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Enhancing plant growth and fiber production by silencing GA 2-oxidase

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Summary

Enhancing plant height and growth rates is a principal objective of the fiber, pulp, wood and biomass product industries. Many biotechnological systems have been established to advance that task with emphasis on increasing the concentration of the plant hormone gibberellin, or on its signalling. In this respect, the most studied gibberellin biosynthesis enzyme is the GA 20-oxidase which catalyses the rate limiting step of the pathway. Overexpression of the gene resulted in an excessively high activity of the gibberellin deactivating enzyme, GA 2-oxidase. Consequently, this feedback regulation limits the intended outcome. We assume that silencing GA 2-oxidase transcription would abolish this antithetical effect, thereby allowing greater gibberellin accumulation. Here, we show that silencing the gibberellin deactivating enzyme in tobacco model plants results in a dramatic improvement of their growth characteristics, compared with the wild type and GA 20-oxidase over-expressing plants. Moreover, the number of xylem fiber cells in the silenced lines exceeded that of GA 20-oxidase over-expressing plants, potentially, making GA 2-oxidase silencing more profitable for the wood and fiber industries. Interestingly, crossing GA 20-oxidase over-expressing plants with GA 2-oxidase silenced plants did not yield consequential additive effects. Our findings unveil the benefits of silencing GA 2-oxidase to substantially increase tobacco growth and fiber production, which suggest using this approach in cultivated forest plantations and industrial herbaceous plants, worldwide.

Introduction

Growing demand for paper and pulp products poses a major hazard to our environment. Fibers, the main commodity of these industries, are predominantly extracted from trees that are grown over numerous years. Excessive logging of trees dramatically disturbs wildlife preservation and profoundly impacts global warming. Possible paths to reduce these negative environmental effects should include elevating fiber yield and the use of non-wood fiber sources, such as herbaceous plants; the rationale for choosing tobacco plants as our model. Unlike *Arabidopsis*, a prime candidate for plant molecular genetic analysis, tobacco's secondary growth is the dominant process along its stem. Its facile transformation and genetic analysis make it a widely used model plant. Accordingly, advances in molecular biology and plant physiology open new horizons for promoting fiber production in plants.

Fibers, components of the sclerenchyma tissue, are elongated cells with tapering ends and thick, usually lignified cell walls. Fiber cells are usually dead at maturity and function as a supporting tissue in plant stems and roots.

The induction of fiber differentiation is controlled by the plant hormones: auxin produced in leaves (Aloni *et al.*, 2003, 2004), gibberellin (Aloni, 1979) and cytokinin which is produced in roots (Aloni, 1982; Aloni *et al.*, 2004, 2006). Auxin and gibberellin also control the formation and structure of lignin in the fiber cell walls (Cheng and Marsh, 1968; Aloni *et al.*, 1990). It has been shown that gibberellin is the specific signal that induces fibers in both the xylem and phloem (Aloni, 1985, 1987; Mauriat and Moritz, 2009).

Gibberellins (GAs) are a large group of tetracyclic diterpenes. Most are precursors of the active gibberellin compounds which are essential endogenous regulators that influence growth and development processes throughout the plant's life cycle, including shoot elongation, the expansion and shape of leaves, flowering, and seed germination (Hooley, 1994; Kende and Zeevaart, 1997). GA-deficient mutants of *Arabidopsis*, maize, and pea represent the importance of GAs in the control of shoot elongation. These mutants have reduced levels of active GAs in comparison to wild-type (wt) plants, resulting in a dwarfed phenotype characterized by shorter internodes, reduced apical dominance, dark green leaves and a bushier plant shape. The seeds in some of these cases fail to germinate and flowering is delayed (Wilson *et al.*, 1992; Koornneef *et al.*, 2002). The phenotype of these mutants can be completely restored by the exogenous application of an active GA (Hedden and Proebsting, 1999). The low levels of GA in these plants originate from mutations in genes involved in the biosynthesis of gibberellin. Other GA-related mutants, which indicate the importance of GA in plant development, exhibit similar dwarf morphologies but do not respond to such a complementation; they are impaired in the GA signal transduction pathway. These are mainly related to the Della genes [(Achard *et al.*, 2007) reviewed in (Fleet and Sun, 2005)] and the GID gibberellin receptor (Ueguchi-Tanaka *et al.*, 2005; Hartweck and Olszewski, 2006; Nakajima *et al.*, 2006). GA operates in a de-repressible system that is moderated by Della domain proteins, which are transcriptional regulators that repress GA responses. In reaction to GA, and triggered by the GID receptor, the Della proteins are rapidly degraded by the ubiquitin–proteosome pathway (Sun and Gubler, 2004; Murase *et al.*, 2008). In this respect, other phytohormones such as ethylene and auxin alter GA responses by influencing Della protein stability (Achard *et al.*, 2003; Fu and Harberd, 2003; Desgagne-Penix and Sponsel, 2008).

The impact of GA on plant growth is also reinforced by its external application (Suttle and Hultstrand, 1987). In contrast to the phenotype observed in GA-deficient mutants, when GA was applied exogenously, the plants have responded to the treatment, by growing larger, faster, and developing longer and more fibers (Stant, 1961, 1963; Digby and Wareing, 1966; Aloni, 1985; Singh *et al.*, 2002).

