Breeding Science 65: 177–191 (2015)
doi:10.1270/jsbbs.65.177

Research Paper

Overexpression of *Arabidopsis thaliana* gibberellic acid 20 oxidase (AtGA20ox) gene enhance the vegetative growth and fiber quality in kenaf (*Hibiscus cannabinus* L.) plants

Samanthi Priyanka Withanage[1)], Md Aktar Hossain[2)], Sures Kumar M.[2)], Hairul Azman B Roslan[3)], Mohammad Puad Abdullah[1)], Suhaimi B. Napis[1)] and Nor Aini Ab. Shukor*[2,4)]

[1)] *Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia*, 43400 UPM, Serdang, Selangor, Malaysia
[2)] *Faculty of Forestry, Universiti Putra Malaysia*, 43400 UPM, Serdang, Selangor, Malaysia
[3)] *Faculty of Resource Science and Technology, University Malaysia Sarawak*, Kota Samarahan, Sarawak, Malaysia
[4)] *Institute of Tropical Forestry and Forest Product, Universiti Putra Malaysia*, 43400 UPM, Serdang, Selangor, Malaysia

Kenaf (*Hibiscus cannabinus* L.; Family: Malvaceae), is multipurpose crop, one of the potential alternatives of natural fiber for biocomposite materials. Longer fiber and higher cellulose contents are required for good quality biocomposite materials. However, average length of kenaf fiber (2.6 mm in bast and 1.28 mm in whole plant) is below the critical length (4 mm) for biocomposite production. Present study describes whether fiber length and cellulose content of kenaf plants could be enhanced by increasing GA biosynthesis in plants by overexpressing *Arabidopsis thaliana* Gibberellic Acid 20 oxidase (AtGA20ox) gene. AtGA20ox gene with intron was overexpressed in kenaf plants under the control of double CaMV 35S promoter, followed by *in planta* transformation into V36 and G4 varieties of kenaf. The lines with higher levels of bioactive GA (0.3–1.52 ng g^{-1} fresh weight) were further characterized for their morphological and biochemical traits including vegetative and reproductive growth, fiber dimension and chemical composition. Positive impact of increased gibberellins on biochemical composition, fiber dimension and their derivative values were demonstrated in some lines of transgenic kenaf including increased cellulose content (91%), fiber length and quality but it still requires further study to confirm the critical level of this particular bioactive GA in transgenic plants.

Key Words: biocomposite, fiber quality, GA20ox gene, *Hibiscus cannabinus*, overexpression, transgenic kenaf.

Introduction

There are increasingly growing demands for the natural fibers which are predominantly obtained from the woody plants of the forests as the main commodity for most of the fiber-based industries. Along with other biocomposite materials, global consumption of paper was estimated to have increased from 300 million tons in 1998 to 425 million tons in 2010 (Labidi *et al.* 2008, Rowell and Cook 1998). However, woody fiber resources are diminishing rapidly through extensive felling of trees for natural fibers. Kenaf (*Hibiscus cannabinus* L.), which belongs to the Malvaceae family, is a non-woody multipurpose annual plant that is very close to cotton and okra (Ayadi *et al.* 2011) and it possesses excellent potential to fulfil this demand without cutting and depleting forest resources. It has also been recognised as the third largest fibrous crop following cotton and jute (Ahmed *et al.* 1998, Pande and Roy 1996), an excellent source of cellulosic fiber for manufacturing large range of paper products (Ayadi *et al.* 2011). Pulping from kenaf requires less energy and chemical inputs for processing compared to other standard wood sources (Bhardwaj *et al.* 2005, Villar *et al.* 2009). It grows very fast even under minimum amount of requirements and provides a wide range of commercial values for the fiber industry. The stalk of this plant consists of outer fiber "bast", while its inner fiber "core" comprises roughly 40% and 60% of the stalk's dry weight, respectively. These refined fibers are compatible for engineering wood, insulator and clothing-grade clothes, security notes, bullet proof jackets, helmets, packing materials, etc. Meanwhile, cut bast fibers are commonly blended with resins for plastic composites that are used in making car interiors

Communicated by D.S. Brar
Received November 7, 2014. Accepted November 28, 2014.
*Corresponding author (e-mail: anishukor@yahoo.com)

known as bolsters for more sustainable vehicles. Since the fibers of this plant are mainly used in various manufacturing industries, the enhanced quality of fibers, as well as species biomass, is a lucrative target of many researchers, manufacturers and investors alike for overall production system. However, the average length of kenaf fibers is 2.6 mm in bast and 1.28 mm in whole plant (Jamaludin 2008), with some exceptions of 3–4 mm in bast (Farshes *et al.* 2011), which is below the critical fiber length for biocomposite (biopolymer and paper industries) manufacturing (Jamaludin 2008). The larger the fiber length means greater mechanical strength than the shorter ones for flexural and impact properties. Studies have shown that flexural strength in specimens is considerably higher by 67% in longer fiber while these decreases significantly with reduced fiber length including the ones below the critical length (Shibata *et al.* 2006) since the end use of fibers primarily depend upon their physical, chemical and structural properties.

Elongation of kenaf plant and fiber is believed to be obtained through environmental manipulation or genetic modification. Plant hormones play critical roles in growth and development of plants. Gibberellin (GA) is a plant hormone of particular interest to researchers due to its broad spectrum of effects on plant growth, elongation, flowering development, seed formation and germination (Davies 2004, Fleet and Sun 2005, Hedden and Thomas 2012, Mutasa-Göttgens and Hedden 2009, Schomburg *et al.* 2003). Exogenous application of gibberellins (GAs) has created a consensus on altering the plant growth and different developmental features. Although a large number of different GA species (136 GA structures in nature) have been identified (Giacomelli *et al.* 2013), only few have been proven to possess biological active GAs. GA1 and GA4 are the most common active forms in higher plants (Giacomelli *et al.* 2013). Since most of the biosynthesis genes in gibberellin biosynthesis pathway have been identified and extensively studied in many species (Hedden and Thomas 2012, Yamaguchi 2008), genetic modification of GA biosynthesis has become an alternative approach targeting the crop breeding (Barboza *et al.* 2013) to alter plant growth. There are a number of genes encoding enzymes in GA metabolism pathway that have been identified and characterized in *Arabidopsis* (Hisamatsu *et al.* 2005, Plackett *et al.* 2011, 2012, Rieu *et al.* 2008), as well as in cucurbita (Lange *et al.* 2012), tomato (Serrani *et al.* 2007), apple (Kusaba *et al.* 2001), tobacco (Biemelt *et al.* 2004), grape (Giacomelli *et al.* 2013), chrysanthemum (Miao *et al.* 2010), sago palm (Jamel *et al.* 2011) and burley (Jia *et al.* 2009, 2011).

In gibberellin biosynthesis pathway, the last steps are catalyzed by soluble 2-oxo-glutarate-dependent dioxygenases, referred to as GA-oxidase (Lange *et al.* 2012, Yamaguchi 2008) which are involved in the production of bioactive GA1 and GA4. GA20 oxidase has been identified as one of the key enzymes for the production of bioactive GAs that controls various aspects of plant development, stem elongation, flower and fruit development and seed germination (Barboza *et al.* 2013, Carrera *et al.* 2000, Hisamatsu *et al.* 2005, Jia *et al.* 2009, 2011, Plackett *et al.* 2012, Rieu *et al.* 2008). Initial studies on *Arabidopsis* showed that regulating GA20 oxidase could successfully modify the gibberellins levels. The enhanced level of GA through their biosynthetic gene expression is known to elongate shoots in many plants while decreasing the level of GA will lead to plant dwarfness. Overexpression of GA20 oxidase gene has been reported to increase the indigenous GAs in plants and elongate the stem in *Arabidopsis* (Hisamatsu *et al.* 2005, Huang 1998, Rieu *et al.* 2008), potato (Carrera *et al.* 2000) and tobacco (Biemelt *et al.* 2004, Vidal *et al.* 2001) that are regulated spatially and temporally during the development. Another study has shown that the antisense expression of GA20 oxidase gene caused earlier tuberization in potato (Carrera *et al.* 2000). Therefore, GA20 oxidase genes are currently being used in plant improvement programmes in a wide range of species, particularly in crop plants. Thus, genetic manipulation of these genes could be the useful tool to generate new crop varieties of desired trait.

Although kenaf is considered as one of the important multipurpose fiber crop plants to have excellent potentials for manufacturing versatile value added products, effort has not been made to enhance the fiber quality and biomass production of the plant till date. There is no report describing the molecular aspect of the plants except for the characterization of ferulate 5-hydroxylase (Kim *et al.* 2013a) and caffeic acid O-methyltransferase (*COMT*) (Kim *et al.* 2013b) gene from kenaf. Therefore, the present research work was the first to specifically investigate whether overexpression of GA20 oxidase can accelerate the stem elongation and fiber quality through enhancing bioactive GA synthesis in transgenic kenaf plants aimed for an overall increment of growth of the species. *Arabidopsis thaliana* GA20 oxidase (AtGA20ox1) gene was overexpressed in two kenaf varieties; G4 and V36. The gateway recombination cloning system was adapted to develop the plant transformation vector. Meanwhile, pMDC32 plasmid vectors were used for the overexpression of AtGA20ox gene under duplicated CaMV 35S promoter in the kenaf plant through *Agrobacterium tumefaciens* mediated transformation system *in planta*.

Materials and Methods

Amplification and molecular cloning of Arabidopsis AtGA20 Oxidase 1 gene

Total genomic DNA was extracted through CTAB method from the young leaves and floral buds of 4–6 weeks old soil grown plants in growth room under standard growth condition. The *Arabidoposis thaliana* GA20 oxidase genomic (AtGA20ox) clone was amplified by PCR with genomic DNA as a template. The gene specific primers for the amplification of the AtGA20ox gene (Forward) 5′-AGGATCCAT GGCCGTAAGTTTCGTAAC-3′ and AtGA20ox (Reverse) 5′TGGATCCTTAGATGGGTTTGGTGAGCC3′ were designed from the published sequence (Accession no:

X83379). The PCR cycling conditions was 5 min of predenaturation at 94°C, followed by 35 cycles of 94°C (1 min), 60°C (1 min) and 72°C (2 min) and final extension at 72°C (5 min). The amplified PCR product was purified and transformed into the competent cell *E. coli* strain DH5α. The plasmid DNA was then extracted from the freshly cultured cells by using the alkaline lysis method and sequenced with pGEM-T vector specific universal primers (T7 promoter and SP 6 promoter).

Construction of expression vectors and transformation of pEXP-AtGA20ox into Agrobacterium tumefaciens strain LBA 4404

After confirmation of successful transformation of gene through PCR amplification and nucleotide sequencing, construct of expression vector was performed using Gateway technology. The construction of pEXP-AtGA20ox vectors was performed following the instruction manual of the Gateway cloning system (Invitrogen, CA, USA). Transformation of pEXP-AtGA20ox into *Agrobacterium tumefaciens* Strain LBA 4404 was performed through electroporation using Gene pulsar apparatus (Biorad USA). The transformant was transferred into 1 mL of LB broth and incubated at 28°C for 4 h at 250 rpm. Around 100 µL of the incubated culture was plated on to LB agar with 50 µg mL^{-1} streptomycin, 25 µg mL^{-1} rifampicin and 50 µg mL^{-1} kanamycin and incubated at 28°C for 72 h in the dark for the selection of inserted colonies. The colonies were then cultured in 3 mL of LB broth with appropriate antibiotics. Plasmid DNA was extracted and the presence of insert was verified through PCR amplification. The orientation of the insert was confirmed by the restriction endonuclease digestion. The clones which were confirmed from the above were used for sequencing. The sequencing reaction was performed by gene specific *att*B primers GA20ox *att*B1 (Forward) 5′GGG GACAAGTTTGTACAAAAAAGCAGGCTATGGVVHTA AGTTTCGTAA3′ and GA20ox *att*B2 (Reverse) 5′GGGGA CCACTTTGTACAAGAAAGCTGGTTTAGSTGGGTTT GGTGAGC3′.

