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ORIGINAL ARTICLE

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Overexpression of xyloglucanase (AaXEG2) accelerates heteroblastic development in mangium leaves

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Abstract Transgenic mangium trees (*Acacia mangium*) overexpressing xyloglucanase (AaXEG2) were generated by spraying flower buds with *Agrobacterium* solution and allowing seeds to develop. The overexpression of xyloglucanase decreased xyloglucan content in the cell walls and increased stem length and diameter. The leaves of the transgenic seedlings exhibited accelerated heteroblastic development, proceeding from the stage of three bipinnate leaves to that of enlarging petiole 2 weeks earlier than wild type seedlings did.

Key words *Acacia mangium* · Heteroblasty · Leaf development · Transformation · Xyloglucan

Introduction

Hemicelluloses are instrumental in the formation of the cellulose microfibril networks in higher plants. Their genetic alteration could either disrupt or enhance cellulose formation. One of the functional hemicelluloses is xyloglucan, which forms bridges between cellulose microfibrils through surface adsorption and chain intercalation.[1] The constitutive degradation of xyloglucan decreased wall pressure and accelerated cell enlargement in growing cells of poplar by overexpression of *Aspergillus* xyloglucanase (AaXEG2).[2] The overexpression also changed leaf morphology, making them greener, thicker, and smaller than those of the wild type. A decrease in wall-bound xyloglucan also resulted in increased wall plasticity, which in turn accelerated leaf growth in transgenic *Arabidopsis* overexpressing poplar cellulase (PaPopCel1).[3] In the case of transgenic sengon (*Paraserianthes falcataria*) overexpressing poplar cellulase,[4] the transgenic expression increased the length and width of stems with larger leaves, which showed a moderately higher density of green color than leaves of the wild type. Furthermore, their leaf movements were somehow disturbed due to a decrease in xyloglucan: transgenic trees opened their leaf pairs at midnight, at the same time as wild-type plants did, but started closing their leaves 30 min later than the wild type and completed closing them more than 1 h later. The opening and closing movements of leaves originate in the leaf bases (petiolule pulvinus) and correspond to the expansion and shrinkage, respectively, of the motor cells.[4] Therefore, the decrease in xyloglucan resulted in not only the enhancement of plant growth but also some changes in leaves.

Mangium (*Acacia mangium*) is a fast-growing tropical forest tree that grows symbiotically with nitrogen-fixing *Rhizobium* and that, for this reason, can colonize infertile areas. It grows well not only in cleared forest sites but also in degraded sites, such as weedy *Imperata* grasslands and mining sites.[5] The tree's attributes include rapid early growth, high rates of cellulose recovery (for pulp, timber, and fuel), and tolerance of a range of soil types and pH values. The specific gravity of the wood is greater than 0.5 because of its high cellulose density with thick walls. Because of its good characteristics, mangium has been selected as one of the Indonesian Industrial Timber Estate (HTI) commodities, and is now promoted as a raw material for wood-based industries. This study was originally started in Indonesia because it was expected that fast-growing and stress-tolerant mangium trees would grow as the faster in the forest plantations of Southeast Asia. The question is, therefore, whether mangium overexpressing xyloglucanase exhibits any phenotypic traits related to wall loosening and leaf development. The wall loosening would be expected to

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allow the plant to grow faster, a trend that could be also assessed through leaf growth, because there was a certain relationship between plant growth and leaf development in poplar,[2] *Arabidopsis*,[3] and sengon.[4] This article describes a transgenic mangium overexpressing xyloglucanase; this increase in xyloglucanase resulted in a decrease in xyloglucan molecules in the walls, allowing us to assess mangium growth and leaf development under conditions of altered wall composition.

Materials and methods

Plant materials

Mother plants were grown in the Germ Plasm Garden at the Research Centre for Biotechnology, Indonesian Institute of Sciences, on the island of Java. The growing conditions for the parent plants were as follows: rainfall, 3400–3600 mm/year; climate type A; temperature range, 28°–32°C; humidity, 50%–60% during the dry season and 80%–90% during the wet season; soil type, red–yellow latosol with a pH range of 4.5–6. All mangium plants for grafting were obtained in January 2007, and grafted trees were grown in a 1:1 mixture of red–yellow latosol and sand in a greenhouse.