As diterpenoids, GAs are synthesized from geranylgeranyl diphosphate (Hedden and Kamiya, 1997). The GA-biosynthetic pathway can be divided into three parts. The first part, which occurs in plastids, leads to the synthesis of the tetracyclic hydrocarbon, ent-kaurene. In the second part of the pathway, which occurs in the endoplasmic reticulum, ent-kaurene is sequentially oxidized to yield the first formed GA, GA12 and its 13-hydroxylated analogue GA53. In the third part of the pathway, which occurs in the cytosol, GA12 and GA53 are further oxidized to other C20-GAs and C19-GAs (Hedden and Kamiya, 1997; Hedden and Proebsting, 1999; Kobayashi *et al.*, 2000). Reactions in the third part of the biosynthetic pathway are catalysed by soluble 2-oxoglutarate-dependent dioxygenases. The first enzyme GA 20-oxidase, being also rate limiting to the pathway, is responsible for the removal of C-20, forming the C19-GA skeleton by sequential oxidations and removal of carbon. Catalysed by GA 3-oxidases (Hedden and Kamiya, 1997), the growth-active GAs GA4 and GA1 are formed by 3β-hydroxylation of GA9 and GA20, respectively.

Catabolism of biological active GAs involves GA 2-oxidases that hydroxylate the C-2 of active GAs (Martin *et al.*, 1999; Thomas *et al.*, 1999). The conversion catalysed by GA 2-oxidases of active GAs and their precursors to inactive forms is irreversible (Sponsel and Hedden, 2004). There are additional mechanisms to reduce the level of bioactive GA such as their conversion to conjugates (Sponsel and Hedden, 2004). However, these reversible processes seem to be marginal in comparison with GA2-oxidase inactivation (Rieu *et al.*, 2008).

Gibberellin levels are mainly regulated by transcriptional control of gibberellin-biosynthesis-genes by various internal and external signals, namely: products of its biosynthesis pathway, hormones (such as auxin and ethylene), light, temperature and day length (Nakaminami *et al.*, 2003; Sun and Gubler, 2004; Fleet and Sun, 2005). Direct end-product repression of bioactive GAs downregulates both GA 20-oxidase and GA 3-oxidase expression (Phillips *et al.*, 1995; Hedden and Kamiya, 1997; Cowling *et al.*, 1998; Xu *et al.*, 1999). Moreover, active GAs have a feed-forward effect on the level of transcripts of the deactivating enzyme GA 2-oxidase (Thomas *et al.*, 1999). In this respect, it has also been found that red light treatment increases GA 3-oxidase gene activity and reduces that of GA 2-oxidase (Nakaminami *et al.*, 2003), implying that a mutual regulator, which adversely affects their transcription, may exist. In pea, GA2ox2 is the main thermoregulator, it is up-regulated by cold temperatures, thereby halting stem elongation (Stavang *et al.*, 2005). As described, many factors regulate the expression of gibberellin biosynthetic genes (Eriksson and Moritz, 2002; Fleet and Sun, 2005). Hence, constant levels of active GAs are maintained respective to the momentary requirements of the plant.

To date, most of the genetic research on fiber yield enhancement has focused on overexpression of GA 20-oxidase. These plants showed higher levels of GAs in both leaves and internodes, which indicate that hormone biosynthesis was enhanced in the plants. Moreover, an 8% increase in xylem fiber length was achieved as well (Eriksson *et al.*, 2000; Eriksson and Moritz, 2002). Complying with the feed-forward regulation of GA 2-oxidase, extremely high levels of inactive GAs were detected in these experiments (up to 10-fold of the inactive GA concentration).

Studies on the *slender* pea mutant, with reduced 2 beta hydroxylase activity (Lester *et al.*, 1999; Martin *et al.*, 1999), have shown an inverted phenotype to GA deficiency. The mutation prevents GA deactivation, resulting in hyperelongation and light green leaves compared with wt plants. The *SLENDER* gene encodes a GA 2-oxidase, revealing its role in controlling GA levels. Overexpression of GA 2-oxidase in *Arabidopsis* reduces active GA levels, thereby producing dwarf phenotypes, typical to GA-deficient mutants (Schomburg *et al.*, 2003).

Here, for the first time, we show that silencing GA 2-oxidase in tobacco plants is a more potent technique to induce both plant growth and fiber production. This manipulation can be further applied to other plants of interest to the forest, pulp and paper industries.

Results

Transformation of tobacco plants with a GA 2-oxidase gene suppression construct

Nicotiana tabacum GA 20 and GA 2-oxidases share similar amino acid sequences, as both are 2-oxoglutarate-dependent dioxygenases that recognize GA precursors. For specific silencing of the deactivating enzyme family, we have conducted multiple alignments to identify a sequence fragment of the gene that does not share any significant similarity with the endogenous GA 20-oxidase gene. Using the ClustalW2 software (www.ebi.ac.uk/Tools/ClustalW) (Larkin *et al.*, 2007), we have singled out a 158 bp fragment that shares high homology with the tobacco GA 2-oxidase genes (GA 2ox1 and GA 2ox2 accession no. AB125232 and AB125233, respectively, shown in Figure 1, and Figure S1, see supporting information). This fragment was used to construct a GA 2-oxidase hairpin RNAi silencing vector which was transformed into tobacco explants. Five independently transformed lines were selected from a pool of transgenic plants silencing the GA 2-oxidase gene for further analysis. The germination of the silenced transgenic plants preceded that of wt plants in about 24 h (wt germination period was 5 days). Moreover, in comparison to wt, GA 2-oxidase silenced tobacco lines grew taller and developed more internodes (Figure 2). These characteristics comply with the aforementioned phenotypes of gibberellin overproduction, or its exogenous application. However, we did not observe a significant slender phenotype or any drastic brightening of leaves.