Agrobacterium mediated transformation in kenaf through co-culture

A. tumefaciens LBA 4404 harboring pEXP AtGA20ox and empty expression clone (pEXP32) were used for transformation of kenaf *in planta* (in vitro) (Herath *et al.* 2005) with some modifications. Kenaf (*Hibiscus cannabinus* L.) Variety 36 (V36) and Guatemala 4 (G4) were used for the transformation. Around 2–3 mm long shoot apexes were excised aseptically from the newly germinated seedlings of both the varieties V36 and G4 and grown in a standard MS medium. After growing up to 2–3 cm in length, the shoots were transferred into the same MS media containing different concentrations of hygromycin B (CALBIOSHEN®, Germany) (0 mg L^{-1}, 10 mg L^{-1}, 20 mg L^{-1}, 30 mg L^{-1}, 40 mg L^{-1}, 50 mg L^{-1} and up to 100 mg L^{-1} with 10 mg L^{-1} increment in each step to determine minimal inhibitory concentration (MIC). The shoots were maintained at 25°C ± 1°C for 2 weeks and MIC of hygromycin was determined on the basis of 50% shoot death. After growing, 3–5 mm long shoot apexes were excised aseptically and cultured in the preculture shoot induction (SI) medium containing vitamins, 3% sucrose (w/v), 8.8 µM Benzyl Adenine (BA) solidified in 0.4% Gelrite at pH 5.8). The cultures were maintained at 25°C ± 1°C for 3 days. The precultured shoot apexes were pierced twice with a sterile needle, just above the cotyledonery nodes, and transferred into *Agrobacterium* innoculum containing 200 µM acetosyringone for 30–45 min. After blotting on sterile Whatmann papers to remove the excess inoculums, the shoot explants were placed on co-culture medium consisting of SI medium with 200 µM of acetosyringone and maintained at 28°C ± 1°C for 3 days in the dark. The co-cultured shoot explants were then washed 3 times with sterile water containing 500 mg L^{-1} carbenicillin to eliminate the overgrowth of Agrobacterium. The washed co-cultured shoot explants were blotted and transferred onto the SI medium containing 500 mg L^{-1} carbenicillin and maintained at 25°C ± 1°C for 7–10 days. After growing 2–3 cm, the explants were transferred into the selection medium containing 50 µg L^{-1} hygromycin B in glass jars and maintained at 25°C ± 1°C under continuous light for 8–10 weeks with subculturing to fresh medium of the same composition every 12 days. A schematic diagram of the transformation of shoot apexes is shown in **Supplemental Fig. 1**.

Rooting of shoots, acclimatization and growth of plant in controlled growth house

Surviving and growing shoot explants from the selection medium were transferred into the root induction medium similar to selection medium without growth regulators (BA). The cultures were maintained until they grew 5–6 roots longer than 2 cm. The rooted putative transformed plantlets were transferred into a pot and acclimatized for 2–3 weeks. The acclimatized plantlets were then transferred into soil with the untransformed plants in clay pots (25 cm × 35 cm) at a control growth house with irrigation carried out on alternate days.

Confirmation of transgenic plants through PCR amplification and southern blot analysis

Genomic DNA extracted from the immature leaves of putative transgenic kenaf and untransformed (UT) plants, following the method described by Edwards *et al.* (1991), was amplified for hygromycin resistant gene and the coding region of AtGA20ox gene, as well as 35S promoter regions with the primer sets Hygro- (Forward) 5′TGCTGCTCCAT ACAAGCCAACA3′; Hygro (Reverse) 5′CGACAGCGTCT CCGACCTGAT3′; GA20ox-I (Forward) 5′GAGCCGCTTC TTTGATATGC3′; GA20oX-I (Reverse) 5′ATGGTCTTGG TGAAGGATGG3′; 35S (Forward) 5′GCTCCTACAAAT GCCATCA3′; 35S (Reverse) 5′GATAGTGGGATTGTGCG TCA3′ to confirm the gene insertion and the presence of 35S promoter for the overexpression of the gene, respectively.

Genomic DNA from plants with positive results from PCR confirmation was used for further confirmation by southern blot analysis. DNA from putative transgenic and UT plants was digested with EcoR V (NEB, UK). Denatured DNA was blotted into the nylon membrane, following cross linking with UV light. The DNA probe (AtGA20ox clone) was labelled by using NEB blot® photo tope® kit (New England Biolabs, USA) according to the users' manual. After hybridization, the membrane was fixed in the cassette to expose it to an X-ray film overnight. The hybridization signal was fixed to the film.

Fiber morphology

Three fresh samples from each plant were taken at 10% (base), 50% (middle) and 90% (top) of individual plant's height respectively (Paraskevopoulou 1987) for fiber morphology analysis. Each sample was separated into a core or pith fraction and a bast fraction. Both core and bast were split separately into a matchstick size of 2 mm in width and 10–20 mm in length. Fibers were disintegrated by using sodium chlorite/acetic acid procedure according to the Technical Association of Pulp and Paper Industry (TAPPI) standard T233-Su-64 (Smook 2003). When the colour of the fiber turned into silver, it was washed gently with distilled water. One or two pieces of loose fiber sticks were transferred into a Macathy bottle with distilled water, which was shaken gently to separate individual fibers. The macerated fiber suspension was stained in one-two drops of 1% Safranin-O for 30 min, placed on a slide glass (Han and Rymsza 1999) and covered with a cover slip. All the samples were viewed under Leica image analysis system (Leitz DMRB). A total of 30 randomly selected fibers were measured from each sample of each transgenic line and their respective UT plants, at three positions; top, middle and basal of stalk for bast and core, separately. The dimensions of fiber including fiber length, diameter, lumen diameter and cell wall thickness were measured. Three derivative values were calculated by using fiber dimensions as slenderness ratio, flexibility co-efficient and Runkel ratio. In particular, slenderness ratio as fiber length/fiber diameter, flexibility ratio (co-efficient) as (fiber lumen diameter/fiber diameter × 100) and Runkel ratio as (2 × fiber cell wall thickness)/lumen diameter (Saikia *et al.* 1997).

Bio-chemical analysis

The transgenic plants and their untransformed control plants were analyzed for Holocelulose, Cellulose, Lignin and Ash content. The harvested plants were air dried for 1–2 weeks and chopped into small pieces, followed by grinding them into powder in a pulverizer and sieved mechanically by a series of mesh. Alcohol-acetone soluble contents of wood such as waxes, fats, resins and certain ether-insoluble components were removed from the samples to prepare the extractive free wood samples for the determination of holocellulose, cellulose and lignin contents. The process was carried out according to the instructions of TAPPI standard Tsox-73. In order to assess the holocellulose, the moisture content of wood was determined for the actual weight of extractive free sample. The holocellulose content in the samples was determined following the procedure described by Wise and Addico (1946). From the actual weight of the holocellulose, the content of the hemicellulose was determined while the lignin content in transgenic plants was determined in three replicates according to the method described by the TAPPI standard T222ox-74. The holocellulose, cellulose and lignin contents were calculated as percentages by weight at the extractive free sample. The ash content in transgenic plants was also determined in three replicates, with air dried saw dust according to the method described by TAPPI standard T15os-58 and calculated as the percentage of wood.

Expression analysis of AtGA20ox gene in transgenic plants by real-time PCR

Total RNA was extracted using total RNA extraction kit the easy-BLUE (iNtRON Biotechnology, Inc, Korea) from kenaf. First-strand DNA was synthesised with QuantiTect® Reverse Transcription kit (Qiagen, USA) following the manufacturer's instructions and stored at –20°C. Primers for the gene AtGA20ox were designed based on their sequences. The house keeping genes from oil palm, 18S and 17S (Gene Bank, respectively), were used to normalize all the test samples. GA20ox (Forward) 5′CGGTTTTGCGAC GACATGAG3′; GA20ox (Reverse) 5′AGATTATTTTGCT CTTGTTTTAACTTTTACCC 3′; 17S (Forward) 5′ATGG CCGTTCTTAGCTGGTG3′; 17S (Reverse) 5′GTACAAA GGGCAGGGACGTA3′; 18S (Forward) 5′ATGGCCGTT CTTAGTTGGTG3′; 18S (Reverse) 5′GTACAAAGGGCA GGGACGTA3′ primers were used for the amplification.

The amplification efficiencies of interested gene and housekeeping genes (18S and 17S) were determined using the 1QTM5 real-time PCR system (BioRad USA) according to the manufacturer's instructions. Approximately 100 ng of the first strand cDNA was used with 1 × Brilliant SYBR Green qPCR master mix (strategene USA) 60 ng of each primers and the final volume was adjusted to 20 µL by sterile water. The amplification was performed with an initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min with four replicates in each sample. The amplification efficiencies between the target and housekeeping genes were validated for the quantification of relative transcript of genes by using the comparative method. The transgenic plant with the highest Ct value was used as a calibrator because the target gene could not be detected and amplified from the untransformed (UT/control) plants.

Quantification of endogenous active gibberellins (GA1 and GA4)

Endogenous GAs was isolated following the method described by Lange (1997). The extractions were dissolved with 100 µL methanol and 2 mL acetic acid (pH 3.2) water

and passed through the column. The GA was eluted with 8 mL of 100% methanol and dried. It was dissolved in 200 µL of 1 : 3 methanol (Methanol: pH 3.2 ddH_2O) and injected into C18 reverse phase column (Chromolith® RP-18e Merck Germany) for fraction of GAs. The extractions were fractioned at 2-min intervals with gradient from 25% methanol in water to 100% methanol in 30 min delivered by the two-pump HPLC system. The fractions were dried and stored at –20°C. HPLC fractions were then dissolved in 50 µL of methanol and dried. MSTFA (N-methyl-N-trimethyl-silyltrifluo-acetamid) was incubated for 30 min at 80°C. The methylated samples were analyzed by Agilent 6850 by using the 5975C MSD ChemStation software. It was injected into a DB-5 capillary column (J and W scientific columns, Agelant Technologies). The GA quantification was performed based on retention time (GA1 was 18.59 min and GA4 was 19.22 min). Known concentrations were prepared in 5X time series of dilutions of standards to develop a calibration curve for endogenous active gibberellins. The endogenous GA1 and GA4 were calculated from the peak area ratios of 506/508 (GA1) and 284/286 (GA4) iron pairs.