Flower buds acquired from 1-year-old grafted mangium (*A. mangium*) trees grown in soil in pots were subjected to transformation. First, vegetative seedlings were obtained from the branched stems of 12-year-old mangium trees (16–20 m tall), 1 m from the shoot top, by the grafting method consisting of peeling the branch skin, removing the cambium, and covering it with wet kadaka fibers and plastic wrap. It took about 3 months to obtain rooting in the plastic wrap.

Transgenic construct and plant transformation

A transgenic construct was generated according to the method described by Park et al.[4] *Aspergillus aculeatus* cDNA for xyloglucanase was amplified from first-strand cDNA as a template by polymerase chain reaction (PCR) using a forward primer containing an *Xba*I site (5P-GCT GCCAGTCTAGAGCCCGCAGCGAC-3P) and a reverse primer containing an internal *Sac*I site (5P-CTCCCGT CAGCCGCGGTCCACGCAAC- 3P) complementary to the DNA sequence of *AaXEG2* (accession number AY160774). The *Xba*I-*Sac*I fragment for *AaXEG2* was subcloned into pBluescript II (SK3). The signal peptide (Met1 to Leu30) of *Populus alba* cellulase was cloned from PaPopCel1 cDNA (accession number D32166) by PCR in a pGEM-T Easy Vector system (Promega, Madison, WI, USA) using a forward primer containing a *Bam*HI site (5P-CTAGTGGATCCTTTGGAG-3P) and a reverse primer containing an *Xba*I site (5P-AGCATAGTC TAGAGAAGTGAAGGC-3P). The *Bam*HI–*Xba*I fragment was ligated into the pBluescript II (SK3) harboring the *Xba*I–*Sac*I fragment for *AaXEG2*. The chimeric DNA for the PopCel1 signal peptide and the XEG2 mature protein was excised with *Bam*HI and *Sac*I and inserted into the *Bam*HI–*Sac*I site of the binary vector pBE2113-GUS under control of the CaMV35S promoter and E12-6 enhancer sequences. The plasmid constructs were electroporated into *Agrobacterium tumefaciens* LBA4404, and the bacterial solution (OD 0.6) was sprayed onto flower buds as described below.

Transformant selection from seeds

The seedlings, like their mother trees, produced flowers according to the normal process of flowering development, in which flower buds form first, followed by inflorescences, immature seeds, and mature seeds. Flower buds formed on the grafted seedlings after 1 year. These were sealed into plastic bags. A hole was made in each bag close to the flower bud, and *Agrobacterium* solution was sprayed into the bag through this hole (Fig. 1). The bag was then sealed and the bacteria and buds were incubated for 3 days. After the bag was removed, seeds formed in the pods over the next 25 to 30 weeks. Other plants were sprayed with a similar solution lacking *Agrobacterium*; the seeds they produced were used as a control.

Mangium seeds derived from the buds were washed with water five times and then soaked in hot water at 80°C for 10 min. The seeds were then sterilized with 2% Benlate for 30 min and 4% Dithane for 30 min followed by 70% ethanol for 3 min and 0.05% $HgCl_2$ for 3 min. The seeds were washed five times with sterilized water and germinated in MS agar medium containing 300 μg ml^{-1} kanamycin. After germination, the meristems of kanamycin-resistant shoots were cultivated in MS medium containing 10 μM 6-benzylaminopurine and 5 μM 1-naphthaleneacetic acid, essentially according to the method of Bon et al.,[6] and were allowed to form multiple shoots. Each shoot was then transferred to MS medium containing 3 μM indole-3-acetic acid and allowed to form roots.

Western blot analysis

The shoot and leaves of each 6-month-old plant, which had a fresh weight of about 0.3 g, were homogenized in 20 mM sodium phosphate buffer (pH 6.2) in a mortar, and the wall residue was washed three times. The wall-bound proteins were extracted from the wall residue with a buffer containing 1 M NaCl. The proteins were then subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrotransferred to a Hybond-C Extra membrane (Amersham Biosciences, GE Healthcare, Chalfont St. Giles, UK), and probed with an antibody against the AaXEG2 sequence and a second antibody that was provided with the Toyobo (Osaka, Japan) ABC High-HRP immunostaining kit.

