Demonstration of improved plant growth and biomass production by At1g73160 promoter-controlled root-preferential expression of the AtGA20ox gene

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Abstract

Background: Overexpression of the *GA20 oxidase* gene has been a recent trend for improving plant growth and biomass. Constitutive expression of *GA20ox* has successfully improved plant growth and biomass in several plant species. However, the constitutive expression of this gene causes side-effects, such as reduced leaf size and stem diameters. To avoid these effects, different tissue-specific promoters were identified and employed for *GA20ox* overexpression. In this study, we demonstrated the potential of At1g, a root-preferential promoter, for *GA20ox* expression to enhance plant biomass in tobacco and *Melia azedarach*.

Results: We examined the utility of the At1g promoter to drive the expression of a GUS (β -1,4-glucuronidase) reporter and the *GA20ox* gene in tobacco and *Melia azedarach*. Histochemical GUS assays in tobacco showed that At1g was a root-preferential promoter whose expression was particularly strong in root tips. The ectopic expression of the *AtGA20ox* gene under the control of the At1g promoter showed improved plant growth and biomass of both tobacco and *M. azedarach* transgenic plants compared to wild-type (WT) control plants. Stem length as well as stem and root fresh weights increased by up to 1.5-3 fold in transgenic tobacco and 2-fold in transgenic *M. azedarach*. Both tobacco and *M. azedarach* transgenic plants showed increases in the root xylem width and xylem over phloem ratio by 50%–100% compared to WT plants. Importantly, no significant differences in the leaf shape or size were observed between the At1g::*AtGA20ox* transgenic and WT plants. Moreover, transgenic *M. azedarach* showed a 135% increase in stem diameter although no change was found in transgenic tobacco.

Conclusions: These results demonstrate the great utility of the At1g promoter for driving the *AtGA20ox* gene to induce growth and biomass improvements in woody plants and potentially some other plant species.

Background

Intense efforts have been made to improve the plant growth and biomass production of important crops as well as forestry tree plants to meet the increased demand of global food, feed, fibre and energy [1–3]. The last two decades have witnessed plant growth and biomass improvements by the transgenic expression of the *GA20ox* gene in different plant species [4–13]. *GA20ox* is a key enzyme involved in the gibberellin biosynthesis (GAs) pathway and it directly controls the content of bioactive Ga in plants [14–18]. Previous studies showed that overexpression of *GA20-oxidase* (*GA20ox*) conferred desirable phenotypes, including accelerated stem growth, increased biomass production, enhanced fibre cell elongation, early flowering, and decreased seed dormancy [4, 12, 19, 20].

Controlling *GA20ox* expression to achieve optimal bioactive GA contents in transgenic plants has attracted increasing attention. The strong and constitutive 35S promoter has been widely used in plant transformation [21]. However, previous reports found that *GA20ox* overexpression under the control of constitutive promoters, particularly CaMV35S, caused undesirable phenotypes such as poor root growth,

low numbers of adventitious roots, a reduced stem diameter, a small leaf area and delayed flowering due to the hyper-accumulation of bioactive gibberellins [5, 9, 12, 20, 22, 23]. These undesirable phenotypes were eliminated when the 35S promoter was replaced by a non-constitutive promoter such as CAD4 or DX15 [3, 20]. Thus, the recent trend has been identifying and employing suitable promoters to drive *GA20ox* expression in order to achieve desirable phenotypes.

The At1g promoter, a 614 bp upstream sequence of the At1g73160 gene, was previously isolated from chromosome 1 of *Arabidopsis thaliana* [24]. This *Arabidopsis* genome locus encodes the UDP-glycosyltransferase superfamily protein. The report showed that the At1g73160 promoter was predicted to drive root-specific expression based on sequence information and GUS assays. This promoter contains several different root motifs such as ASF1 MOTIF CAMV, ARFAT, OSE1, OSE2, RAV1AAT, SURE core and TAPOX1, which are involved in root tissue expression. As the At1g promoter was used to drive the GUS gene, GUS staining was observed only in *Arabidopsis* roots, with the primary root displaying stronger GUS expression than the secondary roots. However, the expression patterns of this promoter have not been fully characterized at the molecular level for the whole *Arabidopsis* plant. Therefore, additional research is needed to validate the function of the At1g promoter and its utility in biotechnological applications and basic research.