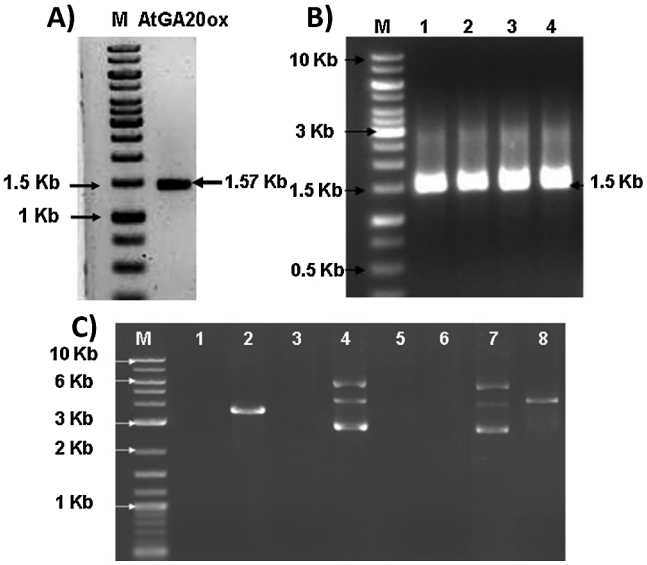

Fig. 1. Molecular cloning of AtGA20 oxidase gene. (a) Isolation of the AtGA20 oxidase gene with introns (AtGA20ox). Verification of the entry clones pENTER/Zeo-AtGA20ox by PCR amplification (b) and restriction endonuclease analysis (c).

Results

Amplification of Arabidopsis thaliana AtGA20ox gene and construction of overexpression vectors pMDC32-AtGA20ox

The AtGA20ox gene that produced 1.57 kb PCR product from genomic DNA (**Fig. 1**a) was cloned in pGEM-T easy vector and its sequences were determined. The selected clones contained deduced amino acid sequence (1576 nucleotides encoded a protein of 377 amino acid residues) were exactly homologue to the AtGA25420.1 and NM118674.1 gene. The selected clones were flanked with *att*B sites as *att*B1-AtGA20ox-*att*B2 and recombined with the donor vector (pDONRTM/Zeo). The correct positive clones were selected by PCR analysis with AtGA20ox gene specific primers which produced 1.57 kb fragments with clone pENTER/Zeo-AtGA20ox (**Fig. 1**b). The orientation of entry clones was confirmed through restriction analysis with enzyme *Bg1* II, which digested one site of gene producing 3.51 kb fragment but not the vector pDONRTM/Zeo. On the other hand, *Eco*RI digested only at one site of vector (pDONRTM/Zeo) and produced 4.2 kb fragments but not the gene (**Fig. 1**c). LR clonase enzyme was used to deliver the gene to destination vector (pMDC32) so as to produce expression clones (pMDC32-AtGA20ox) with *att*B1-AtGA20ox-*att*B2.

In these expression clones, the genes from start codon to stop codon were flanked downstream of duplicated CaMV35S promoter followed by NOS terminator. CaMV35S promoter was included to confer hygromycin resistance, served as a selectable marker to select putative kenaf transformation. Verification of expression clones was performed by using the PCR analysis with gene specific primers showing 1.57 kb fragments for genomic clone pMDC32-AtGA20ox (**Supplemental Fig. 2**) to confirm the insertion. The orientation of the gene was confirmed by using the restriction enzyme analysis with *Xho*I, which produced 4 different sizes of restricted fragments (7.5 kb, 3.1 kb, 1 kb and 0.36 kb) for genomic clones and 3 restricted fragments (7.5 kb, 3.1 kb and 1 kb) for empty expression clone. After confirmation of the expression clone, pMDC32-AtGA20ox and empty vector pMDC32 were transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The clones were reconfirmed by sequencing them with gene specific primers (GA*att*B1/GA*att*B2), which showed the exact homologous to the original sequencing.

Agrobacterium mediated transformation of pMDC32-AtGA20ox expression clones in the kenaf plant

The kenaf variety V36 and G4 were transformed by *Agrobacterium* mediated transformation. The MIC of hygromycin for selecting putative transformed shoot apices was determined using 45 mg L^{-1} hygromycin B which induced around 50% of shoot death compared to 0 mg L^{-1}. Therefore in this study, the putative transformed plants which thrived in the 45 mg L^{-1} hygromycin medium more than 10 weeks were selected. UT plants produced a large number (10–15) of long (3–5 cm), thin and whitish roots rapidly in the MS medium without hygromycin B (**Fig. 2**a) but they died in the selection media containing hygromycin B (45 mg L^{-1}) (**Fig. 2**b). A faster growth and appearance of a few (3–5) thick short roots were the most obvious features of the putative transgenic plants in the selection media (**Fig. 2**c, **2**d). Those T_0 putative transformants were consistently grown on selection and rooting medium with hygromycin B (**Fig. 2**c, **2**d) until they were acclimatized. Thirty one (31) and nine (9) putative transformants were obtained for pMDC32-AtGA20ox and pMDC32 empty clone, respectively, for both varieties,

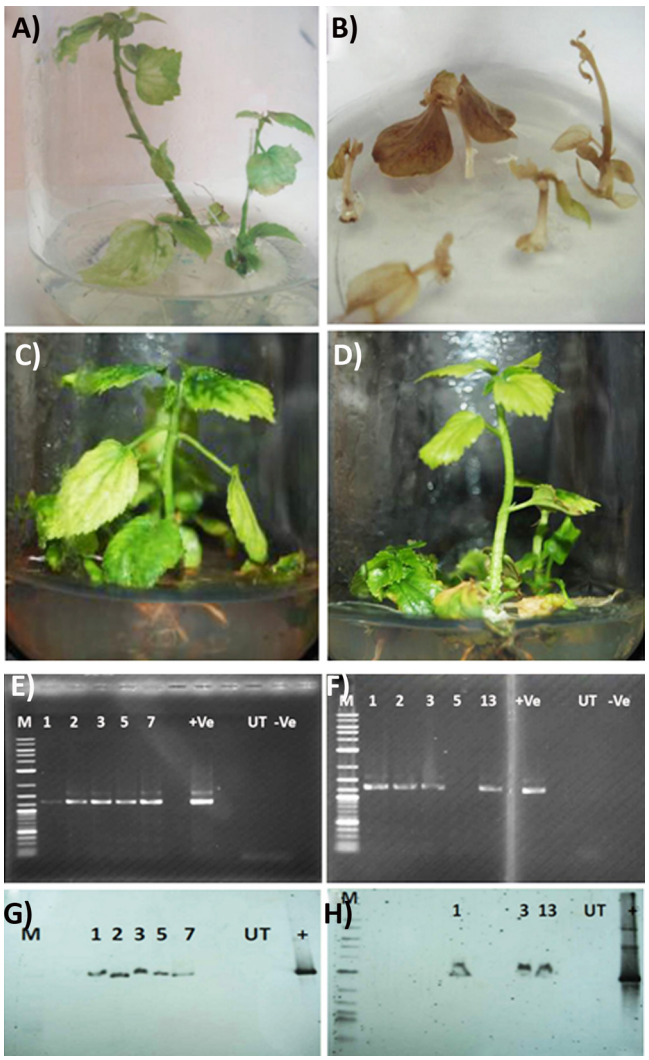

Fig. 2. Selection of putative CaMV35S : AtGA20ox transgenic kenaf plants in MS media containing hygromycin B and the confirmation through PCR amplification and Southern blot analysis. Growth of UT plants in the MS medium without hygromycin B (a) and with hygromycin B (45 µg mL^{-1}) (b). Growing of putative transformants in the MS media with hygromycin B (45 µg mL^{-1}) (c and d). Detection of the transgene encoding AtGA20ox under duplicate 35S promoter in transgenic G4 and V36 kenaf and UT plants either amplified by PCR using GA-R and 35S-F primers (e and f) or southern blot analysis (g and h). Lane M is ladder, lanes 1–3, 5, 7 (e) and 1–3, 5, 13 (f) are putative transformed G4 and V36 plants respectively showing two amplifications 1.3 kb and 1.6 kb, except for line G4-1 (lane 1 in e) which produced only 1.3 kb amplification product; lane UT is the DNA of untransformed plants; lane –Ve is negative control used PCR product without DNA; lane +Ve is the plasmid DNA of expression clone pEXP32-AtGA20ox. Southern blot analysis was performed with genomic DNA (6 µg–30 µg) digested with *EcoR* V, separated on 0.8% agarose gel using biotin labelled ORF of AtGA20ox as probe: lane M is marker; lanes 1–3, 5, 7 are putative G4 transformants; lane UT is the DNA of G4 UT and lane +Ve is plasmid DNA (g). In Figure h, lanes 1, 3, 13 are the putative V36 transformants, lane UT is the DNA from V36 UT plant and lane +Ve is plasmid DNA of pEXP32-AtGA20ox digested with *EcoR* V.

V36 and G4. All the plants were successfully acclimatized and transferred into the controlled growth house after 10 days.

PCR screening of putative transformants and confirmation by southern blot analysis

For the confirmation of T-DNA delivery to the host genome kenaf, the genomic DNA from putative CaMV35S : AtGA20ox plants of the T_0 generation was analyzed by using PCR amplification (**Fig. 2**e, **2**f). The genomic DNA of UT plants and the plasmid DNA of *A. tumefaciens* strain LBA 4404 containing the binary vectors pMDC32-AtGA20ox were used as positive controls for respective transformants. The putative transformants were preliminarily screened with hygromycin resistant gene (HPT) specific Hygro-F and Hygro-R primers, which produced around 700 bp fragment. For the clone pMDC32-AtGA20ox, six and four putative plants were identified in the G4 and V36 varieties, respectively, and the presence of AtGA20ox gene in the transgenic lines was confirmed through southern blot analysis. The transgenic CaMV35S : AtGA20ox kenaf genomic DNA was digested with enzyme *EcoR* V, which produced 2.9 kb fragment. It was hybridized with biotin labelled AtGA20ox genomic probe. The AtGA20ox gene was detected in all the five transgenic plants of the G4 variety in the southern blot analysis (**Fig. 2**g), which had previously found positive results in the PCR amplification. In the V36 variety (with the exception to line V36-2), three other lines showed positive results (**Fig. 2**h). No hybridization signal was observed in the UT plants of both varieties. After concluding all the confirmation results, nine transgenic CaMV35S : AtGA20ox lines were selected from the clone AtGA20ox for the two varieties used and were proceeded for further study. The selected transgenic lines were allowed to grow along with their respective UT plants under the same condition for further study. pMDC32 empty vector, negative control and UT plant of both varieties provided no positive results (**Fig. 2**e–**2**h).

Morphologycal characteristics of CaMV35S : AtGA20ox in T_0 transformants

During acclimatization, the transgenic lines were found to grow faster compared to the UT plants (**Fig. 3**a) and exhibited increased shoot height, which were primarily due to the long internodes. When they were transferred to the controlled growth house, the transgenic line G4-2 revealed the highest internode length. The average internode length in the transgenic plants and UT plant was 5.5 cm and 2.3 cm, respectively (**Fig. 3**b). The plant height of the transgenic lines in both the G4 and V36 varieties varied compared to their respective UT plants. Three distinct phases in the trend of height increment were also observed. Up to the 8th week of transferring in the growth house, the transgenic plants showed a faster growth (1st phase), followed by the 2nd phase up to 29–30th weeks, and the 3rd phase after the 30th week of growth. The transgenic lines were also categorized into four different groups according to their growth development and

morphology as follows: a) short early flowering lines (V36-2 and V36-3), b) short non-flowering lines (G4-5 and G4-7), c) normal flowering lines (G4-1 and V36-13) and d) tall non flowering lines (G4-2, G4-4 and V36-1).