Ectopic overexpression of the *Arabidopsis* GA 20-oxidase gene in tobacco

To compare the phenotypes of GA 2-oxidase silenced plants with previously published results on GA 20-oxidase overexpression, we have generated transgenic 35s::*AtGA20ox1-GFP* tobacco plants. These plants have exhibited phenotypes similar to earlier studies that used the same technique (Biemelt *et al.*, 2004). Transformed lines were taller than the control (Figure 3a) and their internode length was larger in the longest internodes. Fusing the *Arabidopsis* GA 20-oxidase gene to GFP (green fluorescence protein) enabled a

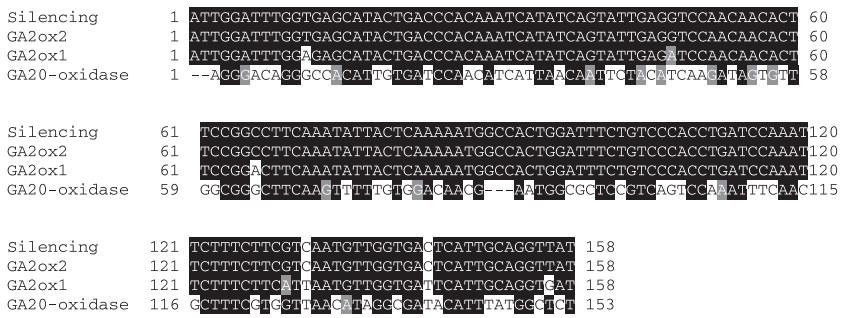

Figure 1 GA 2-oxidase silencing fragment specificity. Alignment of respective fragments of the *Nicotiana tabacum* GA 2-oxidase silencing sequence (noted as silencing) and members of the gene's family. Perfect alignment with the GA 2ox2 gene and only seven mismatches can be detected compared with the GA 2ox1 gene. No significant similarity to the GA 20-oxidase gene conveys the uniqueness of the fragment. Sequences were aligned using the ClustalW2 software.

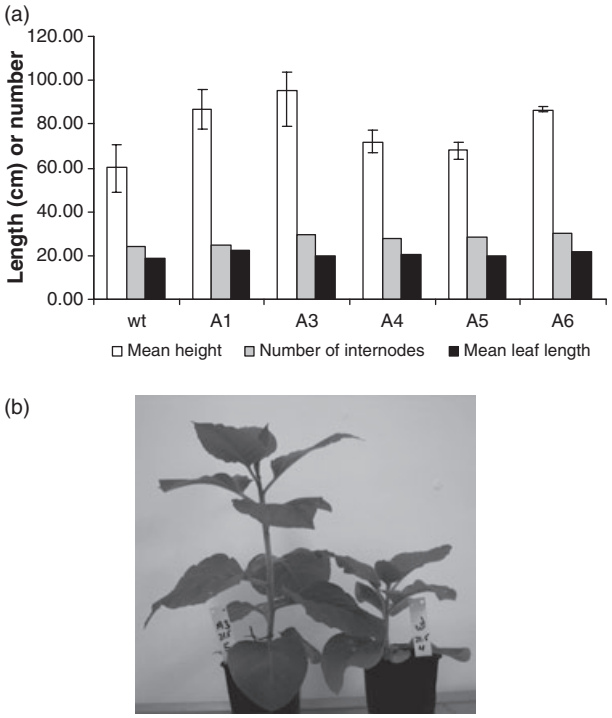

Figure 2 Phenotypic characterization of wt and GA 2-oxidase silenced lines. (a) Chart depicting a respective line comparison of: the height (white), number of internodes (grey), and the mean length of the longest leaf (black). Error-bars indicate maximum and minimum measurements. (b) A representing transgenic line (left) and a control (right) grown 5 weeks in the growth chamber.

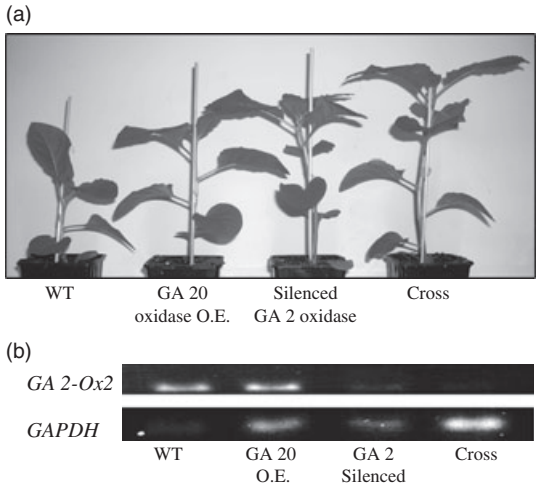

Figure 3 GA 2-oxidase expression level in transgenic tobacco plants and their phenotypic comparisons. (a) Control and transgenic plants after 1.5 months of growth. (b) RT-PCR conducted on cDNA prepared from total RNA of wt, GA 20-oxidase over-expressing, GA 2-oxidase silenced plants, and their crosses (i.e. GA 20-oxidase overexpressed and GA 2-oxidase silenced lines). At the bottom, the expression level of the control GAPDH gene is displayed. The expression level in plants transgenic to the silencing complex was lower than, both, the control and the over-expressing GA 20-oxidase plants.