Melia azedarach Linn is a native forestry plant in Asia, including Vietnam. This plant grows fast and adapts well to a variety of soil and climatic conditions. *M. azedarach* is grown in all ecological regions in Asia as a multifunctional timber tree. Due to its termite resistance and durability, *M. azedarach* wood could be used in construction and furniture as well as input material for energy production. In addition, *M. azedarach* leaves can be used as an insecticide [25], and some of their limonoid compounds show potential for human cancer treatment [26, 27]. Therefore, *M. azedarach* is considered an important plant species for forestry development strategies, not only in Vietnam but also in many other countries. Our previous works succeeded in growth and biomass production improvements of this plant. However, the transgenic plants showed weak stems and narrow leaves as the 35S promoter was used to guide *GA20ox* gene [3]. These undesirable phenotypes of transgenic *M. azedarach* plants were minimized and eliminated as the 35S promoter was replaced by a xylem specific promoter CAD4 [28]. In the current study, we demonstrated the great utility of the root-preferential At1g promoter to control *AtGA20ox* gene expression for plant growth and biomass improvement. We first analysed and ascertained the root-preferential expression of the At1g promoter in transgenic tobacco using a GUS (β -1,4-glucuronidase) reporter gene as a proof of concept for non-host plant. Then, we used this promoter to guide the ectopic *AtGA20ox* expression and found desirable plant morphological alterations, including accelerated plant growth and increased biomass production in both transgenic tobacco and *M. azedarach*. Importantly, these desirable phenotypes were consistently observed in all At1g::*AtGA20ox* transgenic plants compared to non-transgenic plants.

Results

Root-preferential expression of the At1g promoter

Overexpression of the AtGA20ox gene in tobacco under the control of different promoters

The GUS gene under the control of the CaMV35S or At1g promoter was cloned into the binary vector pBI121, which was used to transform the tobacco cultivar K326 using the *Agrobacterium* mediated method (Fig. S3a). Transgenic tobacco plants of the 35S::GUS and At1g::GUS constructs were randomly selected for GUS assays. The GUS gene expression indicated by histochemical blue staining was observed in all tested tissues, including the leaves, stems and roots of the 35S::GUS transgenic lines (Fig. 1, Fig. S4). In contrast, GUS expression was only found in the roots, particularly in the root tips, of the At1g::GUS transgenic tobacco plants. In addition, expression of the GUS gene was stronger in the root tips of At1g::GUS than the 35S::GUS transgenic tobacco. These results demonstrated the root-preferential expression of the At1g promoter in tobacco, especially in the root tips.

We further characterized the At1g::AtGA20ox and 35S::AtGA20ox transgenic tobacco plants. Specific primers for *AtGA20ox* were used to confirm the presence of this transgene in the transgenic lines. All tested plants showed clear PCR bands (1176 bp) in the agarose gels (Fig. S3b), indicating the transgene integration into the tobacco genome. We used RT-PCR to determine the transcript levels of *AtGA20ox* in different tissues, including the root tips, roots (non-tip roots), stems and leaves of the two representative transgenic lines from At1g::AtGA20ox and 35S::AtGA20ox constructs (Fig. 2). The 35S::AtGA20ox transgenic lines showed no significant differences in *AtGA20ox* expression among the roots, leaves and stems. However, the *AtGA20ox* expression varied in the root, stem and leaf tissue (in this descending order) of the At1g::AtGA20ox transgenic plants with the roots, especially the root tips, showing the highest expression. These results indicate the At1g promoter controls root-preferential expression of At1g::AtGA20ox.

Growth and developmental characterizations of AtGA20ox tobacco plants

Previous studies showed that overexpression of *AtGA20ox* increased cell division and elongation and resulted in other morphological alterations in different plant species [12, 17, 29]. In this present study, all *AtGA20ox* transgenic lines carrying either At1g or the 35S promoter exhibited longer stems than the WT tobacco ($p = 0.015-0.037$). In particular, either At1g-7 and At1g-9 or 35S-2 and 35S-5 showed 1.5-fold increases in the stem length compared to WT plants except for the line At1g-5, which had about a 1.25-fold increase in stem length (Fig. 3b). Both the 35S and At1g transgenic tobacco were taller than the WT plants.

However, the stem diameter of the 35S transgenic tobacco, particularly 35S-5, was smaller than the WT plants ($p = 0.031$). On the other hand, there was no observable difference in stem diameter between the At1g::AtGA20ox and WT plants (Fig. 3c). As a result, the stem fresh weights of the At1g::AtGA20ox lines were greater than the 35S::AtGA20ox transgenic lines (Fig. 3d). Of these At1g::AtGA20ox lines, At1g-9 showed the greatest increase in stem diameter (~110%) and stem fresh weight (~200%) compared to the WT plants (Fig. 3c, d).