In the first phase, short early flowering (lines V36-2 and V36-3) and short non-flowering (lines G4-5 and G4-7) lines grew faster than the other lines. At the end of the 7th week, the lines V36-2 and V36-3 initiated flowering that happened much early than UT V36 (usually flowers at 13th–14th weeks). However, both the lines stopped flowering after producing 2–5 small flowers and retarded their growth at the beginning of the 2nd phase by having around 100 cm of height (**Fig. 3**). In contrast, the transgenic lines G4-5 and G4-7 grew faster in the 1st phase but had retarded growth at the beginning of the 2nd phase (at 11th–12th weeks) without any flowering. They survived until harvesting (66th week) without any growth after reaching 158 cm and 173 cm in height, respectively (**Fig. 3**c, **3**d). The transgenic lines with normal flowering, V36-13 and G4-1, followed the growth pattern of their UT plants and initiated flowering accordingly at the 15th and 17th week, respectively. The size of their flowers, number of flowers bloomed, flowering period and life span were normal as that of UT and ended their life by the 29th–32nd week after reaching a height of 225 cm and 256 cm for V36-13 and G4-1, respectively. Meanwhile, V36-UT and G4-UT plants reached the height of 230 cm and 241 cm, respectively (**Fig. 3**c, **3**d).

Compared to all other transgenic lines, tall non-flowering V36-1, G4-2 and G4-4 grew continuously and entered the third phase. Line G4-4 stopped its growth at 40th week without initiating flowering, but reached its maximum height 264 cm and survived until harvesting. Meanwhile, line G4-2

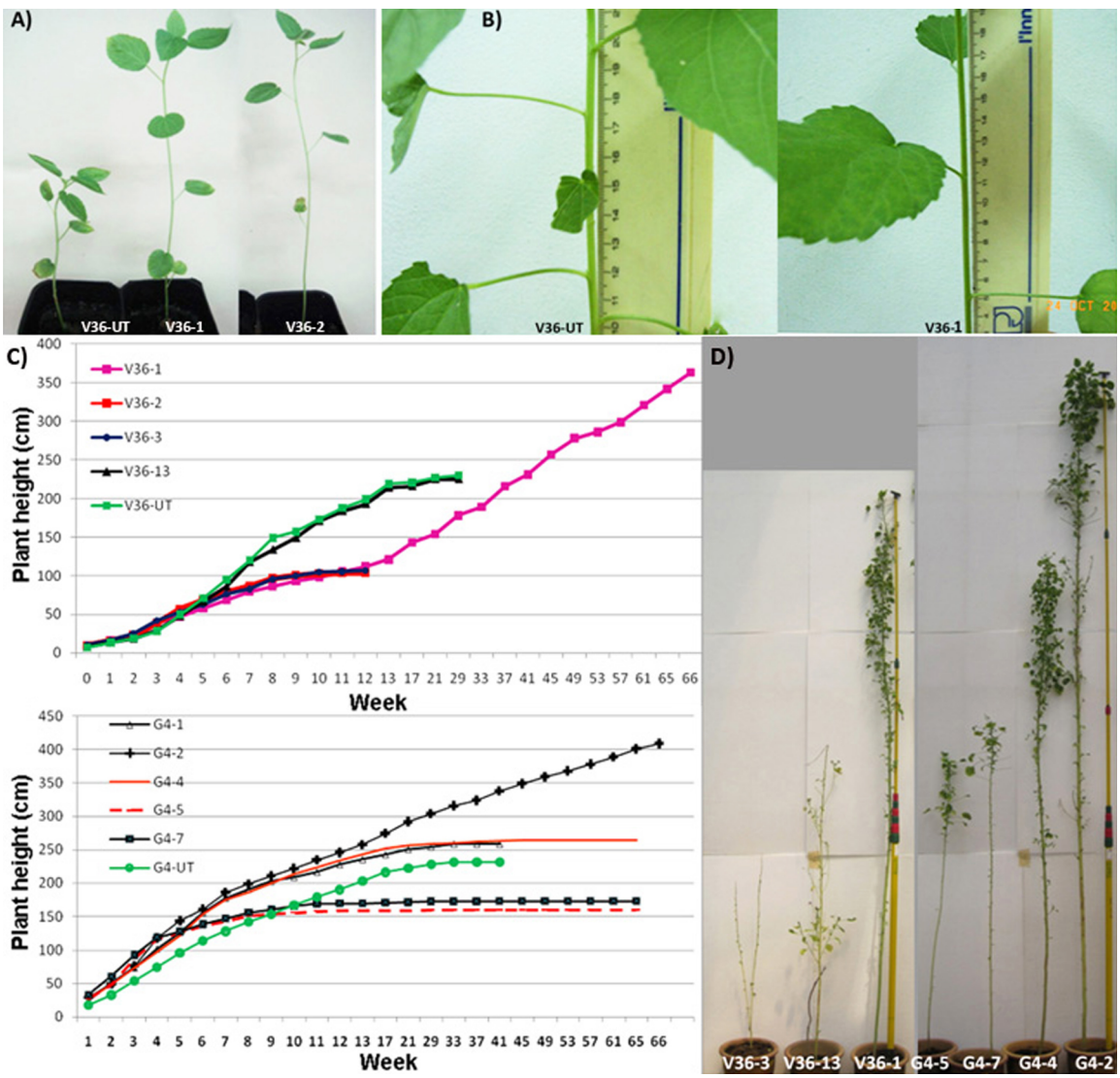

Fig. 3. Acclimatization and growth performance of putative transgenic kenaf plants. Faster growth of putative transgenic lines V36-1 and V36-2 compared to UT plants during the acclimatization process (a). Effects of gibberellic acid on internode length of transgenic kenaf plants (b). Vegetative growth (height) of transgenic lines and UT plants of variety G4 and V36 after 66 weeks of transferring into the soil (c and d).

grew continuously (**Fig. 3**c, **3**d) and showed some different phenotypic features (bushy appearance) compared to UT and other transgenic lines. The transgenic lines G4-2 and V36-1 showed continuous growth even with lesser growth increment compared to earlier phases. Until the 66th week, the plants were monitored and the lines G4-2 and V36-1 reached 409 cm and 321 cm of height, respectively. However, both the lines were supported as they were unable to stand for themselves. Short non-flowering lines G4-5 and G4-7 achieved their maximum basal diameter around 3.8 mm at the beginning of the 10th week. Tall non-flowering lines G4-4 and V36-1 reached their maximum basal diameter (21 mm, 17 mm and 16 mm for the lines G4-2, V36-1 and G4-4, respectively) at 21st week and G4-2 at 40th week (**Supplemental Fig. 3**). Reproductive development was also severely affected in all the transgenic lines. Although four out of 9 lines flowered, lines V36-2 and V36-3 did not reveal normal flowering behaviour. They produced a few small flowers followed by small capsules with very few seeds. They also produced abnormal seeds with low weight and low germination percentage compared to the UT seeds, while the lines G4-1 and V36-13 produced normal seeds like UT.

Fiber morphology of T_0 generation

Fiber dimensions: The fiber length of the transgenic kenaf plant was significantly varied from the UT plant of both the varieties for bast, but not for the core. The mean bast fiber length of line G4-1 and G4-2 showed the highest values 2.80 mm compared to their UT plants (2.6 mm). The mean bast fiber length of V36-1 was 3.22 mm compared to 1.5 mm for their UT plants (**Fig. 4**a, c). The average length of the core fiber for all the transgenic lines (above 1 mm) was greater than that of the UT plants (around 0.8 mm) (**Fig. 4**a). Nonetheless, no significant ($p \leq 0.05$) difference was observed in the fiber diameter of the transgenic lines from the UT line for bast and core (except the V36-1 in core) in both varieties although they were slightly higher to UT (**Fig. 4**b). In contrast to fiber diameter, the lumen diameter of all the transgenic lines in both G4 and V36 was comparatively lower for core than the UT plants but there was no significant difference in bast compared to UT (**Fig. 5**). The core lumen diameter of the transgenic lines of G4-1 ranged from 13.7 µm to 15.7 µm compared to 21.4 µm in the UT. All the transgenic lines showed higher cell wall thickness for both bast and core compared to their respective controls (**Fig. 5**). Accordingly, lines G4-1, G4-2, G4-4, G4-5, V36-1 and V36-13 had core cell wall thicknesses that were highly significant at $p < 0.05$. In the bast cell wall, lines G4-2, G4-5 and G4-7 showed significantly higher thicknesses whereas these were marginally different in other lines.

Fiber derivative values: Both the bast and core flexibility

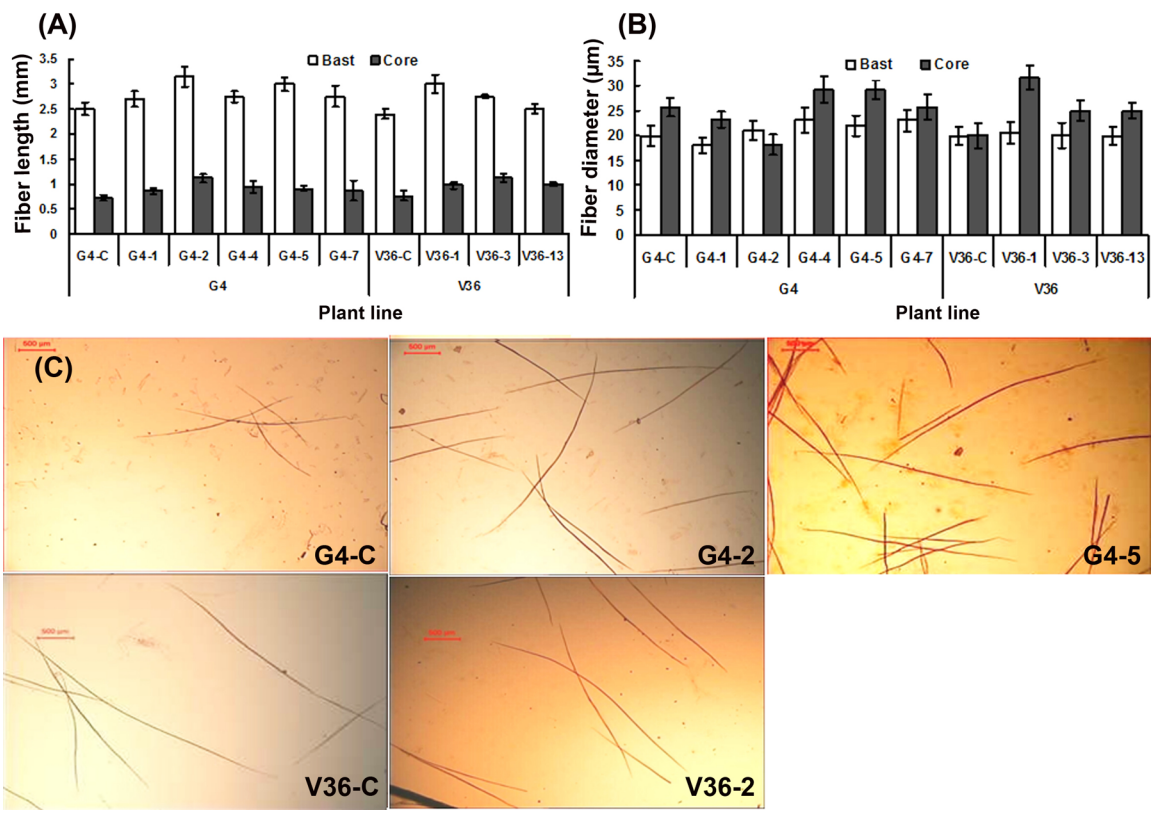

Fig. 4. Effects of gibberellins on fiber length and diameter in transgenic kenaf and their respective UT plants. Length (a) and diameter (b) of bast and core fibers of transgenic G4 and V36 lines and their respective UT. Microscopic images of bast fiber length of the transgenic lines compared to their respective UT(c).