time efficient cross-fertilization of the transgenic plants to further analyse their possible synergetic effects. To this end, we have chosen a representing line overexpressing the GA 20-oxidase gene to fertilize four independent GA 2-oxidase silenced lines. Progeny (crosses) were tested both for GFP luminescence (Figure S2) and reduced GA 2-oxidase transcription by RT-PCR (Figure 3b). In addition to the phenotypes corresponding to GA 20-oxidase overexpression, RT-PCR and western blot analysis were conducted to analyse the effectiveness of its expression as a result of its fusion with GFP. Figure S3 illustrates the efficient overexpression of the GFP fused *AtGA20ox1* gene. GA 2-oxidase expression is positively regulated by higher concentrations of bioactive GAs, described previously. Indeed, the analysis revealed that its expression was elevated in the GFP fused GA 20-oxidase over-expressing plant (Figure S4).

Shoot growth and stem anatomy of transgenic lines and their crosses

To further understand the effects of the distinct manipulations on tobacco morphology, we have grown transgenic tobacco lines over-expressing GA 20-oxidase, Silencing GA 2-oxidases, their crosses (i.e. GA 20-oxidase overexpressed and GA 2-oxidase silenced) and wt controls. The plants were grown 8 weeks during which their height and internodes were measured and counted in specific time intervals (Figure 4). During a 5-week period, the transgenic lines have grown taller than their wt counterparts, as depicted in the aforementioned experiments (Figure 4a). Overexpression of GA 20-oxidase resulted in an estimated 50% increase in plant height while GA 2-oxidase silenced plants grew approximately twice the height of the control plants. Surprisingly, in the crosses, the overexpression did not have any additive effect on the silencing phenotype. Analysing the growth rate during a period of 2 weeks, beginning 5 weeks after germination revealed a steady increase between the wt, overexpressing, silenced, and crossed lines, respectively (Figure 4b). Although comparing the crossed lines with the GA 2-oxidase silenced plants revealed a slightly faster growth rate that can explain the phenotype observed in Figure 3a, the significance of the result was not proven statistically, as was the case with their heights (Figure 4a). Therefore we do not regard it as significant. The number of internodes increased in a similar manner (Figure 4c). The stems of these plants were then dissected and their cambial and xylem tissues analysed

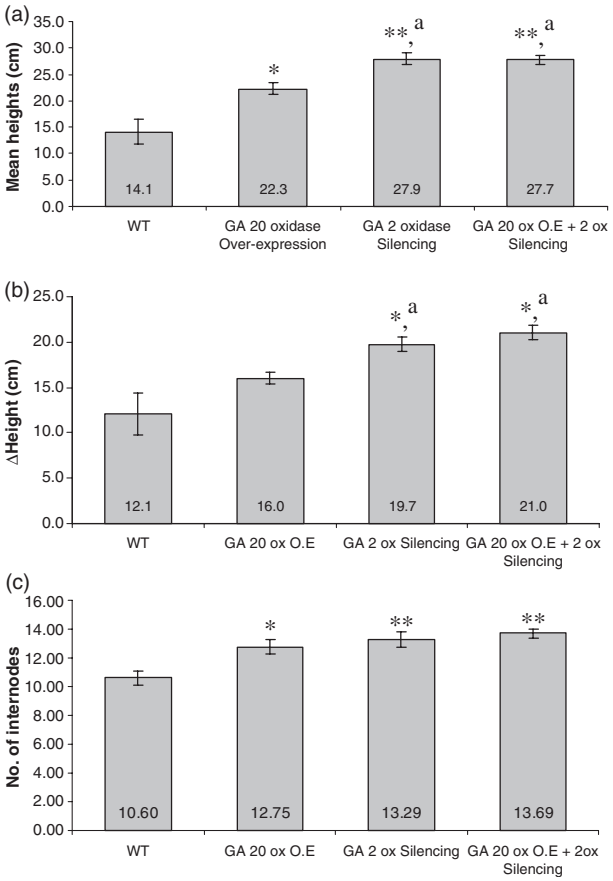

Figure 4 Effects of GA 20-oxidase overexpression and GA 2-oxidase silencing on (a) plant height (b) growth rate and (c) number of internodes. The numbers of plants used for the measurements were as followed: five controls, five GA20 O.E, seven GA 2 silenced and thirteen crosses; *significantly different from the wild type (*t*-test: $P < 0.05$); **significantly different from the wild type (*t*-test: $P < 0.01$); [a]significantly different from GA 20 O.E (*t*-test: $P < 0.01$). (a) Difference (mean values) between transgenic plant line's and wild type control growth during a period of 5 weeks. The overexpression of GA 20-oxidase increased the mean height of the plants by about 50% but had a weaker effect than the silencing of GA 2-oxidase. There was no significant synergistic effect of GA 20-oxidase overexpression to the silencing of GA 2-oxidase. (b) Difference (mean values) between transgenic plant line's growth and that of the wild type control during a period of 2 weeks (beginning a month after germination). It is evident that silencing GA 2-oxidase expression promotes faster stem growth as compared with both wt and the overexpression of GA 20-oxidase. (c) Comparison of internode numeration. It is evident that GA induces internode creation. The predominant effect is induced by the silencing of GA 2-oxidase and a slight additive effect induced by the co-overexpression of GA 20-oxidase. The error bars represent standard error.