Histological analysis of stem cross-sections of the 3rd tobacco internode with toluidine blue showed no significant difference in xylem width and cell number between the 35S::*AtGA20ox* transgenic tobacco and the WT plants. However, there were increases in both xylem width and cell number in certain At1g::*AtGA20ox* lines (Fig. 3e, f, g). As a result, these At1g::*AtGA20ox* transgenic tobacco lines had had a higher stem fresh weight than the 35S::*AtGA20ox* transgenic and WT plants ($p = 0.013\text{--}0.043$) (Fig. 3d).

In addition, the 35S::*AtGA20ox* lines had smaller, curling leaves relative to the WT plants (Fig. 4a). The leaf area of these lines was approximately 210 cm² in the 35S::*AtGA20ox* transgenic plants compared to 285 cm² in the WT plants ($p = 0.012, 0.017$) (Fig. 4b). Despite this, there was no observable difference in leaf area between the At1g::*AtGA20ox* and WT plants.

Finally, under greenhouse conditions, all 35S::*AtGA20ox* transgenic tobacco lines showed a delayed flowering compared to the WT plants (Fig. S5). Their flower buds failed to emerge within 100 days (from 104 days to 107 days) after planting compared to the flower bud emergence of WT plants approximately 90 days after planting (from 89 days to 92 days). Interestingly, there was no significant difference in flowering time between the At1g::*AtGA20ox* transgenic tobacco and WT plants (Fig. S5). Together, these results demonstrated the desirable phenotypes of the At1g::*AtGA20ox* transgenic plants.

Root growth and morphology of transgenic tobacco

All tested transgenic tobacco plants showed a faster root growth than the WT plants (Fig. 5). Of these transgenic plants, the At1g::*AtGA20ox* transgenic lines showed a large increase in the root diameter by up to 2.2-fold (At1g-9) compared to the WT plants ($p = 0.011$). In contrast, the root diameter of the 35S::*AtGA20ox* lines was only 1.25-fold increased compared to WT (Fig. 5c). Analysis of the root cross-section displayed a faster root development in the *AtGA20ox* transgenic plants with larger xylem zones and higher xylem/phloem ratios than the WT (Fig. 5d, e, f). The xylem width of the At1g::*AtGA20ox* roots was 2- to 2.5-fold increased whereas that of the 35S::*AtGA20ox* roots was increased 1.2- to 1.5-fold; compared to the WT roots (Fig. 5e). The xylem/phloem ratio was from 1.9 to 2.2 in At1g::*AtGA20ox* and from 1.5 to 1.75 in the 35S::*AtGA20ox* transgenic lines, while that of the WT was only 1.45 (Fig. 5f). Consequently, the root fresh weight of the At1g::*AtGA20ox* transgenic tobacco (from 11.4 g to 16.8 g in the three tested lines) increased from 2 to nearly 4 times compared to the 35S::*AtGA20ox* (7 g and 7.7 g) and WT plants (4.7 g), respectively (Fig. 5b).

Root-preferential expression of At1g::*AtGA20ox* in a woody plant (*M. azedarach*)

Expression of AtGA20ox in transgenic M. azedarach

Transgenic *M. azedarach* of At1g::*AtGA20ox* were generated using the *Agrobacterium*-mediated method (Dong et al. 2011) with modifications (Fig. S6a). Approximately 250 hypocotyl fragments were used for inoculation, and more than 30 transgenic lines were produced on the selection medium. We randomly selected 13 lines to confirm the presence of the *AtGA20ox* gene. Of these, 12 plants showed the expected PCR bands on agarose gels, indicating the integration of the *AtGA20ox* gene in the *M. azedarach* genome

(Fig. S6b). Semi-quantitative RT-PCR and agarose gels were performed using the *GADPH* gene as an internal control to evaluate the *AtGA20ox* transcript level in transgenic *M. azedarach* (Fig. 6a). All tested plants showed increased transcript levels of the *AtGA20ox* gene compared to the WT plants. In addition, the expression levels of the *AtGA20ox* gene were higher in the root than in the leaf and stem of all *M. azedarach* transgenic lines. Different tissues of the transgenic line At1g-1, including leaves, stems, roots and root tips, were also analysed by quantitative real-time-PCR to examine the expression patterns of the *AtGA20ox* gene, which were highly consistent with those in At1g::*AtGA20ox* transgenic tobacco. The highest expression level of *AtGA20ox* was in the root tissue, especially the root tips (Fig. 6b), which was elevated 1.4- and 2-fold compared to that in the root elongation zone and young stem. The *AtGA20ox* expression in the leaves was much lower than in the other tissues. Together, these results again indicated the preferential root expression of the At1g promoter.