Assay of xyloglucanase activity

Each enzyme preparation was obtained from the wall residue of the shoot and leaves of a 6-month-old plant with

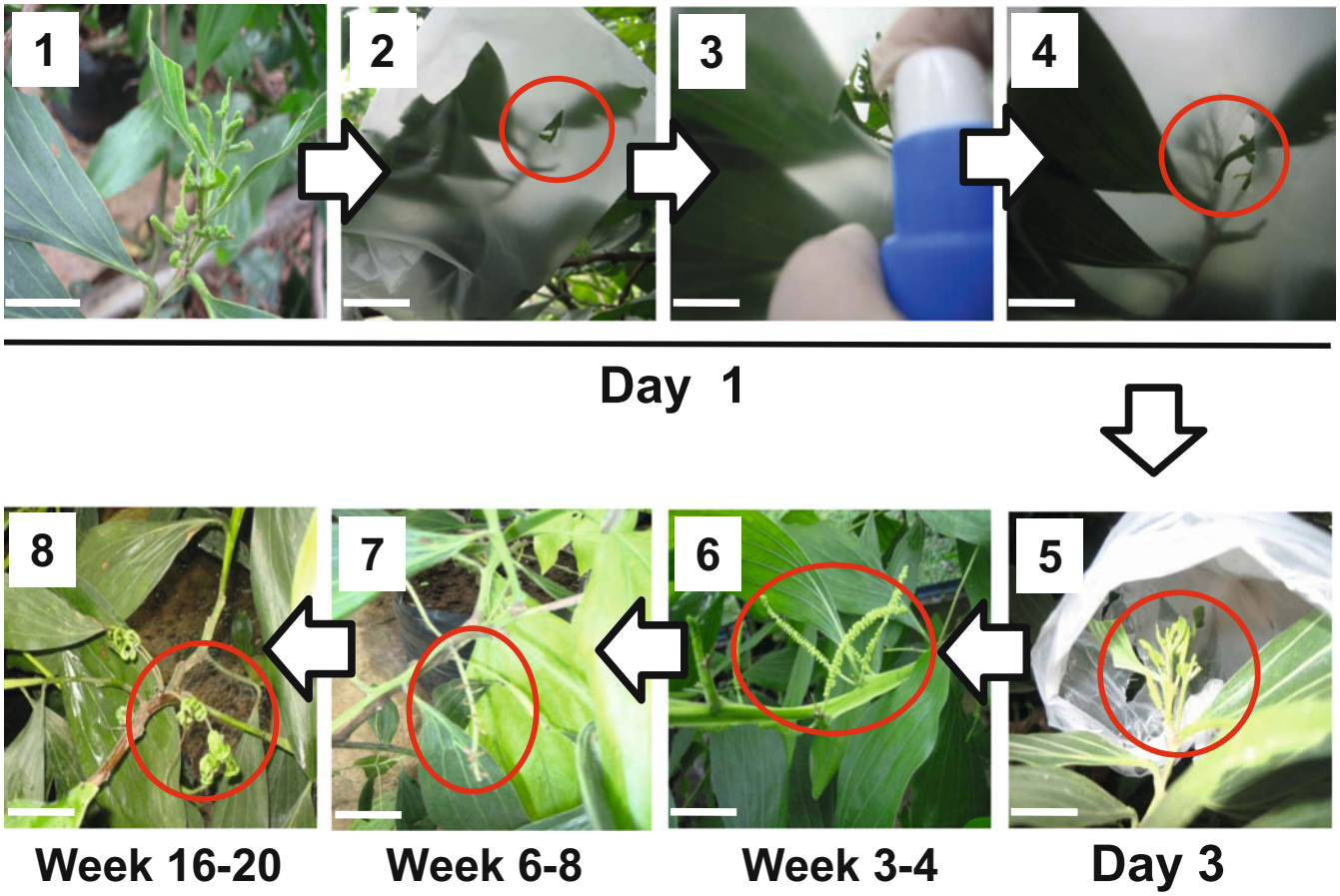

Fig. 1. *Agrobacterium*-mediated transformation method for mangium involving spraying the flower buds. Stages of flower development: *1*, flower bud; *2*, when the branch was covered with flower buds, a hole was made in the bag containing buds; *3*, *Agrobacterium* solution was sprayed onto flower buds; *4*, the hole was sealed; *5*, the cover was removed; *6*, flowering stage; *7*, pod-forming stage; *8*, mature-pod stage