microscopically. Three distinctive internodes were characterized from the upper, central and lower parts of each plant (Figure 5). In higher (younger) internodes of the transgenic plants, a detectably higher number of internodes in their primary developmental phase were identified, given they did not exhibit fiber initiation that was observed in the same internodes of the wt control plants. This suggests that GA may accelerate plant growth by enabling more cells to differentiate simultaneously. Lower (older) internodes have demonstrated extremely high fiber production, particularly in GA 2-oxidase silenced plants and their crosses (Figure 5). The number of xylem fiber layers was slightly higher in the transgenic lines although their circumference was much larger, accounting for the higher fiber yield. Moreover, these fibers were almost twice as large as the wt fibers (Figure 6; note the magnification of the wt photo is twofold larger than the corresponding cross sections). Fiber size is a major feature for fiber quality evaluation, and is highly valuable for the respective industries. In addition, cambial activity was more eminent in the crossed lines.

Discussion

World demand for pulp and fibers is constantly growing and their various uses continually expand. Consequently, fiber resources are diminishing. A major disadvantage of traditional tree breeding, especially of forest tree species, is its slow development that requires a long regeneration time. So far no practical remedy has been suggested, despite the large number of studies that have been conducted to increase the hardwood fiber yield. Therefore, new and improved sources of fibers need to be found. The gibberellin hormone has a broad positive impact on plant development and specifically promotes fiber production (Aloni, 1979, 1987). Therefore, elevating its concentrations would insure multiple metabolite expression and de-repression processes of plant growth. We have focused on a biotechnological approach to hinder the hormone's homeostasis with the intent of elevating, both, the plants' fibers quality and yields. We conducted our study on tobacco plants; modelling the effectiveness of our technology on herbaceous plants. We assume herbaceous plants constitute a great potential to minimize world reliance on forest trees. Previous publications have used tobacco plants as model systems researching gibberellin signalling and its impact on growth (Eriksson *et al.*, 2000; Schomburg *et al.*, 2003; Gallego-Giraldo *et al.*, 2007). These have shown that ectopic expressions of *Arabidopsis* genes have similar impacts on both non-wood plants and trees, and specifically used tobaccos as a model for gibberellin induced biomass accumulation (Biemelt *et al.*, 2004).

Accumulating evidence suggests that the dioxygenases, which catalyse the later steps in GA biosynthesis – namely