Increased stem and root growth

At three months under greenhouse conditions, all tested transgenic plants exhibited a faster growth rate than the WT plants (Fig. 7). The stem length increased two-fold in the transgenic plants compared to the WT plants (Fig. 7c). In addition, the stem diameter was greater in all tested At1g::*AtGA20ox* transgenic *M. azedarach* ($p = 0.036-0.04$) (Fig. 7d). Stem cross-sectional analysis showed a large xylem zone and greater xylem cell numbers in the transgenic *M. azedarach* than in the WT plants (Fig. 7f, g). As a result, the transgenic lines At1g-1, At1g-5 and At1g-14 displayed around a two-fold increase in the stem fresh weight compared to the WT plants (Fig. 7e). This result is highly consistent with that of the transgenic *AtGA20ox* tobacco.

Similar to the observations of At1g::*AtGA20ox* tobacco, all tested transgenic *M. azedarach* exhibited a faster root growth and bigger root system than WT plants (Fig. 8). The bigger xylem zone and more numbers of xylem cells were observed in all transgenic plants. The xylem zone of transgenic plants varied from 1622 μm (At1g-14, $p = 0.03$) to 1847 μm (At1g-5, $p = 0.016$), while that for WT plants was approximately 1249 μm (se = 69.36). The xylem cell number increased from 60 in the WT plants to up to 93 in the At1g::*AtGA20ox* plants ($p = 0.013$) (Fig. 8e, f). Furthermore, the xylem/phloem ratio was much higher in the transgenic lines than the WT plants (Fig. 8d, g). Importantly, the transgenic *M. azedarach* plants showed an over two-fold increase in root fresh weight compared to the WT (Fig. 8b). The highest root fresh weight was obtained in transgenic line At1g-5 ($p = 0.044$), which had the largest xylem zone and the highest xylem cell number. Consequently, all transgenic plants had a much higher dry root weight (Fig. 8c). These results demonstrated the great utility of At1g::*AtGA20ox* root-preferential expression for root growth and root biomass production of *M. azedarach* and potentially other woody plants.

Discussions

To overcome the various undesirable limitations of using a constitutive promoter, the employment of a tissue-specific promoter instead has become the approach of choice and has achieved more desirable results. Jeon and colleagues used the DX15 promoter (a xylem tissue-specific promoter) to drive

PdGA20ox1 gene expression in transgenic poplar plants, which enhanced plant growth with the partial elimination of the unwanted phenotype [20]. However, the leaf area and root mass of these transgenic plants were still lower than those of the WT plants. In addition, other studies using root-specific promoters for *AtGA20ox* overexpression demonstrated its superiority over the constitutive promoters [30–32]. In agreement with previous reports, the *At1g::AtGA20ox* transgenic plants showed enhanced plant growth and root development without comprising leaf area and flowering time compared to the WT. Therefore, our results here illustrate the superiority of using tissue-preferential promoters instead of constitutive promoters in overexpressing *AtGA20ox* for plant biomass improvement.

Prior to our study, Vijaybhaskar and colleagues indicated that the *At1g* promoter was a root-specific promoter based on GUS assays in *Arabidopsis* [24]. However, no transgene expression at the transcription levels under the control of the *At1g* promoter had been characterized before. In our research, we visually observed GUS activity only in the root tips of *At1g::AtGA20ox* transgenic tobacco instead of the entire root, different from that reported by Vijaybhaskar et al. [24]. This difference in GUS expression may be due to the differences in promoter activities from plant to plant. For example, the *rolD* promoter was described as a root-specific promoter in tobacco [33]. Nevertheless, this promoter showed a strong expression in both the roots and shoots of pea and *Gladiolus* plants [34, 35]. In the report by Vaughan et al., the *FaRB7* promoter showed root-specific expression in strawberry but constitutive expression in tobacco plants [36]. In our work, the qRT-PCR results exhibited expression of *AtGA20ox* throughout the entire plant under the control of the *At1g* promoter (Fig. 6c). In these transgenic plants, the transcription levels were in the descending order of root tips, root elongation zones, stems and leaves. This result agrees with that of Chen et al., who used a “root-specific” promoter *GmPrP2* for soybean transgenic plants [30]. Their qRT-PCR results showed that the transgene was expressed strongly not only in roots but also in stems, leaves, flowers, seeds and hypocotyls, suggesting a root-preferential rather than a root-specific expression. A more recent study reported that *At1g* was a root-specific promoter in *Arabidopsis*, mainly based on GUS assays [24]. However, transcription analysis, which is more sensitive than reporter gene analysis, was not conducted in their study. Here, we have demonstrated that *At1g* is a root-preferential promoter with its expression level varying in different plant tissues. Thus, our study has provided more insightful information about the *At1g* promoter function, employing not only reporter genes but also the gene of interest.