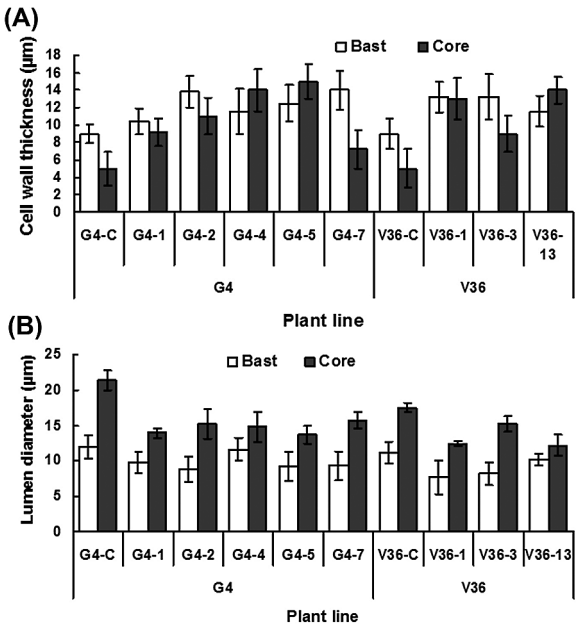

Fig. 5. Cell wall thickness (a) and lumen diameter (b) of the bast and core fibers in transgenic and untransformed control plants. The mean is followed by ± standard error of means (SE).

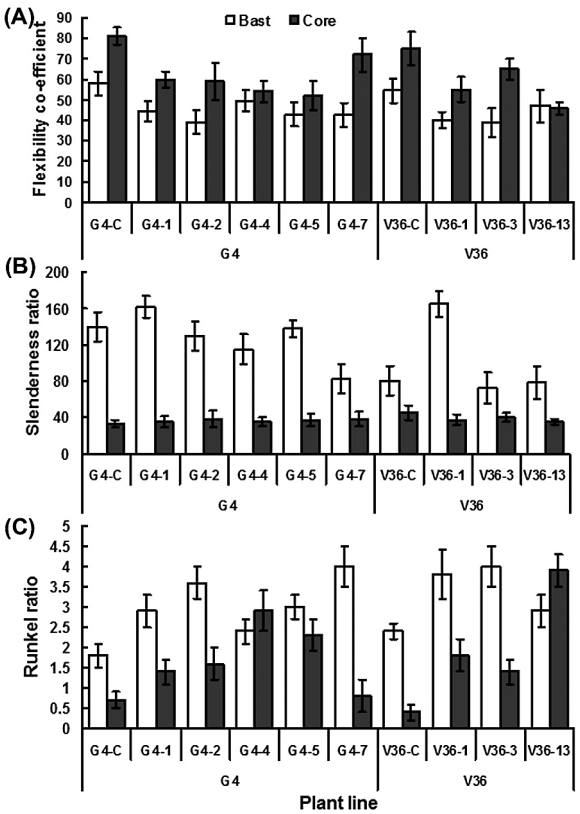

Fig. 6. Fiber derivative values flexibility co-efficient (a), slenderness ratio (b) and runkel ratio (c) of bast and core fibbers of transgenic G4 and V36 kenaf plants and their respective UT.

ratios of all the transgenic line of the varieties G4 (except line G4-4) and V36 (except V36-13) were significantly ($p \leq 0.05$) lower than those of the UT (**Fig. 6**). In bast slenderness ratio, lines G4-1 and V36-1 showed higher values (162 and 165, respectively) compared to their respective UT plants, with only V36-1 was significantly different. In core, all the lines in G4 showed slightly higher values (ranging from 35 to 38) compared to UT (33) but they did not vary meaningfully. In the variety V36, the slenderness ratio was slightly lower in the transgenic lines (37 to 40) compared to its UT (45) although with no significant difference ($p < 0.05$) (**Fig. 6**). The value of the runkel ratio for bast and core in both the varieties was remarkably higher in all the transgenic lines compared to their UT plants (**Fig. 6**). The highest runkel ratio (4) was observed in line G4-7 and V36-3 in bast and in V36-13 (3.9) for core compared to their UT plants (0.7 and 0.4 for G4 and V36, respectively).

Biochemical analysis of CaMV35S : AtGA20ox T_0 lines

Transgenic lines and UT plants were analyzed for Holocellulose, cellulose, lignin and ash content and details are summarized in **Fig. 7**. Lines V36-1, G4-2 and G4-4 showed significantly higher holocellulose (91% and 90%, respectively) contents but lines G4-1, G4-5 and G4-7 (84%) showed lower holocellulose contents compared to the UT plants, with line V36-1 (91%) particularly showed the highest holocellulose content (**Fig. 7**). The transgenic line G4-2 possessed the highest alpha cellulose content (65%), followed by V36-1 (60%) and the lowest content was recorded for V36-3. The lignin contents in all the transgenic lines in G4 and V36 were significantly lower than their UT plants (**Fig. 7**). In particular, line G4-2 showed the lowest value

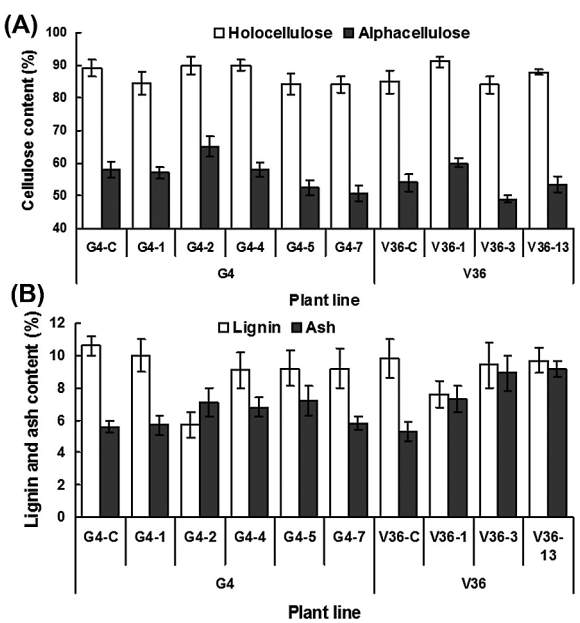

Fig. 7. Changing of biochemical composition, holocellulose and alphacellulose (a) and lignin and ash (b) content of transgenic and UT G4 and V36 kenaf plants.

(5.7%) compared to their UT (10.6%). Lines G4-2, G4-4, G4-5, V36-1, V36-2 and V36-3 obtained significantly higher amounts of ash compared to the UT plant (**Fig. 7**).

Expression analysis of the T_0 populations of CaMV35S : AtGA20ox kenaf

The expression level of AtGA20ox gene in kenaf transgenic genome was analyzed by using real-time PCR adopting the ΔΔCt method. The AtGA20ox gene could not be detected and amplified with UT plants (**Fig. 8**). Therefore, the transgenic lines G4-4 and V36-13, which showed the highest Ct values for their respective variety, were used as calibrators to compare the expression level of the AtGA20ox gene in other transgenic lines for the first two phases of their growth. When analyzing the third phase, line G4-2 produced the highest value and there was no more plants left for the variety V36. In phase one, lines G4-5 and G4-7 (short non-flowering lines) and V36-2 and V36-3 (short early flowering lines) showed the highest expression level (**Fig. 8**), 26.73, 29.19, 22 and 33.38 folds higher than their calibrators, respectively. The tall non-flowering plants G4-2 and V36-1 showed 1.87 and 15 folds higher relative expressions than the calibrator, respectively. The normal flowering line G4-1 produced 2.16 folds higher expression compared to G4-4 calibrator. With the same pattern of the expression in phase two, the fold differences were slightly reduced in lines G4-5, G4-7 and V36-2 while increased up to 39.9 folds in line V36-3 (**Fig. 8**). Line G4-1 produced 4.5 folds higher but line V36-13 remained the same. The tall non-flowering G4-2 and V 36-1 possessed the values of 1.23 and 4.95 folds, respectively. In the third phase, only three lines (G4-2, G4-4 and V36-3) remained. In particular, line G4-2 could maintain its lower values at 1.45 folds higher expression of G4-4 but the expression level in V36-3 line was insignificant (**Fig. 8**).

Quantification of endogenous bioactive GA levels in the transgenic lines

Endogenous GA1 and GA4 levels in the transgenic lines in the three phases from non-13-hydroxylation and early 13-hydroxylation branches (respectively) were analyzed. In the first phase (**Fig. 9**), all the transgenic lines of both G4 and V36 produced significantly higher amounts of bioactive GA1 and GA4 level than the UT plant. In this phase, short early flowering (V36-2 and V36-3) and short non-flowering (G4-5 and G4-7) lines showed the highest levels of GA1 and GA4 compared to the UT plants. The GA1 content of tall non-flowering lines (G4-2 and V36-1) was slightly higher than the UT plants and G4 content in G4-2 was less than GA1, and it could not be detected in V36-1 and UT plants. Normal flowering lines G4-1 showed slightly higher GAs than both UT and tall none flowering lines but it was nearly UT in line V36-13. In the second phase, drastic reductions of GA1 and GA4 content were observed in all the transgenic plants (**Fig. 9**). The GA1 and GA4 contents of lines V36-2 and G4-7 were even not under detectable level whereas in lines V36-3 and G4-5, only GA4 was detected although at a much lower level compared to that in the first phase. In relation to the tall non-flowering lines (G4-2, G4-4 and V36-1), the content of active GAs was considerably different from the first phase. The transgenic lines which showed normal flowering were observed to have slight reduction of GAs, whereby only GA4 content was detected at the lower level in both lines G4-1 and V36-13 compared to the first phase. In the UT plants, GA1 was only observed at the lower level compared to all the transgenic lines. In phase three, the tall non-flowering lines G4-2 and V36-1 were able to maintain their GA1 level low but no bioactive GAs were detected in G4-4 (**Fig. 9**).

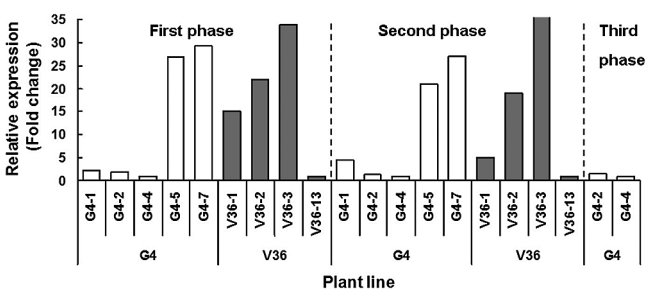

Fig. 8. Relative expression of AtGA20ox gene in T_0 transgenic kenaf plants in three stages of their growth. Tissues were harvested at different phases of their growth as first (8–10 weeks), second (28–30 weeks) and third (after 30 weeks) phases and total RNA was amplified by real-time PCR using AtGA20ox sequence specific primers to measure the transcript level. Expression levels were normalised against 18S and 17S.

