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Plant polar growth in tobacco disturbed by γ-tubulin gene silencing

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Abstract

To further understand the functions of γ-tubulin in plant cells, we conducted a study in which the γ-tubulin gene was down-regulated in tobacco plants (obtained by the *Agrobacterium*-mediated method). This involved transforming the target fragments, in which the sense and antisense partial γ-tubulin cDNA fragments were ligated together, into *Nicotiana tabacum* var. Samsun NN. The γ-tubulin down-regulated transformants developed multiple meristems or branches with trumpet-shaped leaves; their root generation also appeared abnormal, with the taproots undeveloped, whereas lateral roots were developed. In addition, the content of indole-3-acetic acid (IAA) and expression of polarity transportation vector PGP1 were aberrant. These results suggest that γ-tubulin gene silencing disturbed the polar growth of tobacco plants, and that this phenomenon was probably correlated with the IAA content and the polar transportation process.

Keywords: Tobacco; γ-Tubulin; Gene silencing; Polar growth

1. Introduction

The γ-tubulin gene is highly conserved in eukaryotes, and the encoded protein is localized to centrosomes and other defined microtubule organizing centers (MTOCs). γ-Tubulin plays an important role in microtubule nucleation, cell cycle regulation and spindle formation in animal and fungal cells [1–7]. However, because there is no centrosome in higher plant cells, the role of γ-tubulin in these cells is still unclear and is a current hotspot for research by many cell biologists. The γ-tubulin gene is an important conserved gene therefore its disruption would be lethal to the organism, making it difficult to discover its function by traditional methods. To address this, researchers have recently been exploring negative genetic techniques. In *Arabidopsis*, the γ-tubulin gene (*TUBG1*) has been successfully down-regulated by inducible expression of RNA silencing constructs. The results showed that although cells with decreased levels of γ-tubulin could progress through mitosis, cytokinesis was strongly affected. Step-wise diminution of γ-tubulin revealed some of its roles in plant development, such as organization of cell files, anisotropic and polar tip growth, and stomatal patterning [8]. Another report on isolated γ-tubulin mutants by T-DNA insertions in each gene (*TUBG1* and *TUBG2*) and the analysis of two combinations of γ-tubulin double mutants showed that the two genes had redundant functions. Disruption of both γ-tubulin genes caused aberrant spindle and phragmoplast structures, and altered nuclear division in gametophytes, ultimately leading to lethality three weeks after germination [9]. In our previous study, we found that the γ-tubulin gene was silenced in *Nicotiana tabacum* by a virus (potato virus X, PVX). The mutants exhibited narrower and curved leaves, and all buds fell off the plants at the flowering stage [10]. The different phenotypes caused by the low expression of the γ-tubulin gene in *Arabidopsis* and *N. tabacum* fail to clarify its role in plant development, and further study is still necessary. In the present study, we transformed sense

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and antisense fragments into *N. tabacum* using the *Agrobacterium*-mediated method to obtain γ-tubulin gene-silenced plants. The phenotypes of the silenced plants were analyzed to reveal more about the role of γ-tubulin in plant growth and development.

2. Materials and methods

2.1. Materials

The tobacco plants (*N. tabacum* var. Samsun NN) used in this study were grown in greenhouses at 22–28 °C and 60–70% relative humidity, with a 14/10 h light/dark regime. *Escherichia coli*, *Agrobacterium tumefaciens* strain LBA4404 and plasmid pCAMBIA1301 were stored in our laboratory under standard conditions.

2.2. γ-Tubulin cDNA fragment cloning

Total RNA was extracted from young tobacco inflorescences using the Trizol (Invitrogen Co.) reagent. RT-PCR amplification was carried out using primers P1 and P2 (Table 1), which were designed according to the γ-tubulin cDNA sequence (GenBank Accession No. AJ278739) using the RT-PCR kit (Invitrogen Co.) according to the manufacturer's instructions. The reaction parameters were one cycle of 45 min at 45 °C and 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 40 s at 57 °C and 2 min at 68 °C, and 7 min at 68 °C. The 50 μl RT-PCR products were separated on an agarose gel and were extracted from the gel (QIAquick Gel Extraction Kit), then cloned into the pMD-18T vector (Takara Company, China) using the method described by Sambrook et al. [11]. The positive clone (pMD18-T_t) was extracted by the QIAprep Spin MINIprep Kit for sequencing using the BigDye-terminator reaction (Applied Biosystems).

2.3. dsRNA vector construction and transformation

Primers P3 and P4 were designed and synthesized as shown in Fig. 1. PCR amplification was carried out using the plasmid pMD18-T_t as the template. The PCR product and pCAMBIA1301 were first digested with Nco I and Bgl II, and cross-ligated. The positive recombinant plasmid containing the sense γ-tubulin cDNA fragment was named as pCA1301_a; then the PCR product and pCA1301_a were digested by Bst EII and Pml I and ligation was performed. Thus, the new positive recombinant plasmid containing both sense and antisense γ-tubulin cDNA fragments was named as pCA1301_d, and used in the next experiment as the dsRNA vector. Both the empty vector (pCAMBIA1301) and the dsRNA vector (pCA1301_a) were transformed into LBA4404, and the *Agrobacterium*-mediated leaf-disk method was used to transfer them into *N. tabacum* var. Samsun NN sterile shoots. Resistant shoots were selected on the regeneration media with hygromycin 20 mg/l and carbenicillin sodium 500 mg/l.