Previously, different root-specific promoters had been isolated and analysed for spatiotemporal expression using reporter genes [24, 30, 33, 35, 37]. However, very few researchers later observed the effect of root-specific expression of their target genes on the phenotype, growth and development of transgenic lines as Jeong et al. reported [38]. Most publications on root-specific promoters have been confined to the analysis of promoter expression through the GUS gene [24, 30, 35, 37, 39]. In our study, we evaluated *AtGA20ox* expression and function under the control of the *At1g* promoter. The gene expression profile was highly correlated with plant morphology and biomass production, which included enhanced stem and root growth and improved biomass production. The desired phenotype could be explained by a moderate level of *GA20ox* expression in the transgenic lines since a root specific promoter was utilized [3, 12, 22]. Moderate bioactive gibberellin levels promote plant cells to enlarge and divide,

enhancing plant growth. This is in comparison to the observed plant morphology when the constitutive promoter 35S was used to drive *AtGA20ox* gene. The activity of bioactive gibberellins may affect plant tissues differently, causing changes in certain tissues such as the xylem zone [40]. Furthermore, the increased plant growth and biomass production of *At1g::AtGA20ox* overexpression transgenic lines may be due to the specificity of the *At1g* promoter, which enhances root system development, resulting in better nutrient absorption efficiencies [38, 41]. The desirable phenotypes observed in both tobacco and *M. azedarach* demonstrated the great potential of this promoter for gene-preferential expression in root tissue for plant biomass improvement. Additional research is necessary to evaluate the effects of *At1g::AtGA20ox* root-preferential expression on the drought tolerance of transgenic plants.

Conclusions

Our results showed that the *At1g* promoter drove strong expression of the *AtGA20ox* target gene in roots. The root system of the transgenic lines was better developed than in the control lines, in terms of both volume and weight. The stems and leaves also expressed the *AtGA20ox* gene, but to a much lesser degree. This minimized the undesirable phenotypes caused by the strong expression of target genes in all parts of transgenic lines with the 35S promoter. The *At1g* promoter has great utility in genetic engineering to improve growth and biomass or to enhance stress tolerance of woody plants and potentially some other plant species.

Materials And Methods

Plant materials and growth conditions

The *in vitro* plants of the *Nicotiana tabacum* cultivar K326 were generated in the Institute of Biotechnology, Vietnam Academy of Science and Technology. Mature seeds of *M. azedarach* L. were supplied by the Vietnam National University of Forestry. Plants were grown under greenhouse conditions (27 ± 2 °C, 70% humidity, 14 h photoperiod, $350 \mu\text{mol m}^{-2} \text{s}^{-1}$) using mixed soil (1 Tribat (Saigon Xanh, Vietnam): 1 TN1 (Research Center for Fertilizers and Plant Nutrients, Vietnam) in plastic pots (E280×260) for tobacco and FAA530 (450×300×300) for *M. azedarach*.

Vector construction

The specific forward and reverse primers (*At1gF/At1gR*) were designed based on the sequence of the *At1g73160 Arabidopsis* promoter [24] (Table S1) and used to amplify this promoter from *Arabidopsis* genomic DNA. The *AtGA20ox* gene was amplified from *Arabidopsis thaliana* genomic DNA using primer pairs specific to this gene sequence [3] (Table S1). The amplified *AtGA20ox* gene and the *At1g* promoter were confirmed by Sanger sequencing and inserted into the binary vector pBI121. To do so, the 35S promoter region of pBI121 was replaced with the *At1g* promoter as a *HindIII/ SmaI* fragment while the GUS gene was replaced by the *AtGA20ox* gene, resulting in the transformation construct pBI121/*At1g::AtGA20ox* vector (Fig. S1, S2).

Tobacco and M. azedarach transformation

Leaves of tobacco *in vitro* seedlings were used as explants for *Agrobacterium*-mediated transformation following the protocol of Topping (1998) with modifications [42]. Briefly, a single *A. tumefaciens* C58 colony carrying the transformation constructs was cultured on LB medium [43] supplemented with appropriate antibiotics and kept at 28°C, 250 rpm until the bacterial density reached an OD₆₅₀ of 0.6. The bacterial cells were harvested by centrifugation at 5,000 rpm at room temperature for 10 min and then suspended in ½ MS liquid medium containing 200 µM acetosyringone (Sigma-Aldrich, Inc.) (inoculation medium). The *in vitro* tobacco leaf disks (1×1 cm²) were prepared and soaked in the bacterial suspension with gentle agitation for 30 min. The infected leaf disks were placed on the co-cultivation media (CCM-K) at 25±2 °C in the dark for 2 days. After co-cultivation, the leaf disks were washed with sterile water, blotted by filter papers and cultured on selection medium (SM-K) containing 150 mg/L kanamycin. Healthy shoots that formed from the leaf disks were transferred to root induction medium (RM-K). Rooted plants were moved to mixed soil under greenhouse conditions.