a buffer containing 1 M NaCl; using Cannon Instruments (State College, PA, USA) semi-microviscometers, the enzyme activity was assayed viscometrically at 28°C for 4 h using 0.1 ml of the enzyme preparation plus 0.9 ml of 10 mM sodium phosphate buffer (pH 6.2) containing 0.5 % (w/v) tamarind xyloglucan. One unit of activity was defined as the amount of enzyme required to cause a 0.1% loss in viscosity in 2 h under certain conditions.[7] Protein was measured using the Coomassie Plus protein assay reagent (Pierce Protein/Thermo Scientific, Rockford, IL, USA) according to the method described by Bradford.[8]

Wall analysis

A whole leaf including either the rachis of pinna with petiole or the phyllodium was ground in liquid nitrogen and successively extracted four times with 10 mM sodium phosphate buffer (pH 7.0) and three times with 24% KOH containing 0.1% $NaBH_4$ at less than 45°C for 3 h in an ultrasonic bath. Noncellulosic polysaccharides were determined using 24% KOH extracts and the phenol/sulfuric acid method.[9] Xyloglucan was determined by the iodine/sodium sulfate method.[10]

Hemicelluloses were successively extracted three times from the xylem of 6-month-old plants with 24% KOH containing 0.1% $NaBH_4$. The insoluble wall residue (cellulose fraction) was washed twice with water and solubilized with ice-cold 72% sulfuric acid. Cellulose was determined in the solubilized fraction by the phenol/sulfuric acid method.[9] The alkali-soluble fraction was neutralized, dialyzed, and freeze-dried for methylation analysis. Partially methylated alditol acetates were analyzed using an Agilent gas chromatography-mass spectrometer apparatus with a glass capillary column (DB-225, 0.25 mm i.d. × 15 m).[11] Each alditol acetate was identified by its retention time and mass spectrum. Lignin content was determined by the Klason method.[12]

Growth measurement

The growth of the transgenic plants was monitored starting after the plants had been transplanted in soil under nonsterile conditions. When the stems had grown to approximately 15 cm long, which took about 3 weeks after the 2-week habituation period, each was marked at a height of 5 cm; the marks were subsequently used as a reference point for measuring its height and diameter every third day. The

length of the stem was defined as the distance from the top to the reference point. The timing of leaf development was determined by observing the development every day for 14 weeks after the 2-week habituation period, starting when the stem heights were around 2 cm above the soil.

Results and discussion

Grafted seedlings

Seedlings were obtained from 12-year-old mangium trees. Seedlings from the upper branches of the trees (i.e., from the top 2.5 m of the main stem) exhibited a rooting efficiency of about 80%, whereas seedlings from the lower branches exhibited an efficiency of less than 50%. Similarly, upon transfer to soil, seedlings derived from upper branches exhibited survival rates of 60%–80%, while those from lower branches exhibited survival rates of less than 50% (data not shown). Both higher rooting efficiencies and higher vegetative plant seedling survival rates were seen in branches originating in the top 2.5 m of the main stem. After 1 year, the grafted seedlings were determined to be ready for transformation because their stems could flower stably and repeatedly.

Transformation

One-year-old grafted seedlings that had formed flower buds were sprayed with *Agrobacterium* solution in closed bags in a closed greenhouse (Fig. 1) and then permitted to produce seeds. Only 4 of 149 independent transgenic seeds sprayed with *Agrobacterium* solution grew in MS medium in the presence of 300 μg ml^{-1} kanamycin (Fig. 1). Control seeds, which were not transformed, showed a germination rate of only 65.5% in MS medium in the absence of kanamycin. Kanamycin-resistant seeds were permitted to germinate and develop into normal seedlings. The data obtained for transgenic plants indicates a transformation efficiency of about 4.1%; transformation efficiencies obtained by other groups for acacia plants grown by tissue culture via organogenesis using selectable and reporter genes have ranged from 30% (*A. mangium*)[13] to 4% (*A. sinuate*)[14] and 7% (*A. crassicarpa*)[15]. The transformation efficiency we obtained was therefore relatively low; we may be able to increase the efficiency in the future by altering the timing of the administration of the *Agrobacterium* spray.