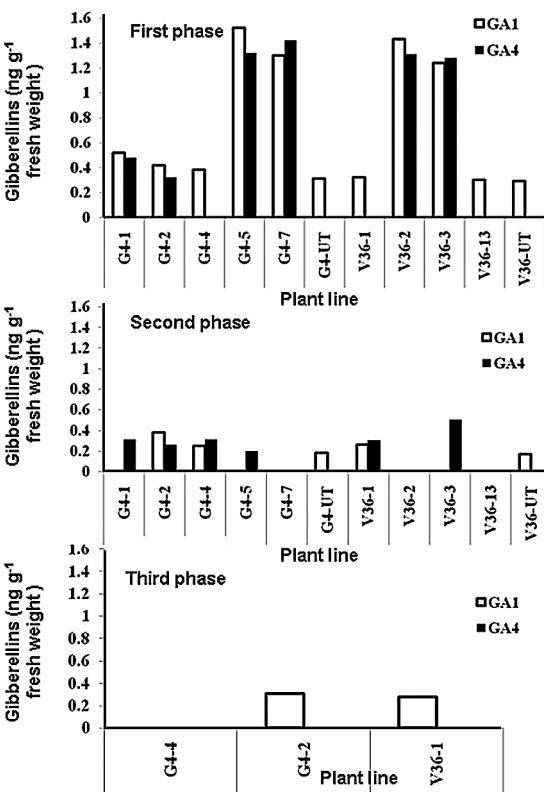

Fig. 9. Endogenous bioactive gibberellins (GA1 and GA4) level in T_0 transformed kenaf plants. After, GAs (GA1 and GA4) were extracted and purified from the tissues of first (8–10 weeks), second (28–30 weeks) and third (after 30 weeks) phases of plants' growth and analyzed by using GC-MS.

Breeding Science
Vol. 65 No. 3 BS

Discussion

Impact of bioactive gibberellins on vegetative growth and reproductive development of CaMV35S : AtGA20ox in kenaf plant

Manipulation of GA metabolism has become an alternative method to the use of chemical regulators for altering plant growth, development and quality. Several genes encoding the enzymes of GA biosynthetic pathway have been identified in many species, especially in *Arabidopsis* (Hisamatsu *et al.* 2005, Plackett *et al.* 2011, 2012, Rieu *et al.* 2008). Although till date, the functions and regulations of these genes have not been understood clearly, GA20 oxidase, being a key enzyme in the GA metabolism, has been studied and cloned from/in many plant species such as *Arabidopsis* (Plackett *et al.* 2011, 2012, Rieu *et al.* 2008), cucurbita (Lange *et al.* 2012), tomato (Serrani *et al.* 2007), apple (Kusaba *et al.* 2001), tobacco (Biemelt *et al.* 2004), citrus and grape (Giacomelli *et al.* 2013), chrysanthemum (Miao *et al.* 2010), sago palm (Jamel *et al.* 2011) and burley (Jia *et al.* 2009, 2011). In order to develop transgenic kenaf (*H. cannabinus*) aimed with long cellulosic fibers, the *Arabidopsis thaliana* GA20 oxidase gene 1 (AtGA20ox1) was cloned and introduced into two varieties of kenaf. AtGA20 oxidase overexpressed transgenic kenaf in this study displayed four phenotypes and categorized as short early flowering, short non-flowering, normal flowering and tall non-flowering. Regardless of their phenotypic category, at the early developmental stage (up to 10 weeks), all lines grew fast and showed increased internode length compared to the UT plants (**Fig. 3**b). This increment was more than twice compared to the UT plant, which was prominent phenotypic trait associated with increased GA in plants (Hedden and Proebsting 1999). This has been observed in most transgenic plants with elevated endogenous gibberellins. At cellular level, GAs stimulates both cell division and cell elongation (Kende and Zeevaart 1997). It has been proven in transgenic *Arabidopsis* which overexpressed AtGA20 oxidase, increased shoot growth, early flowering and 2–3 fold increase of GA4 (Coles *et al.* 1999). Dayan *et al.* (2010) recorded 50 percent increase of plant height by overexpressing GA20 oxidase gene in tobacco plant. Similar elongation was also observed in overexpressed AtGA20 oxidases tobacco (Bimelt *et al.* 2004), hybrid aspen (Eriksson *et al.* 2000), citrus (Vidal *et al.* 2003) and water melon (Kang *et al.* 1999) due to the increase in bioactive GAs. On the other hand, GA deficient mutants of *Arabidopsis*, maize and pea (Schomburg *et al.* 2003) showed dwarf phenotypes. Similar results were exhibited in the GA2 oxidases gene expressing transgenic lines in tobacco (Bimelt *et al.* 2004, Dayan *et al.* 2010). However, when the GA2 oxidase gene was silenced, the transgenic tobacco plant grew almost twice of the height of the control plant indicating overexpression of GA20 oxidase gene or silencing the GA2 oxidase gene plays vital roles in plant growth and development.

However, when these highly expressed transgenic kenaf lines entered the second phase, the bioactive GAs was drastically reduced and displayed GA deficiency phenotypes onwards. Short non flowering lines (G4-5 and G4-7) were not even initiated the flowering. Lines V36-2 and V36-3 initiated flowering but showed dramatic reproductive abnormalities which included a few small-sized pods, abnormal unfilled seeds and low germination percentage. Similar results were also observed in GA deficient *Arabidopsis* mutants (Koornneef and Vanderveen 1980). In the same token, GA2 oxidase overexpressed rice showed delayed reproductive development and flowering (Sakamoto *et al.* 2001), while transgenic Arabidopsis and tobacco formed small flowers with reduced number of seed capsules (Bimelt *et al.* 2004). The transgenic kenaf lines which obtained slightly higher amount of GAs compared to UT plants did not reveal any significant additive effect in their phenotype other than the initiation of flowering 2–3 weeks earlier although they overexpressed the gene. In contrast to all those lines, the tall non-flowering lines (G4-1, G4-2 and V36-1) maintained their bioactive gibberellins above the UT plants and the normal flowering lines but much lower to short early flowering lines and short non-flowering lines. Although at the beginning, they showed increased internodes length, this lengthening was not observed later. Interestingly, they continuously maintained their GA20 oxidase gene expression and bioactive GAs level until they were purposively subjected to harvest after 66 weeks. Studies in perennials, mainly with fruit crops (reviewed by Wilkie *et al.* 2008) showed inhibitory effect of GAs on flowering although it generally promotes vegetative growth. A similar finding was also observed in this study. The non-flowering plants continuously maintained their low bioactive GA1 and GA4 levels compared to the early flowering lines but above the normal flowering ones. Following the results in this study, it is therefore necessary to do further investigation so as to clearly understand how endogenous bioactive GA levels correlate with the initiation of flowering in kenaf through balance of inhibitory and promoting effect.

Higher levels of bioactive gibberellins altered the chemical composition of the transgenic kenaf plants

The chemical composition of the fiber crop which is a critical phenomenon in the fiber industries (specifically in the pulp and paper industries) concerns more with paper strength particularly when producing security papers. The strength of paper largely depends on the lignin and cellulose contents of raw materials and tensile strength which are directly proportionate to the cellulose content (Madakade *et al.* 1999). Holocellulose, alpha cellulose, lignin and ash content in transgenic kenaf lines seemed to be influenced by the GAs in this study. Regardless of the variety, all tall non-flowering plants achieving higher holocellulose content and following the similar trend for alpha cellulose continuously maintained lower level of bioactive GAs. In contrast to these plants, short lines revealed the lowest amount of holocellulose and alpha cellulose content relevant to their short period of GAs

peak. Normal flowering lines obtained a bit higher amount of holocellulose compared to UT. This situation was further confirmed by the ash content, which was maximum in short lines and minimum in tall lines, indicating the effects of sudden reduction of GAs on biomass synthesis.

In general, the harvesting age of kenaf under normal agronomical conditions significantly affects most yield parameters including total biomass. Total biomass has been shown to continuously and significantly increase over the age (Webber III and Bledsoe, 2002) by decreasing holocellulose and increasing cellulose content. The lowest lignin content was shown in tall non-flowering G4-2 and V36-1 lines compared to UT plants whereas the highest lignin content was shown in short early flowering and short non-flowering lines. Furthermore, it has been demonstrated that due to low lignin content, tall non-flowering G4-1 and V36-1 lines were unable to stand on their own and were supported during their growth. Change of lignin composition in rachis and pedicles of grape through manipulation of auxin and gibberellins has been reported (Hellgren *et al.* 2004). Israelsson *et al.* (2003) found upregulation of some lignin biosynthesis genes in wood forming tissues under overexpression of GA20ox in hybrid aspen and suggested the regulation of lignin biosynthesis by GAs. Meanwhile, Bimelt *et al.* (2004) observed a 2-fold increase of lignin in GA20 oxidase overexpressed transgenic tobacco and a decrease in GA deficient tobacco lines, suggesting that the increase of lignin by transcriptional activation of lignin biosynthesis might be due to long-term adaptation of altered GA content. Therefore, the tall non-flowering line obtained the highest amount of cellulose, which could be due to the high vegetative growth (Webber III and Bledsoe 2002) and maintenance of bioactive GAs for a long period of time.

The CaMV35S : AtGA20ox kenaf showed higher levels of endogenous bioactive gibberellins (GAs)

Expression analysis of AtGA20ox gene was performed by using real-time PCR for the three distinct growth phases of transgenic kenaf lines (First phase at 8–10 weeks, second phase at 10–28 weeks and third phase at 30–66 weeks). The AtGA20ox transcript was not detected in the UT plants in both the varieties. Therefore, the transgenic line G4-1 and V36-13, which produced the highest Ct values, were used as calibrators in the present study. All the transgenic kenaf lines showed AtGA20ox expression at different levels. In many plant transformation studies, a great variability in the level of expression among individual plants has been reported (Jones *et al.* 1987). Therefore, different expression levels among transgenic lines in this study were expected. However, the reason for differential expression level in this study has not been revealed. In the first phase, all short early flowering (V36-2 and V36-3) and short non-flowering (G4-5 and G4-7) lines showed the highest expression, which was 25–33 folds higher than the calibrator lines G4-1 and V36-13 (**Fig. 9**). Following the expression, they reached the highest bioactive GAs (GA1 and GA4) compared to other transgenic lines and UT plants. The next highest expression, i.e. around 15 folds, was shown in tall non-flowering V36-1 line. Although other tall non-flowering lines G4-2 and G4-1 showed around 2-fold increases, all the three lines obtained lower, almost the same level of bioactive GAs of the short early flowering and short non-flowering lines. The normal flowering transgenic lines (G4-1 and V36-13 that were used as calibrators) followed a similar trend and produced the lowest amount of bioactive GAs with lower relative expression. Thus in the first phase, there was a strong positive correlation between AtGA20ox gene expression and the production of bioactive GAs. It is evident that the transgenic kenaf in which AtGA20ox has been integrated into the kenaf genome is intact, allowing the expression and increase of gibberellins. This confirmed the earlier findings that overexpression of GA20ox-1 gene could enhance the endogenous gibberellin content in fibers and ovules (Xiao 2010). Direct influence of GA20 oxidase expression on the level of GA was reported by Erricsson *et al.* (2002) and Xiao *et al.* (2010). Transgenic *Arabidopsis* with overexpressed AtGA20 oxidases led to a 2–3 fold increase of GA4 level (Coles *et al.* 1999), while Erricsson *et al.* (2000) reported a 20-fold increase in hybrid aspen. Overexpression of GA20 oxidase in tobacco and citrus (Vidal *et al.* 2001) also showed increased level of bioactive GAs. However, there are also reports on reduction of bioactive GAs level through antisense expression of GA20 oxidase gene in *Arabidopsis* and potato (Coles *et al.* 1999, Carrera *et al.* 2000) which collectively showed the positive correlation between GA20 oxidase and GAs production. When transgenic lines entered into the second phase (10–28 weeks), the situation was changed. The lines (V36-2, V36-3, G4-5 and G4-7) which produced the highest level of bioactive GAs showed a drastic reduction in both GA1 and GA4 contents but maintained their transcript levels almost similar to or slightly higher as in the first phase (**Fig. 9**). However, other transgenic lines maintained the bioactive GA1 and GA4 levels parallel to the relative transcription level. In the third phase (28–66 weeks), only the tall non-flowering (G4-2, G4-4 and V36-1) lines were survived and subjected to analysis. Their bioactive GAs level and transcripts level did not deviate much from the first and second phases.