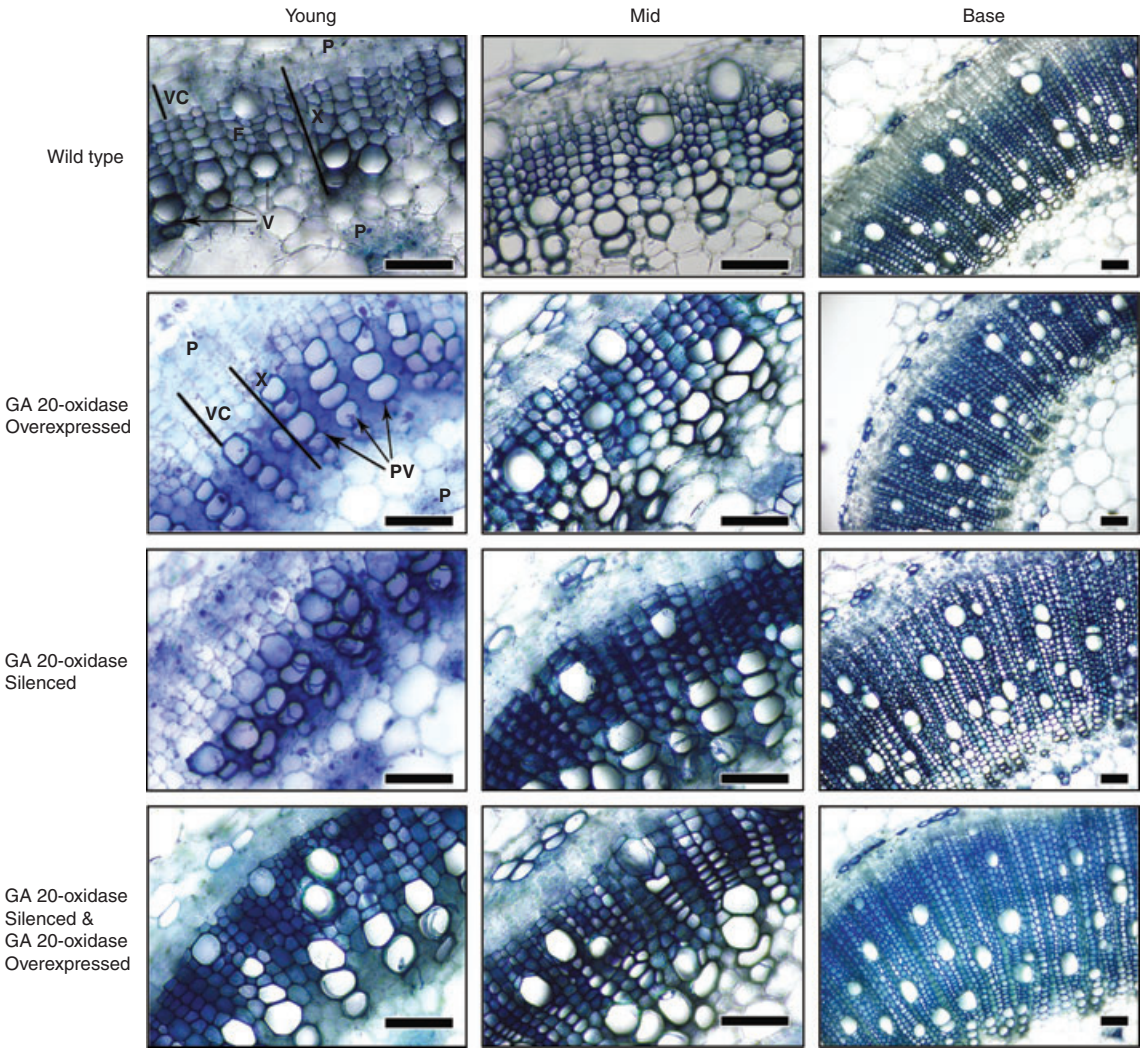

Figure 5 Stem anatomy of wild-type control and transgenic tobacco plants over-expressing GA 20-oxidase, silencing GA 2-oxidase and their crosses. Three columns separate between different regions of the dissected stems (corresponding to young, middle and basal internodes). It is evident that the silenced plants have higher cambial activity with thicker xylem zones. In the young cross sections, a delay in xylem differentiation is depicted in the transgenic lines, prolonging the developmental stage, and allowing cell elongation. High xylem fiber production is observed in the base internodes of the silenced lines and their cross-fertilization with GA 20-oxidase over-expressing plants. The middle internodes do not exhibit any significant difference in cambial activity and xylem development. X, xylem; P, phloem; VC, vascular cambium; V, vessels; PV, primary vessels; F, fibers. Magnification bars represent 80 μm.

GA 20-oxidases, GA 3-oxidases, and GA 2-oxidases, are key regulators of GA-related plant growth (Yamaguchi, 2008). In our study, we have engaged in both, the silencing of the main deactivation catalyst of GA, and in reproducing previous studies findings by constitutively expressing the *Arabidopsis* GA 20-oxidase gene in tobacco plants. Our study demonstrates that GA 2-oxidase silencing is an effective mechanism, accelerating plant growth and enhancing its elongation (Figures 3 and 4a). The phenotype of the transgenic plants obtained is consistent with those observed in previous studies, incorporating both transgenic approaches and mutants containing higher GA levels. The GA over-production phenotype is characterized by longer hypocotyls, increased internode length, and pale-green leaves. These phenotypic effects were attained by overexpression of endogenous GA 20-oxidase genes in *Arabidopsis* (Huang *et al.*, 1998; Coles *et al.*, 1999), potato (Carrera *et al.*, 2000), tobacco (Biemelt *et al.*, 2004) and also, in most part, in our study by the heterologous expression of the *Arabidopsis* GA 20-Oxidase. In contrast to these studies, we have not detected a slender phenotype. In this respect, it was shown that the elevation

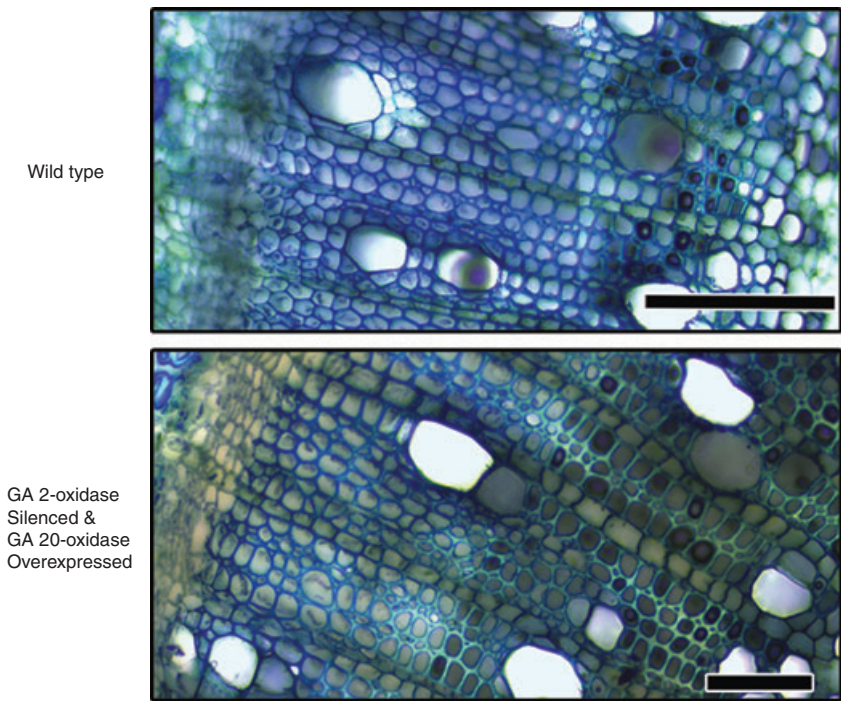

Figure 6 Magnification of basal internodes of the wt and crossed transgenic lines (Top and bottom, respectively; note the magnification of the wt plants is twofold larger than its transgenic counterpart). These magnified cambial and xylem regions clearly demonstrate the increased fiber size in the transgenic lines. Magnification bars represent 80 μm.

of GA concentrations in hybrid-aspen over-expressing GA 20-oxidase results in a steady increase of stems' diameter (Eriksson *et al.*, 2000), as observed in our study.