This transformation was originally derived from the vacuum infiltration method[16] used for the production of transgenic *Arabidopsis*. When mangium flower buds were subjected to the original vacuum infiltration method, however, no flowering and no seed production occurred. Through experimentation with variations on the method, we arrived at the present transformation method, in which mangium flower buds are sprayed with *Agrobacterium* solution in plastic bags, where they are kept in place for 3 days subsequently. It may be possible to find a transformation method that works better for this plant in the future. Kanamycin was used to select the transformants in these experiments, although the mangium explants used had resistance to kanamycin that extended to 250 μg ml^{-1} in the medium. There is a possibility that some mangium plants are resistant to up to 300 μg ml^{-1} of kanamycin in the medium.[13]

Although we initially obtained four independent transgenic mangium lines, we were only able to propagate three lines to the point where each generated multiple shoots followed by roots. Five to six seedlings were obtained from each of these lines. In order to check whether live *Agrobacterium tumefaciens* existed in the transgenic mangiums, each transgenic plant was homogenized and incubated in yeast extract and beef medium (0.5 g beef extract, 0.1 g yeast extract, 0.5 g peptone, 0.5 g sucrose, 30 mg $MgSO_4 \cdot 7H_2O$ in 100 ml water) at 28°C for 48 h. The bacterium *A. tumefaciens* was not found on the medium plated with the homogenates of the plant tissues, indicating that the bacterium was not alive and did not remain in the transgenic mangium.

Xyloglucanase expression

Each of the three independent transgenic lines expressed a xyloglucanase from *Aspergillus aculeatus* (AaXEG2) under the control of a constitutive promoter and the PopCel1 signal sequence. To assay the expression of the transgene, we used an antibody against the *AaXEG2* gene product, which appeared as a single 28-kDa band on Western blot analysis. Xyloglucanase was present in the leaves and petioles of our transgenic plants, occupying a position corresponding to the size of mature xyloglucanase (Fig. 2A). No

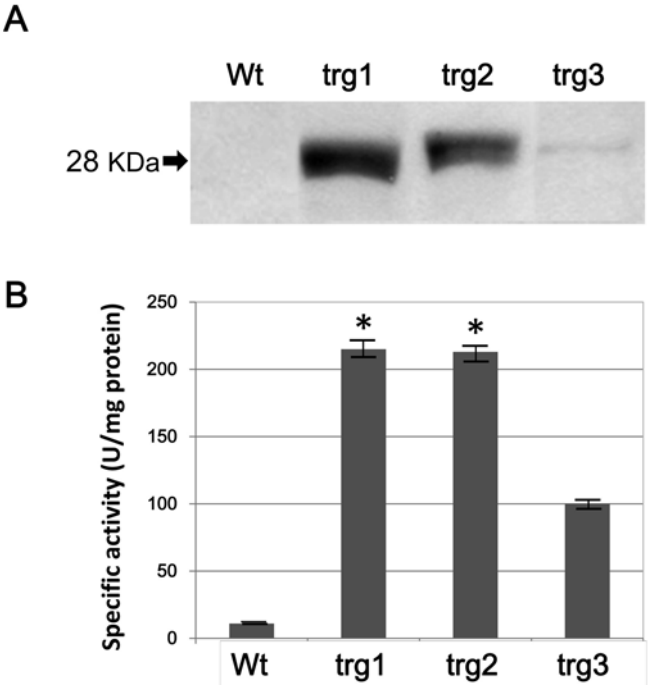

Fig. 2. Western blot analysis of *AaXEG* gene product (**A**) and the level of xyloglucanase activity (**B**) in mangium. *Arrow* indicates the 28-kDa gene product. Activity values represent the means ± SE of three independent plants of a single line. *Asterisks* represent a statistically significant difference (student's *t* test, $P < 0.06$) in comparison with the wild type (*Wt*). *trg1–3*, transgenic lines

Table 1. Xyloglucan content (μg/mg dry weight) in mangium leaves including rachis of pinna with petiole

Genotype	Xyloglucan	Noncellulosic polysaccharides	Content[a] (%)
Rachis of pinna with petiole			
trg1	2.1	152.4	1.4
trg2	14.1	158.2	8.9
trg3	21.5	165.1	13.0
Wild type	33.4	160.1	20.9
Phyllodium			
trg1	13.3	222.2	6.0
trg2	22.5	230.0	9.8
trg3	29.0	228.0	12.7
Wild type	37.6	235.0	16.0