The incidences of overexpression of gibberellin metabolism genes in short non-flowering and short early flowering lines are not surprising because the information on how the transgenic plant limits or controls the biosynthesis rate has already been revealed (Hedden and Phillips 2000). In some situations, down regulation of GA20 oxidase (Phillips *et al.* 1995, Xu *et al.* 1999) or feed forward regulation of GA2ox gene (GA deactivation gene) (Hedden and Phillips 2000) by the level of bioactive GAs was shown. In this study, however, there is limited evidence to suggest feedback regulation of AtGA20ox gene as there were no such changes in the transcripts level even under low content of bioactive GAs. This is because in *Arabidopsis*, a high level of AtGA20ox-1 transcripts in GA deficit background was down regulated with

external application of GAs (Xu *et al.* 1999). On the other hand, GA deactivation genes AtGA2ox-1 and AtGA2ox-2 were upregulated by GA treatments (Thomas *et al.* 1999). In most transgenic plants, the activation of these genes has been confirmed by quantification of bioinactive GA products (Radi *et al.* 2006). In OsGA2ox overexpressed rice, increased transcriptional activity of biosynthesis genes was a result triggered by low levels of GA1 and GA4 (Sakamoto *et al.* 2001). Evaluation of the studies in other crops has shown that although the overexpression of AtGA20ox in kenaf caused overproduction of bioactive GAs, it could be subjected to gibberellin homeostasis' situation once the level of GAs overcame the critical level. This condition might be due to the activation of some GA deactivation genes. This critical level of bioactive GAs and GA deactivation genes involved in kenaf remain to be revealed.

Influence of gibberellins on fiber dimensions and their derived values in transgenic kenaf

Several researchers have pointed out the significance of fiber dimensions in predicting wood pulp mechanical properties. Among other, Seth and Page (1988) have demonstrated that tearing resistance of pulp and paper depends strongly on fiber length under certain conditions. Studies also showed that flexural strength in the specimens was considerably higher by 67% in longer fiber while decreased significantly with reduced fiber length including those fibers below the critical length (Shibata *et al.* 2006). Alterations of fiber dimensions and their derived values in all the transgenic lines were evaluated by comparing them with their respective UT plants. All the transgenic lines have shown higher values in both bast and core for fiber length, diameter, cell wall thickness and lower values for lumen diameter compared to UT plants, although some values were not statistically different. In bast, fiber length of tall non-flowering V36-1 line showed significantly higher than the UT. Core fiber length was also above 1 mm compared to less than 0.8 mm in the UT plants. It has been reported that the induction of fiber differentiation is controlled by plant hormones such as auxin, cytokinin (Aloni *et al.* 2004) and gibberellins (Aloni 1979). Mauriat and Moritz (2009) have identified gibberellins as specific for the induced fiber in both xylem and phloem. GA deficient mutants have produced longer fiber when they were applied with exogenous gibberellin (Aloni 1985, Singh *et al.* 2002). The positive influence of gibberellins on fiber elongation was further confirmed by transgenic poplar by revealing 8% increase of xylem fiber length compared to untransformed lines (Eriksson *et al.* 2000).

In case of cell wall thickness, all transgenic lines showed higher cell wall thickness compared to UT for both bast and core. It has been found that GA sensitive lines showed strong response to external GA application by increasing cell wall thickness in culms fiber of wheat (*Triticum aestivum* L.) (Yadun *et al.* 1999). Agronomically, cell wall thickness has been explained by the fact that cell wall growth is reliant on the accumulation of metabolism products (Cellulose, hemicellulose, lignin, wax, etc.) which increases with plants' maturity (Vallet *et al.* 1996). Although fiber diameter was not significantly different in transgenic lines, lumen diameter was much lower than the UT plant due to the high cell wall thickness.

Collectively, the changes in fiber dimensions influenced their derived values. The flexibility coefficient (lumen diameter/fiber diameter × 100) depends on lumen diameter and fiber diameter. Having low lumen diameter, all the transgenic lines showed low flexibility coefficient values for both bast and core fiber. Veriveris *et al.* (2004) pointed out a similar flexibility coefficient for both kenaf bast and core. On the other hand, tall non-flowering line V36-1 was shown with the highest slenderness ratio for bast. Bast fiber usually has a very good slenderness ratio (Neto *et al.* 1996) with no significant difference in the slenderness of the core fiber. This is partly due to short and thick fibers (Sacks *et al.* 1997). Core fibers, however, obtained good runkel ratios and bast fibers in all transgenic lines, thus complementing higher mechanical strength. Therefore, under those alterations, the whole stem could produce good quality and strength. Nevertheless, the overall picture of fiber dimensions and their derivative values of transgenic kenaf showed positive impact made by gibberellins on the quality parameters. Although sharp margins or proportionate effect of gibberellins and their concentrations are not clear, it is possible to achieve favourable changes. Finally, it is concluded in this study that enhanced bioactive GA is extremely important to increase the length of kenaf fiber, which can be obtained by overexpressing AtGA20ox in kenaf plants although a further study is still required to confirm the critical level of this particular bioactive GA in the transgenic plants. The findings can be used as a basis for further improvement of the quality and quantity of kenaf for the industries.

Acknowledgement

The present research work was partially supported by the Ministry of Higher Education, Malaysia, through the research project number 5523658 entitled "Overexpression of Gibberellin 20 oxidase gene for increase of cellulose fiber length in Kenaf (*Hibiscus cannabinus* L.)". The authors are also grateful to Prof. L. Mander of Australian National university Canberra, Australia, for providing the internal standards ([2H2] GA1 and [2H2] GA4).

Literature Cited

Ahmed, A., G. Scott, M. Akhtar and G.C. Myers (1998) Biokraft pulping of kenaf and its bleachability. Proc. of the North American Nonwood Fiber Symposium, pp. 231–238, Atlanta, GA, USA, 1998.

Aloni, R. (1979) Role of auxin and gibberellin in differentiation of primary phloem fibers. Plant Physiol. 63: 609–614.

Aloni, R. (1985) Plant growth method and composition. United States of America Patent Number (4): 507–144.

Aloni, R., M. Langhans, E. Aloni and C.I. Ullrich (2004) Role of cytokinin in the regulation of root gravitropism. Planta 220: 177–182.

Ayadi, R., L. Hamrounia, M. Hananac, S. Bouzidb, M. Trifib and M.L. Khoujaa (2011) In vitro propagation and regeneration of an industrial plant kenaf (*Hibiscus cannabinus* L.). Ind. Crops Prod. 33: 474–480.

Barboza, L., S. Effgen, C. Alonso-Blanco, R. Kooke, J.J.B. Keurentjes, M. Koornneef and R. Alcázar (2013) Arabidopsis semidwarfs evolved from independent mutations in GA20ox1, ortholog to green revolution dwarf alleles in rice and barley. Proc. Natl. Acad. Sci. USA 110: 15818–15823.

Bhardwaj, H.L., C.L. Webber III and G.S. Sakamoto (2005) Cultivation of kenaf and sunn hemp in the mid-Atlantic United States. Ind. Crops Prod. 22: 151–155.

Biemelt, S., H. Tschiersch and U. Sonnewald (2004) Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. Plant Physiol. 135: 254–265.

Carrera, E., J. Bou, J.L. Garcia-Martinez and S. Part (2000) Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. Plant J. 22: 247–256.

Coles, J.P., A.L. Phillips, S.J. Croker, R. Garcia-Lepe, M.J. Lewis and P. Hedden (1999) Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. Plant J. 17: 547–556.

Davies, P.J. (2004) Plant Hormones: Biosynthesis, Signal Transduction, Action! Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 204–220.

Dayan, J., M. Schwarzkopf, A. Avni and R. Aloni (2010) Enhancing plant growth and fiber production by silencing GA 2-oxidase. Plant Biotech. J. 8: 425–435.

Edwards, K., C. Johnstone and C. Thompson (1991) A simple and rapid method for the preparation of genomic plant DNA for PCR analysis. Nucleic Acids Res. 19: 1349–1354.

Eriksson, M.E., M. Israelsson, O. Olsson and T. Moritz (2000) Increased gibberellins biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. Nat. Biotechnol. 18: 784–788.

Eriksson, M.E. and T. Moritz (2002) Daylength and spatial expression of a gibberellin 20-oxidase isolated from hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.). Planta 214: 920–930.

Farshes, A.T., M.D. Firouzabadi and S. Mahdavi (2011) Properties of kenaf (*Hibiscus cannabinus* L.) bast fiber reinforced bagasse soda pulp in comparison to long fiber. World Appl. Sci. J. 14: 906–909

Fleet, C.M. and T.P. Sun (2005) A DELLAcate balance: The role of gibberellin in plant morphogenesis. Curr. Opin. Plant Biol. 8: 77–85.

Giacomelli, L., O. Rota-Stabelli, D. Masuero, A.K. Acheampong, M. Moretto, L. Caputi, U. Vrhovsek and C. Moser (2013) Gibberellin metabolism in *Vitis vinifera* L. during bloom and fruit-set: functional characterization and evolution of grapevine gibberellin oxidases. J. Exp. Bot. 64: 4403–4419.

Han, J.S. and T.A. Rymsza (1999) Determining the minimum conditions for soda-anthraquinone pulping of kenaf bast, core, and whole stalk fibers. fiber for our future: Second Annual American Kenaf Society Conference (25–26). San Antonio: American Kenaf Society.

Hedden, P. and W.M. Proebsting (1999) Genetic analysis of gibberellins biosynthesis. Plant Physiol. 119: 365–370.

Hedden, P. and A.L. Phillips (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci. 5: 523–530.

Hedden, P. and S.G. Thomas (2012) Gibberellin biosynthesis and its regulation. Biochem. J. 444: 11–25.

Hellgren, J.M., K. Olofsson and B. Sundberg (2004) Patterns of auxin distribution during gravitational induction of reaction wood in poplar and pine. Plant Physiol. 135: 212–220.

Herath, S.P., T. Suzuki and K. Hattori (2005) Factors influencing *Agrobacterium* mediated genetic transformation of kenaf. Plant Cell Tissue Organ Cult. 82: 201–206.

Hisamatsu, T., R.W. King, C.A. Helliwell and M. Koshioka (2005) The involvement of gibberellin 20-oxidase genes in phytochrome-regulated petiole elongation of Arabidopsis. Plant Physiol. 138: 1106–1116.

