plants confirmed the copy number of *AtGA20ox* in *M. azedarach* at 1–2 sites. The transgenic plants had longer internodes and petioles, larger stem diameter, and faster growth rate compared to the nontransformed plants. The wood formation was also improved through the higher xylem cell number and wider xylem zone in all the transgenic plants. The histological staining of stem cross-sections also revealed that the stem and stem diameter were enhanced as a result of the xylem cell division that increased in both the vertical and horizontal directions. Our result is the first report in the improvement of plant growth rate, biomass production, and wood quality of *M. azedarach* due to the expression of ectopic *AtGA20ox* under the control of the CAD4 promoter. These promising results have the potential to increase forestry plant growth and quality and reduce the timeframe for cultivar improvement.

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