[a] Xyloglucan content (%) in noncellulosic polysaccharides. The rachis of pinna with petiole was obtained at 4 weeks for trg1 and trg2 and at 6 weeks for wild type and trg3 during development as shown in Fig. 4A. The phyllodium was obtained at 14 weeks for all the plants as shown in Fig. 4A. Each data point represents the mean value for three independent plants of a single line, with individual values of fractions varying from the mean by less than 4.5%

Table 2. Composition of mangium wood (mg/100 mg wood)

	Line			Wild type
	trg1	trg2	trg3	
Cellulose	62.62	63.19	59.75	59.98
Lignin	22.18	21.01	24.55	24.02
Hemicellulose	15.20	15.80	15.70	16.00
Fucosyl terminal	0.02	0.03	0.04	0.16
Arabinosyl terminal	0.23	0.08	0.18	0.04
Xylosyl terminal	0.05	0.07	0.04	0.10
4-Linked	13.18	13.14	13.40	13.18
Glucosyl 4-linked	0.64	1.20	1.03	1.08
4,6-Linked	0.01	0.05	0.19	0.24
Galactosyl terminal	0.13	0.19	0.08	0.18
4-Linked	0.02	0.15	0.03	0.02
3,6 Linked	0.50	0.53	0.52	0.84
Mannosyl 4-linked	0.31	0.22	0.33	0.16

Each data point represents the mean of three independent plants of a single line, with individual values of fractions varying from the mean by less than 2%

xyloglucanase protein was detected in control plants. The three transgenic plants exhibited different expression levels: the transgenic lines called trg1 and trg2 both gave stronger signals than trg3 did. They also exhibited different levels of xyloglucanase activity, although they all had about 10–20 times more xyloglucanase activity than the control plants did (Fig. 2B).

Our carbohydrate analysis of whole leaves, including rachis of pinnate leaves with petioles, revealed that the transgenic plants contained less wall-bound xyloglucan among noncellulosic polysaccharides than the wild-type plants did (Table 1). Nevertheless, xyloglucan contents increased to 6.0% and 9.8% from 1.4% and 8.9% in trg1 and trg2, respectively, in their phyllodium after the stage of rachis of pinnate leaves. The level of xyloglucan in the walls could be adapted to the size and structure of successive organs during leaf development. Secondary walls with high levels of expression (Table 2) exhibited relatively small decreases in hemicellulose and lignin accompanying the increase in cellulose. Methylation analysis revealed that the proportion of 4,6-linked glucose on the xyloglucan backbone was low in the transgenic lines, although 4-linked xylose was one of the major sugars in the hemicellulose, on account of the xylan.[17]

Growth response

Plants from the two transgenic lines with high expression levels of xyloglucanase (trg1 and trg2) grew faster in terms of both height and stem diameter than wild-type plants did, although one line with a low expression level (trg3) grew at a similar speed to the wild type plants (Fig. 3A,B). Growth of trg1 and trg2 was accelerated between 5 and 25 weeks (between 0 and 20 weeks in Fig. 3) after transfer into soil. However, the growth levels were almost identical for all the transgenic and wild-type plants for 5 weeks after transfer into soil. This in turn accelerated the timing of heteroblastic development, the process by which petioles enlarge and