For *M. azedarach* transformation, sterilized seeds were germinated on MS medium [44] containing 3% (w/v) sucrose and 7.5 g/L agar in 250 ml glass flasks. The cultures were kept in the dark at 24 ± 1°C for 2 days and then transferred to a 14 h photoperiod. After 2 weeks on seed germination medium, the hypocotyl was cut into 1 cm fragments and used for *Agrobacterium*-mediated transformation following the procedure described by Dong et al. [28].

Confirmation of transgene integration and expression

Genomic DNA was isolated from young leaves following the CTAB method [45] and was used for PCRs using specific forward and reverse primers *GA20ox*-F/R (Table S1) to confirm the integrated transgenes. The PCRs were performed as follows: Initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 50 s, followed by a final extension at 72°C for 7 min. Then, the PCR products were analysed by electrophoresis using 0.8% agarose gels.

To confirm transgene expression, total RNA was extracted from the root tips, roots (non-tip roots), stems and leaves of 45-day-old tobacco and 90-day-old *M. azedarach* plants using TRIzol™ reagent following the manufacturer's protocol (Thermo Fisher Scientific-Promega, Madison, WI, USA). The RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used to synthesize the cDNA from the total isolated RNA. Semi-quantitative RT-PCR was performed with 30 cycles to analyse the expression of the *AtGA20ox* gene using the *Nicotiana tabacum Actin* or *M. azedarach GADPH* gene as an internal control. qRT-PCR was carried out utilizing the Advanced TM Universal SYBR® Green Supermix. The primers for RT-PCR and qRT-PCR are given in Table S1. Gene expression levels were normalized and analysed using three clonal plants of each event as biological replicates.

Plant growth measurements

These transgenic lines were *in vitro* propagated to generate multiple plants for phenotyping. At1g::AtGA20ox transgenic and wild-type (WT) plants were transferred to pots of mixed soil under greenhouse conditions, and their stem length was measured every 5 days for tobacco and every 15 days for *M. azedarach* plants. Stem length was measured from the stem above ground to the top of the shoot without removing any leaves. Other morphological characteristics such as stem and root fresh weights, stem diameters, and leaf areas were also measured on the tobacco 45 days after being transferred to the greenhouse. Stem fresh weight only measures the weight of the stem, excluding leaf weight. The tobacco leaf area is measured by the average area of three tested leaves (the 3rd, 4th and 5th leaves from the top) of each plant. The stem diameter is measured at a position 10 cm above the ground. For *M. azedarach*, the measurements were taken 90 days after being transferred to the greenhouse. The data were collected from at least 5 propagated plants from each transgenic line.

Histological analysis

Stem and root cross-sections of tobacco and *M. azedarach* plants were collected and histologically analysed. The cross-sections were stained with 0.05% toluidine blue for 1 min. Images were observed and captured by a Meiji Techno MA151/MT05 C-mount 0.5× Microscope (at 4×, 10× and 40×). Xylem and phloem width were identified from the images using the method described by Jeon et al. [20].

Data analysis

A complete random design was applied to the experimental units under tissue culture and greenhouse conditions. All treatments were conducted in at least three replicates. Data were collected and analysed by SPSS software Version 20 (Chicago, IL) and Microsoft Excel.

Declarations

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Authors' contributions

LL, DP and CH designed the study; NP, CH and PN conceived and supervised the study; LL and BT conducted the experiments; LA provided initial training and assisted with the histological analysis; LL and BT analysed the data; LL and DP wrote the manuscript; ZZ and CH revised and proof-read the manuscript.

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Availability of data and materials

All of the data used in this study are mentioned within the article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interest.