The slight increase in the number of internodes (Figure 4C) may imply that a disturbance to the plastochron exists (the interval between two successive initiations of leaf primordia). It has been shown that shortening the plastochron elongates the vegetative phase in rice (Itoh *et al.*, 1998; Kawakatsu *et al.*, 2006). This could account for both the increased number and elongation of the internodes in the transgenic plants. Another protein that may be related to this mechanism is the KNOX homeodomain protein that represses GA biosynthesis of corpus cells in the shoot apical meristem but is down-regulated in the adjacent cells (Sakamoto *et al.*, 2001). Furthermore, it is assumed that GA promotes both cell division and cell elongation. The underlying mechanisms were thoroughly studied in deepwater rice (Sauter *et al.*, 1993). However, we did not observe internode lengthening throughout the whole stem of the manipulated plants. These findings need more detailed studies to identify the causes for such a phenotype. In addition, the sections in Figure 5 illustrate delayed fiber differentiation in the young internodes of the transgenic lines while in the middle internodes the differentiation is similar; implying that at this stage, the cambial activity of the GA 2-oxidase lines accelerates to produce higher fiber content.

Tissue elongation ceases at maturation; indicating that a delay in fiber differentiation and maturation might be a prerequisite for internode elongation. However, as could be expected, significant internode elongation was only observed in the longest internodes, but not in all of them.

Enhanced stem growth in mature tissues of the transgenic plants coincided with increased cambial activity and an elevated number of fibers in both the phloem and xylem tissues (Figure 6). This GA over-production phenotype is in accordance with an inverted study on eucalyptus saplings (Ridoutt *et al.*, 1996) where a treatment with a GA biosynthesis inhibitor led to reduced fiber lengths and a decrease in the number of cambial cells and differentiating fibers. Our cambial and fiber findings confirm early studies, which found that gibberellin stimulates cambial cell division and cell expansion, and that fiber differentiation and lignification was also facilitated by auxin (Wareing, 1958).

An important conclusion drawn from our results relates to the role of the two enzymes in maintaining GA levels. As GA 2-oxidase silenced lines grew faster and taller than lines over-expressing GA 20-oxidase, it is concluded that the deactivating metabolic role is the dominant factor in maintaining the gibberellin homeostasis. This conclusion is

reasonable, because GA 2-oxidase acts on the products of GA 20-oxidase. Similar to various abiotic signals that induce GA production, other signals would induce its deactivation to meet a growth that is appropriate to the conditions at any given time. We presume that once GA synthesis is induced, its high levels are preserved, subject to the silenced deactivation background. This rational may explain the ameliorated growth caused by the silencing of a deactivating enzyme. Potentially, it could also explain the minimal additive effect observed in the crossed lines. Further research, especially GA precursor quantifications are needed to substantiate this hypothesis.

Many signals, such as day length and temperature, control GA action and influence plant development. These could impact any manipulation of GA metabolism. In this respect, studies conducted on an *Arabidopsis ga2ox* quintuple mutant, revealed that under certain conditions GA signalling is close to saturation, thereby reducing phenotypes that were exaggerated under GA deficient conditions, including an earlier flowering phenotype that was much less pronounced in long days than under short day conditions (Rieu *et al.*, 2008). On the other hand, although they had not observed enhanced vegetative stem elongation, the mutant's inflorescence stem has grown taller and comprised more internodes under normal growth conditions.

Variations in the response and control of GA signalling can occur among different plant species, as was the case for the *Arabidopsis ga2ox* quintuple mutant. Although, both our study on tobacco and the study on Arabidopsis conquer, while at different levels, that GA 2-oxidase silencing positively affects GA signalling.

Analysis of different abiotic conditions on different GA 2-oxidase silenced industrial crops may lead to an amazing ability of widening their exploitation around the globe.

Silencing GA 2-oxidase has many more prospective roles that their investigation exceeds the scope of the current research. It is likely that a metabolic analysis of such plants would reveal undisclosed deactivation and regulation mechanisms that are overshadowed by the principal role of the enzyme.

Experimental procedures

Construction of expression plasmids

AtGA20ox1 (accession no. NM_118674) was amplified by PCR from. *Arabidopsis thaliana* ecotype Columbia-0, genomic DNA using the following primers: ATG GCC GTA AGT TTC GTA ACA AC (forward) and GAT GGG TTT GGT GAG CCA ATC TG. It was then cloned in the sense orientation upstream of the *GFP* gene into the binary vector pBINPLUS (Vanengelen *et al.*, 1995) between the 35SΩ promoter containing the translation enhancer signal and the Nos terminator. The resulting *35::AtGA20ox1:GFP* construct was electroporated into *Agrobacterium tumefaciens* GV3101 and the bacteria used for stable tobacco transformations.

For the silencing constructs a 158bp fragment of the tobacco GA2ox2 (amplified using forward (ATT GGA TTT GGT GAG C) and reverse primers (CCT GCA ATG AGT CAC C), Figure 1) was cloned into the pKANNIBAL vector (Wesley *et al.*, 2001) in both the sense and antisense orientations, flanking the Pdk intron. The construct was digested and sub-cloned into the pART27 binary vector (Gleave, 1992). The construct was introduced into *A. tumefaciens* GV3101 and used for transforming tobacco plants.