Huang, S., A.S. Raman, J.E. Ream, H. Fujiwara, R.E. Cerny and S.M. Brown (1998) Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in Arabidopsis. Plant Physiol. 118: 773–781.

Israelsson, M., M.E. Eriksson, M. Hertzberg, H. Aspeborg, P. Nilsson and T. Moritz (2003) Changes in gene expression in the wood forming tissue of transgenic hybrid aspen with increased secondary growth. Plant Mol. Biol. 52: 893–903.

Jamaludin, J.B. (2008) Effects of fiber size modification on the mechanical properties of kenaf fiber reinforced polyester composite. B.Sc. Thesis submitted to the Universiti Teknikal Malaysia Melaka.

Jamel, B., M.H. Hussain, M.A. Salleh and N. Busri (2011) Isolation and characterization of the GA 20-oxidase cDNA from sago palm (*Metroxylon sagu* Rottb.). AsPac J. Mol. Biol. Biotec. 19: 83–93.

Jia, Q.J, J.J. Zhang, S. Westcott, X.Q. Zhang, M. Bellgard, R. Lance and C. Li (2009) GA-20 oxidase as a candidate for the semidwarf gene sdw1/denso in barley. Funct. Integr. Genomics 9: 255–262.

Jia, Q.J., X-Q. Zhang, S. Westcott, S. Broughton, M. Cakir, J. Yang, R. Lance and C. Li (2011) Expression level of a gibberellin 20-oxidase gene is associated with multiple agronomic and quality traits in barley. Theor. Appl. Genet. 122: 1451–1460.

Jones, J.D.G., D.E. Gilbert, K.L. Grady and R.A. Jorgrnsen (1987) T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefacience* C58 derivatives. Mol. Gen. Genet. 207: 478–485.

Kang, H.G., S.H. Jun, J. Kim, H. Kawaide and Y.G. Kamiya (1999) Cloning and molecular analyses of a gibberellin 20-oxidase gene expressed specifically in developing seeds of watermelon. Plant Physiol. 121: 373–382.

Kende, H. and J. Zeevaart (1997) The five "classical" plant hormones. Plant Cell 9: 1197–1210.

Kim, J., B. Choi, Y-H. Park, B-K. Cho, H.S. Lim, S. Natarajan, S-U. Park and H. Bae (2013a). Molecular characterization of ferulate 5-hydroxylase gene from kenaf (*Hibiscus cannabinus* L.). Scientific World Journal. Article ID 421578.

Kim, J., B. Choi, B-K. Cho, H-S. Lim, J.B. Kim, S. Natarajan, E. Kwak and H. Bae (2013b) Molecular cloning, characterization and expression of the caffeic acid O-methyltransferase (*COMT*) ortholog from kenaf (*Hibiscus cannabinus*). Plant Omics J. 6: 246–253.

Koornneef, M. and J.H. van der Veen (1980) Induction and analysis of gibberellins sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. 58: 257–263.

Kusaba, S., C. Honda and Y. Kano-Murakami (2001) Isolation and expression analysis of gibberellin 20-oxidase homologous gene in apple. J. Exp. Bot. 52: 375–376.

Labidi, J., A. Tejado, A. Garcıa and L. Jiménez (2008) Simulation of tagasaste pulping using soda-anthraquinone. Bioresour. Technol. 99: 7270–7277.

Lange, M.J.P., N. Knop and T. Lange (2012) Stamen-derived bioactive gibberellin is essential for male flower development of *Cucurbita maxima* L. J. Exp. Bot. 63: 2681–2691.

Lange, T. (1997) Cloning gibberellin dioxygenase genes from pumpkin endosperm by heterologous expression of enzyme activities in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 94: 6553–6558.

Madakade, I.C., T. Radiotis, J. Li, K. Goel and D.L. Smith (1999) Kraft pulping characteristics and pulp properties of warm season grasses. Bioresour. Technol. 69: 75–85.

Mauriat, M. and T. Moritz (2009) Analyses of GA20ox- and GID1-over-expressing aspen suggest that gibberellins play two distinct roles in wood formation. Plant J. 58: 989–1003.

Miao, H., B. Jiang, S. Chen, S. Zhang, F. Chen, W. Fang, N. Teng and Z. Guan (2010). Isolation of a gibberellin 20-oxidase cDNA from and characterization of its expression in chrysanthemum. Plant Breed. 129: 707–714.

Mutasa-Göttgens, E. and P. Hedden (2009) Gibberellin as a factor in floral regulatory networks. J. Exp. Bot. 60: 1979–1989.

Neto, P., D. Fradinho, M.A. Coimbra, F. Dominques, D. Evtuguin, A. Silvestre and J.A.S. Cavaleiro (1996) Chemical composition and structural features of the macromolecular components of *Hibiscus cannabinus* grown in Portugal. Ind. Crops Prod. 5: 189–196.

Pande, H. and D.N. Roy (1996) Delignification kinetics of soda pulping of kenaf. J. Wood Chem. Technol. 16: 311–325.

Paraskevopoulou, A. (1987) Variation of wood structural features of cypress (*Cupressus sempervirens*) in Greece. Ph.D. Thesis, Institute of General Botany, University of Athens.

Phillips, A., D. Ward, S. Uknes, N. Appleford, T. Lange, A.K. Huttly, P. Gaskin, J. Graebe and P. Hedden (1995) Isolation and expression of three gibberellin 20-oxidase cDNA clones from Arabidopsis. Plant Physiol. 108: 1049–1057.

Plackett, A.R.G., S.G. Thomas, Z.A. Wilson and P. Hedden (2011) Gibberellin control of stamen development: A fertile field. Trends Plant Sci. 16: 568–578.

Plackett, A.R.G., S.J. Powers, N. Fernandez-Garcia, T. Urbanova, U. Takebayashi, M. Seo, U. Jikumaru, R. Benlloch, O. Nilsson, O. Ruiz-Rivero *et al.* (2012) Analysis of the developmental roles of the Arabidopsis gibberellin 20-oxidases demonstrates that GA20ox1, -2, and -3 are the dominant paralogs. Plant Cell 24: 941–960.

Radi, A., T. Lange, N. Tomoya, M. Koshioka and L.M.P. Pimienta (2006) Ectopic expression of pumpkin gibberellin oxidases alters gibberellin biosynthesis and development of transgenic *Arabidopsis* plants. Plant Physiol.140: 528–536.

Rieu, I., O. Ruiz-Rivero, N. Fernandez-Garcia, J. Griffiths, S.J. Powers, F. Gong, T. Linhartova, S. Eriksson, O. Nilsson, S.G. Thomas *et al.* (2008) The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. Plant J. 53: 488–504.

Rowell, R.M. and C. Cook (1998) Types and amounts of nonwoods fiber available in the US. *In*: TAPPI America Nonwood Fiber Symposium, Chicago, IL, pp. 43–47.

Sacks, R., A. Warszawski, C.I. Ogbonnaya, H. Roy-Macauley, M.C. Nwalozie and D.J.M. Annerose (1997) Physical and histochemical properties of kenaf (*Hibiscus cannabinus* L.) grown under water deficit on a sandy soil. Ind. Crops Prod. 7: 9–18.

Saikia, S.N., T. Goswami and F. Ali (1997) Evaluation of pulp and paper making characteristics of certain fast growing plants. Wood Sci. Technol. 31: 467–475.

Sakamoto, T., M. Kobayashi, H. Itoh, A. Tagiri, T. Kayano, H. Tanaka, S. Iwahori and M. Matsuoka (2001) Expression of a gibberellins 2-oxidase gene around the shoot apex is related to phase transition in rice. Plant Physiol. 125: 1508–1516.

Schomburg, F.M., C.M. Bizzell, D.J. Lee, J.A.D. Zeevaart and R.M. Amasino (2003) Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. Plant Cell 15: 151–163.

Serrani, J.C., R. Sanjuán, O. Ruiz-Rivero, M. Fos and J.L. García-Martínez (2007) Gibberellin regulation of fruit set and growth in tomato. Plant Physiol. 145: 246–257.

Seth, R.S. and D.H. Page (1988) Fiber properties and tearing resistance. Tappi J. 71: 103–107.

Shibata, S., I. Fukumoto and Y. Cao (2006) Effects of fiber compression and length distribution on the flexural properties of short kenaf fiber-reinforced biodegradable composites. Polym. Compos. 27: 170–176.

Singh, D.P., A.M. Jermakow and S.M. Swain (2002) Gibberellins are required for seed development and pollen tube growth in Arabidopsis. Plant Cell 14: 3133–3147.

Smook, G.A. (2003) Handbook for Pulp and Paper Technologists. 3rd Ed. Angus Wilde Publications, Vancouver, B.C.

Thomas, S.G., A.L. Phillips and P. Hedden (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc. Natl. Acad. Sci. USA 96: 4698–4703.

Vallet, C., B. Chabbert, Y. Czaniski and B. Monties (1996) Histochemistry of lignin deposition during schlerenchyma differentiation in alfalfa stems. Ann. Bot. 78: 625–632.

Ververis, C., K. Georghiou, N. Christodoulakis, P. Santas and R. Santas (2004) Fiber dimensions, lignin and cellulose content of various plant materials and their suitability for paper production. Ind. Crops Prod. 19: 245–254.

Vidal, A.M., C. Gisbert, M. Talón, E. Primo-Millo, L. López-DíazJosé and I. García-Martínez (2001) The ectopic overexpression of a citrus gibberellin 20-oxidase enhances the non-13-hydroxylation pathway of gibberellin biosynthesis and induces an extremely elongated phenotype in tobacco. Physiol. Plant. 112: 251–260.

Villar, J.C., E. Revilla, N. Gómez, J.M. Carbajo and J.L. Simón (2009) Improving the use of kenaf for kraft pulping by using mixtures of bast and core fibers. Ind. Crops Prod. 29: 301–307.

Webber, III. C.L. and V.K. Bledsoe (2002) Plant maturity and kenaf yield components. Ind. Crop Prod. 16: 81–88.

Wilkie, J.D., M. Sedgley and T. Olesen (2008) Regulation of floral initiation in horticultural trees. J. Exp. Bot. 59: 3215–3228.

Wise, M. and D. Addieco (1946) Chlorite holocellulose, its fraction and bearing on summative wood analysis and on studies on hemicelluloses. Paper Trade J. 122: 35–43.

Xiao, Y., L. De-Mou, M. Yin, X. Li, M. Zhang, Y. Wang, J. Dong, J. Zhao, M. Luo, X. Luo *et al.* (2010) Gibberellin 20-oxidase promotes initiation and elongation of cotton fibers by regulating gibberellin synthesis. J. Plant Physiol. 167: 829–837.

Xu, Y.L., L. Li, D.A. Gage and J.A.D. Zeevaart (1999) Feedback regulation of *GA5* expression and metabolic engineering of gibberellin levels in Arabidopsis. Plant Cell 11: 927–935.

Yadun, L.S., A. Beharav, R. Di-nur and S. Abbo (1999) Gibberellic acid (GA) increases fiber cell differentiation and secondary cell-wall deposition in spring wheat (*Triticum aestivum* L.) culms. Plant Growth Regul. 27: 161–165.

Yamaguchi, S. (2008) Gibberellin metabolism and its regulation. Ann. Rev. Plant Biol. 59: 225–251.