Abbreviations

+ and -: Positive and negative control, respectively; A and S: transgenic plants generated from At1g::*AtGA20ox* and 35S::*AtGA20ox* constructs, respectively; At1g::GUS and 35S::GUS: transgenic tobacco with GUS gene guided by At1g promoter and 35S promoter, respectively; At1g pro: *A. thaliana* 1 glycosyltransferase 73160 promoter; CCM: co-cultivation medium; *GA20ox*: *Gibberellin (GA) 20-oxidase* gene; GUS: β -1,4-Glucuronidase gene; 35S pro: 35S promoter; LB: left border; NeoR/KanR: Neomycin phosphotransferase II gene; NOS pro: Nopaline synthase promoter; NOS ter: Nopaline synthase terminator; RB: right border; RM: root formation from shoots on rooting medium; SM: seed-derived seedlings on MS medium; SM1, SM2: shoot induction on the first and second selective medium containing kanamycin, respectively; T0-1: a representative putative transgenic line in soil; WT: non-transgenic plants

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Supplementary Materials

Additional file 1: Figure S1. Diagram of the T-DNA regions of the transgene constructs used in this study. NOS ter, Nopaline synthase terminator; *GA20ox c-myc*, Gibberellin (GA) 20-oxidase gene; GUS, β -1,4-Glucuronidase gene; 35S pro, 35S promoter; At1g pro, *A. thaliana* 1 glycosyltransferase promoter; NeoR/KanR, Neomycin phosphotransferase II gene; NOS pro, Nopaline synthase promoter; LB and RB, T-DNA left and right border, respectively. **Figure S2.** Construction of vector pBI121/At1g::AtGA20ox. (a)

Amplification of the At1g fragment from the total DNA of *A. thaliana* using At1gF/R primers. (b) Amplification of the At1g fragment from white colonies in blue-white screening using M13F/R primers. (c) Treatment of pBT/At1g and pBI121 by *Hind*III and *Sma*I. (d) Amplification of the At1g fragment from *E. coli* DH5 α . (e) Treatment of pBI121/At1g by *Hind*III and *Sac*I. (f) Amplification of the At1g fragment from *A. tumefaciens* C58. (g) Treatment pBI121/At1g::GUS and pBT/*GA20ox* by *Sma*I and *Sac*I. (h) Amplification of *GA20ox* gene from *E. coli* DH5 α . (i) Treatment pBI121/At1g::*GA20ox* by *Bam*HI and *Sac*I. (j) Amplification of At1g and *GA20ox* from *A. tumefaciens* C58. M. GeneRuler™ 1 kb DNA Ladder; 1-10. Colony lines. **Figure S3.** Tobacco transformation procedure and transgene verification. **a** Tobacco transformation using the pBI121/At1g::*AtGA20ox* construct. Seed-derived seedlings on MS medium (MS); 1×1 cm² leaf pieces on co-cultivation medium (CCM); Shoot induction on the first selective medium containing 100 mg/L kanamycin (SM1); Shoot proliferation on the second selective medium containing 150 mg/L kanamycin (SM2); Root formation from shoots on rooting medium (RM); A representative putative transgenic line in soil (TN1). **b** PCR - gel running used specific primers for the *AtGA20ox* gene. Lanes 1–13, At1g::*AtGA20ox* transgenic tobacco lines; Lanes S1–S5, 35S::*AtGA20ox* transgenic tobacco lines; + and -: Positive and negative control, respectively. **Figure S4.** Expression patterns of the GUS gene in the whole tobacco plants. WT, non-transgenic wild type plant; At1g::GUS_1, At1g::GUS transgenic line 1; 35S::GUS_1, 35S::GUS transgenic line 1. **Figure S5.** Delayed flowering time of 35S::*AtGA20ox* transgenic tobacco lines compared to WT and At1g::*AtGA20ox* transgenic lines at 110 days under greenhouse conditions. WT, non-transgenic wild type plant; A9, propagated plants of At1g::*AtGA20ox* transgenic line 9; S2, propagated plants of 35S::*AtGA20ox* transgenic line 2. Student's t-test showing significance compared with the WT at *P < 0.05 (n=5). **Figure S6.** *Melia azedarach* transformation procedure and transgene verification. **a** *Melia azedarach* transformation using the pBI121/At1g::*AtGA20ox* construct. Seed-derived seedlings on MS medium (SM); 1-cm-length hypocotyl and cotyledon segments on co-culture medium (CCM); shoot formation and development on the first selective medium containing 50 mg/L kanamycin (SM1) and the second selective medium containing 200 mg/L kanamycin (SM2), respectively; root formation on rooting medium (RM); Transgenic lines are grown in soil (TN1). **b** PCR- gel running used specific primers for the *AtGA20ox* gene. Lanes 1–14, different transgenic *Melia azedarach* lines; + and -: Positive and negative control, respectively. **Table S1.** The primers used in this research.

Figures

Figure 1

Expression patterns of the GUS gene in different plant tissues. The tobacco whole roots (left) including root elongation zones and root tips, the cross-sections of the stems (middle) and the leaves (right) were used for GUS staining. Pictures were captured under the 1.0× Parfocal objective of Olympus SZX12 Optical Co., Ltd. Microscopy (made in Japan). Upper panels: non-transgenic wild-type plant tissues; middle panels: At1g::GUS transgenic line 1; bottom panels: 35S::GUS transgenic line 1.