Plant transformation

Nicotiana tabacum cv *Samsun* plants were transformed as previously described (Horsch *et al.*, 1985). Briefly:

Tobacco plants were grown under sterile conditions in Magenta boxes containing solidified (0.6% Plant Agar, Duchefa, The Netherlands) MS medium. Leaves were cut to small rectangles and incisions were made towards the main vein. The leaves were then incubated for 20 min in an overnight culture of *A. tumefaciens* containing the desired vector diluted in MS liquid medium to a final OD_{600} of 0.5. After blotting on sterile paper the leaves were co-cultivated for 2–3 days on a solidified MS medium supplemented with 2 mg/mL kinetin and 0.8 mg/mL IAA (Sigma-Aldrich Inc., St. Louis, MO, U.S.A). Then the leaves were transferred to solidified MS medium containing 2 mg/mL kinetin, 0.8 mg/mL IAA, 400 mg/L claforan and 100 mg/mL kanamycin. Calli were transferred to fresh medium every 2 weeks until shoot regeneration. Regenerating shoots were then transferred to a solidified MS medium supplemented with 100 mg/L kanamycin and 400 mg/L claforan. Regenerated shoots that rooted in the presence of 100 mg/L kanamycin were potted. Positive kanamycin resistant plants were examined for the presence of the inserted gene by PCR and Southern blot analysis.

RT-PCR analysis of tobacco GA 2ox2 and GA 20ox expression

Total RNA was extracted from apical leaves of 3 to 4-week-old tobacco plants (100 mg) using a SV Total RNA Isolation Kit (Promega, Madison, WI, USA). Two micrograms of RNA were converted to cDNA using the M-MLV reverse transcriptase kit (Promega). Two microliters of each reverse transcriptase reaction were used as a template in a 50-μL PCR reaction containing specific primers. A 418 bp fragment of the GA2ox2 cDNA was amplified using forward (ATT GGA TGT AAT GGC GAT TC) and reverse (GTG GGA CAG AAA TCC AGT GG) primers. The PCR conditions were as follows: 10 min at 94 °C, 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C, for 33 cycles, followed by 10 min at 72 °C. A 221 bp fragment of the GA20ox cDNA was amplified using the following primers: CCT CAT TGT GAT CCA ACA TC (forward) and AAG AAT GCA AGT GAT TTC CT (The product of these primers performed on

genomic DNA results in fragments 324 bp and about 500 bp long for *Arabidopsis* and tobacco, respectively). The PCR conditions were similar to the ones used for GA2ox2 except for the annealing temperature which was 55 °C. GAPDH specific primers: ATG CTC CCA TGT TTG TTG TGG GTG (forward) and TTA GCC AAA GGT GCA AGG CAG TTC, and actin specific primers: AAA GAT TGT ACT CAG TGG CG (forward) and AGA AGC ATT TTC TGT GCA CAA TGG (reverse) were used as control for the GA2ox2 and GA20ox RT-PCRs, respectively. –RT reactions (i.e. no cDNA synthesis) were used to rule out genomic contaminations (negative controls).

Protein extraction and Western blot analysis

Ten-day-old seedlings of the respected tobacco plant lines were ground using liquid nitrogen. The ground materials were mixed with 100 μL of sample buffer (100 mM Tris pH 6.8, 20% glycerol, 5% beta mercapto, 4% SDS, and 0.4% bromophenol blue). Samples were mixed for 5 min and centrifuged at RT at 15 000 g for 5 min. Twenty-five microliter of plant proteins were loaded on 10% SDS-polyacrylamide gels and protein gel blot analyses were performed (Ausubel *et al.*, 2003) using a 1 : 1000 dilution of rabbit anti-GFP antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A), and a 1 : 10 000 dilution of hrp conjugated goat anti-rabit antibodies. The membranes were developed with a chemiluminescent detection method (EZ-ECL, Beit-Haemek, Israel) and subjected to autoradiography.

Plant material and growth conditions

All the work was carried out using *N. tabacum* cultivar Samsun NN. Seeds were grown in tissue culture under 16 h light/8 h dark regime (irradiance 150 μmol quantam/m/s) on Murashige Skoog medium containing 1.5% (w/v) sucrose and the appropriate selective solid media (250 μg/mL kanamycin fro the transgenic lines) and transferred to soil 1–2 weeks after germination. For growth characterization, tobacco plants were cultivated in soil in a growth chamber at 25 °C under a 16 h light/8 h dark regime (irradiance 100 μmol quantam/m/s).

Growth measurements and sampling

After a 5 week growth period, plants were repeatedly measured. Data included plant height, internode length, number of internodes and leaf length (only in part of the experiments). Actively growing internodes were identified according to these data. This was used as a reference point for plant segmentation into three categories; upper, central and lower internodes. As the number of internodes differed between the plants as well as the number of developing internodes, when in doubt, we have examined the more stringent cross section to keep results objective.

Confocal microscopy and histochemical visualization

Green fluorescence protein expressing leaves were analysed using a 510 Zeiss confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

For histochemical analysis of stem cross sections, hand-cut transverse sections from the middle of most of the internodes were analysed. Prior to cross-section preparation, plants were dehydrated in 70% alcohol. Lacmoid staining procedures were essentially performed the same as previously described (Aloni, 1979). Sections were analysed using a light microscope (model BH-2; Olympus, Tokyo, Japan).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Homology of the silencing fragment in the tobacco GA 2-oxidase gene family.

Figure S2 GFP analysis indicating successful cross-fertilization.

Figure S3 35s::AtGA20ox1-GFP expression profile.

Figure S4 Over-expression of the GFP fused GA20-oxidase induces GA2ox2 expression.

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