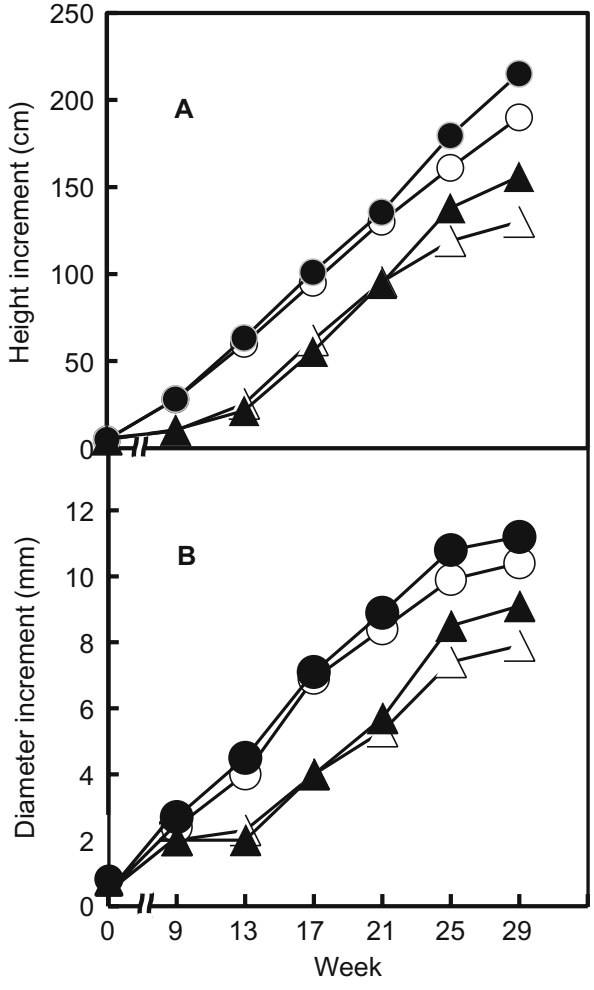

Fig. 3. Effect of *AaXEG* transgenes on stem elongation (**A**) and diameter growth (**B**). *Solid circles*, trg1; *open circles*, trg2; *solid triangles*, trg3; *open triangles*, wild type. Stem growth was shown after the plants had been transplanted into soil. Each data point represents the mean of three different experiments, with individual values varying from the mean by less than 6.6%