Figure 2

Transcription levels of *AtGA20ox* gene in different tissues of transgenic tobacco. A9, At1g::AtGA20ox transgenic line 9; S2, 35S::AtGA20ox transgenic line 2 RT-PCR was performed for *AtGA20ox* gene using different plant tissues including the root tips (isolated root tips with sizes <1mm), roots (root elongation zone around 3 cm from root tips), stems and leaves of the two transgenic lines A9 and S2. *Nt.Act* (*Nicotiana tabacum* actin gene) was used as the internal control.

Figure 3

Transgenic tobacco phenotypes under greenhouse conditions. a Tobacco plants at 45 days under greenhouse conditions. Scale bar = 30 cm. b, c, d Stem length and diameter as well as fresh weight of the tobacco plants. e Stem cross-sections stained by toluidine blue. Scale bar = 100 μm. f Xylem width of stem cross-sections. g Number of stem xylem cells per 40 μm². WT, non-transgenic wild type plant; A5-A9, propagated plants of different At1g::AtGA20ox transgenic lines; S2, S5, propagated plants of 35S::AtGA20ox transgenic line 2 and 5. Student's t-test showing significance compared with WT at *P < 0.05 (n=5).

Figure 4

Leaf morphology of overexpressed AtGA20ox tobacco. a Leaf shapes and sizes. Scale bar = 10 cm. b Leaf area of tobacco plants. Leaf area was measured by the average area of three tested leaves (the 3rd, 4th and 5th leaves from the top) of each plant. WT, non-transgenic wild type plant; A5-A9, propagated plants of different At1g::AtGA20ox transgenic lines; S2, S5, propagated plants of 35S::AtGA20ox transgenic line 2 and 5. Student's t-test showing significance compared with WT at * $P < 0.05$ ($n=5$).

Figure 5

Root phenotype and morphology of transgenic tobacco. a The whole root systems of tobacco under greenhouse conditions. Scale bar = 10 cm. b Root fresh weight. c Root diameters. d Root cross-sections stained by toluidine blue. Scale bar = 500 μm . e Xylem width of root cross-sections. f Ratio of xylem over phloem zones. WT, non-transgenic wild type plant; A5-A9, propagated plants of different At1g::AtGA20ox transgenic lines; S2, S5, propagated plants of 35S::AtGA20ox transgenic line 2 and 5. Student's t-test showing significance compared with WT at $*P < 0.05$ ($n=5$).

Figure 6

Transcription levels of *AtGA20ox* in different tissues of transgenic *Melia azedarach*. A1, A5 and A14, propagated plants of different *At1g::AtGA20ox* transgenic *M. azedarach* lines. a RT-PCR of the *AtGA20ox* gene (above) and *GADPH* gene (bottom) in the whole roots (root elongation zone and root tips), stems and leaves of transgenic *M. azedarach*; . b Realtime-qPCR (above) and RT-PCR (bottom) of the *AtGA20ox* gene in root tips (isolated root tips with sizes < 1mm), roots (root elongation zones around 3 cm from root tips), stems and leaves of the transgenic A1 line. Student's t-test showing significance compared with WT at * $P < 0.05$ (n=5). The *GADPH* gene was used as the internal control.

Figure 7

Plant morphology of transgenic *Melia azedarach*. a *M. azedarach* at 3 months under greenhouse conditions. Scale bar = 20 cm. b Stem cross-sections stained by toluidine blue. Scale bar = 500 μm. c Stem length. d Stem diameter at 10 cm from the ground. e Stem biomass. f Xylem width of stem cross-sections. g Number of xylem cells per pile. WT, non-transgenic wild type plant; A1, A5 and A14, propagated plants of different *At1g::AtGA20ox* transgenic lines. Student's t-test showing significance compared with WT at *P < 0.05 (n=5).

Figure 8

Root morphology of transgenic *Melia azedarach*. a *M. azedarach* root system under greenhouse conditions. Scale bar = 20 cm. b and c Root fresh and dry weight, respectively. d Root cross-sections stained by toluidine blue. Scale bar = 1000 μm . e Xylem width of root cross-sections. f Number of xylem cell per pile. g Ratio between xylem and phloem zones of root cross-sections. WT, non-transgenic wild type plant; A1, A5 and A14, propagated plants of different *At1g::AtGA20ox* transgenic lines. Student's t-test showing significance compared with WT at $*P < 0.05$ ($n=5$).

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