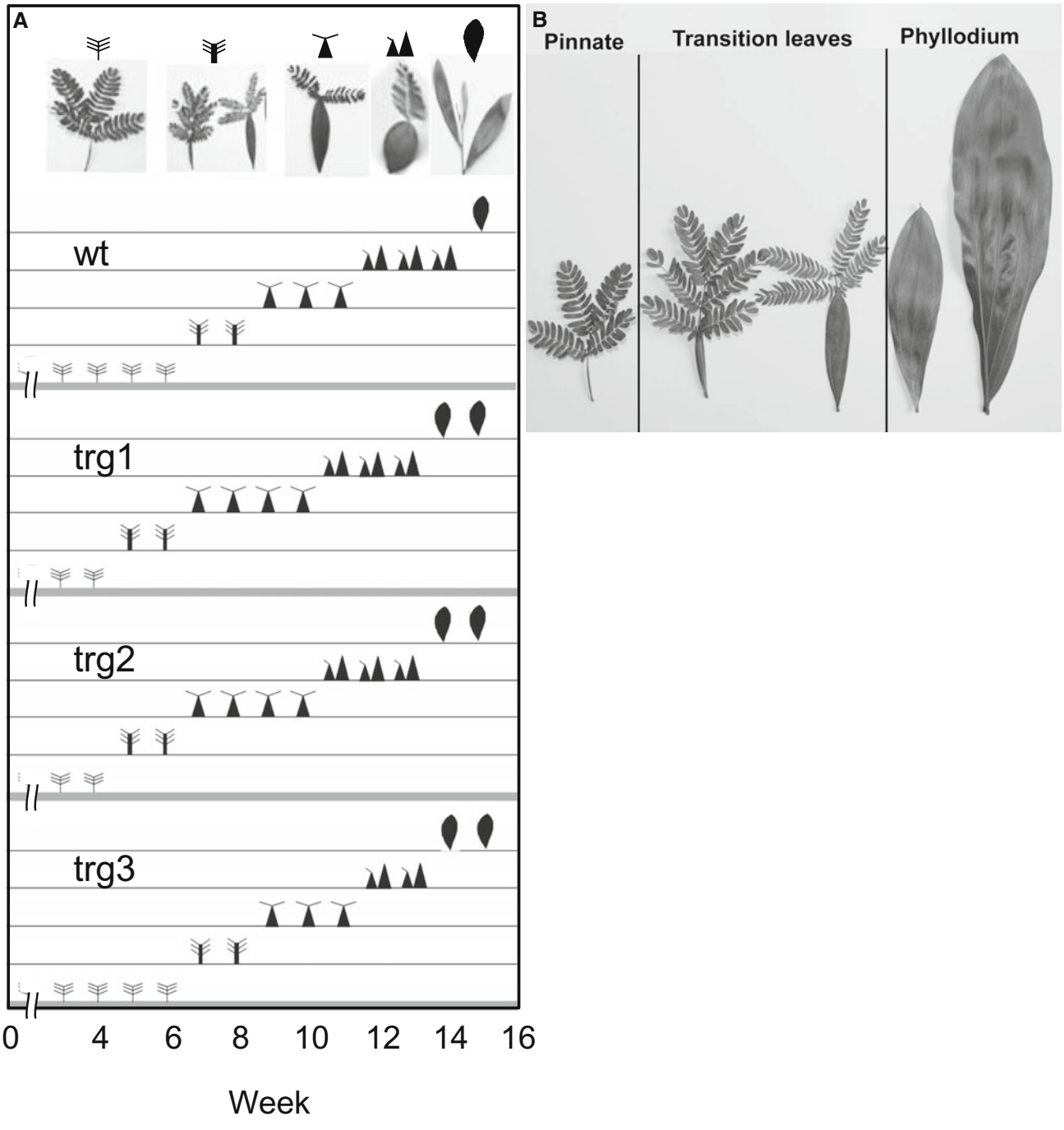

Fig. 4. Effect of *AaXEG* transgenes on leaf development (**A**). The stages of heteroblastic development are shown in the five photographs (from the *left*): two to three bipinnates, enlarged petiole with three bipinnates, early phyllodium with one bipinnate, phyllodium with one pinnate, and phyllodium. The developmental stage indicated for each week represents the mean of three different experiments. The timing of development was shown after the plants had been transplanted into soil. Each line shows the precise timing of development, with individual values varying by 1 or 2 days. Leaf changes (**B**): three bipinnates (*left*), leaves with enlarging petioles (*middle*), and phyllodium (*right*)

become phyllodes, in trg1 and trg2 (Fig. 4A,B).[18,19] This process represents the transition of the compound leaves of the transgenic *Acacia* plants from the juvenile pinnate stage (with first one, then two and three bipinnates) to the adult phyllode stage. This transition occurred earlier in transgenic seedlings trg1 and trg2 (Fig. 4A), which proceeded from the stage of three bipinnate leaves to that of enlarging petiole identically at 5 weeks after transfer into soil. The trg3 transgenic line progressed through development at almost the same rate as the wild-type plants, although this line reached the adult phyllode stage 1 week earlier than the wild-type plants did. The rates of development did not correspond to stem height and diameter in the three transgenic lines and the wild type. It seems likely that some signal induced at an early stage of growth regulates development, because development proceeded step by step during growth between 3

and 13 weeks regardless of the different plant sizes of the transgenic lines trg1 and trg2 and the wild type (Fig. 3).

Thus, heteroblastic development is accelerated in transgenic mangium probably because of growth acceleration, although variation in the sizes and structures of successive organs does not exactly result in the same juvenile and adult phases of shoot development. Yu and Li[19] have analyzed these physiological changes, and pointed out that true leaves enhance the relative growth rate of mangium seedlings, while the phyllodes that develop subsequently could help plants cope with higher irradiance, as phyllodes are better adapted to high irradiance than true leaves are. Phyllodes were also found to contain more water at full turgor and to use less water in turgor control, and therefore to be better adapted to drought.[20] Under conditions of low light, however, the timing of heteroblastic development is delayed.[21] Forster and Bonser[22] have also shown that exposure to low levels of blue light delays heteroblastic development under conditions of low rainfall, although the light exposure increases stem elongation. Based on observations of the genera *Aristotelia*, *Hoheria*, *Pseudopanax*, and *Melicope*, Gamage and Jesson[23] suggested that heteroblastic seedlings possess a leaf morphology that is advantageous under conditions of abundant light but are not adapted for low-light conditions. The maturation of mangium leaves from compound true leaves to phyllodes can thus be accelerated or delayed by their growth level and environmental factors.

Xyloglucan contents were increased in the phyllodium in trg1 and trg2 compared with those in the rachis of pinnate leaves during a progressive change in the size and structure of successive organs (Table 1 and Fig. 4A). It is possible that the observed acceleration in heteroblastic development of mangium leaves for trg1 and trg2 was evoked by the large decrease in xyloglucan content during the stage of the rachis of pinnate leaves. Since plant growth and morphology are controlled by cell walls, acceleration of heteroblastic development could be due to the degradation and reconstruction of cell walls.

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