Fig. 1. Sequences of primers P3 and P4. The PmlI/BglII and NcoI/BstEII cutting sites are located on primers P3 and P4.

2.4. PCR detection of tobacco transformants

Genomic DNA was extracted by the DNA extraction kit (Tiangen Co.) from all putative transformants. PCR amplifications were performed to detect both sense and antisense inserted fragments using the sense primers (P19 and P21, Table 1) and antisense primers (P24 and P25, Table 1), respectively. The PCR conditions for the sense inserted fragment was 5 min at 95 °C; 30 cycles of 30 s at 94 °C, 30 s at 59 °C and 1 min at 72 °C; and a final extension at 72 °C for 8 min. The PCR conditions for the antisense inserted fragments were 5 min at 95 °C; 35 cycles of 30 s at 94 °C, 30 s at 53 °C and 1 min at 72 °C; with finally 10 min at 72 °C. All the PCR products were separated on 1% agarose gels, and the transformants were identified according to the target band.

2.5. RT-PCR detection of tobacco transformants

RNA was extracted from young leaves of the transformants. The SuperScript™ III first-strand synthesis system

Table 1
Primers used in this study.

Primer	Sequences	Amplified products
P1	5′-GGACAATGCGGGAACCAGA-3′	A 1512 bp γ-tubulin cDNA fragment
P2	5′-AGCCCCAAATTGAATCACATACAG-3′	
P19	5′-AGTCGGCGGCTTTTCTGCT-3′	Sense inserted fragment
P21	5′-GCAAGACCGGCAACAGGATTC-3′	
P24	5′-GGACCCCCACCCACGAG-3′	Antisense inserted fragment
P25	5′-GTCACGCGCTTTCCCACCAAC-3′	
P18S1	5′-GCCGGCGACGCATCATTCAAA-3′	Partial fragment of 18S rRNA gene
P18S2	5′-GGCCGGCCCATCCCAAAGTC-3′	
P5	5′-GAGACAAACAACG(A)AAGATGAGG-3′	Partial cDNA fragment of PGP1 gene
P6	5′-CG(A)AAGCCATCAGGT(A)AGTTTG-3′	

for RT-PCR (Invitrogen Co.) was used for reverse transcription, followed by PCR amplification of partial γ-tubulin cDNA and 18S rDNA fragments, using primer pairs P3/P4 and P18S1/P18S2 (Table 1), respectively. PCR conditions for both genes were 5 min at 95 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C; with a final 8 min extension at 72 °C. The products were then run on 1% agarose gels to compare the γ-tubulin gene expression at the RNA level in different transformants by a semi-quantitative method.

2.6. Western blot hybridization of γ-tubulin

Proteins were extracted from the apical and upper leaves of transformants with the empty vector and dsRNA vector, and were separated by SDS–PAGE with 12% separating gel and 4% stacking gel at 150 V for 1 h. A transfer system (BIO-RAD Life Science) was used to transfer proteins onto nitrocellulose membranes at 4 °C, 100 mA for 7–8 h. Western blotting of γ-tubulin and α-tubulin was performed as described in Ref. [11]. Monoclonal antibodies recognizing γ-tubulin and α-tubulin were used as probes. Alkaline phosphatase labeled IgG (rabbit anti-mouse) was used as the secondary antibody. For the detection of the proteins, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma) was used as the substrate in the reaction.

2.7. Phenotype observation

The phenotypes of target transformants were observed and recorded every two days. Roots were cut from the control and target transformants for paraffin sectioning [12], and photographed by a multi-functional microscope (Leica DMRE).

2.8. Analysis of IAA content and PGP1

Two grams of the young apical tissue were cut, and the endogenous IAA was extracted by the previously reported method [13,14], followed by chromatography through a C18 column and elution by 40% acetonitrile. High performance liquid chromatography (HPLC, HITACHI L-2200) was used to measure the IAA quantity, and the IAA absorption value was compared with that extracted from control plants.

For the detection of PGP1, primers P5 and P6 (Table 1) were designed according to its sequence, and an RT-PCR was carried out using total RNA as the template to compare the expression of PGP1 in different transformants. The PCR conditions were one cycle of 45 min at 45 °C and 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C, and 10 min at 72 °C. The products were run on a 1% agarose gel, and the 18S rDNA fragment was also amplified as the internal control. Semi-quantitative analysis was used to compare the PGP1 expression in different transformants.

3. Results

3.1. γ-Tubulin cDNA cloning and construction of the dsRNA vector

The 1512 bp γ-tubulin cDNA fragment was amplified successfully by RT-PCR using primers P1 and P2 (Fig. 2a), and was cloned into the pMD18-T vector. The positive recombinant plasmid was named as pMD18-T_t. The γ-tubulin cDNA fragment was sequenced and deposited into GenBank (Accession No. DQ471451). Sequence analysis showed that it was very similar to the known γ-tubulin cDNA sequence (GenBank Accession No. AJ278739). After removing the 3′ untranslated region, the sequences of the cDNA fragment (1395 bp) and amino acids were 98.8% and 100% homologous to the known sequences, respectively. Thus the sequenced cDNA fragment was verified as a partial γ-tubulin gene.

A 454 bp band was obtained by PCR using pMD18-T_t as the template and primers P3 and P4 (Fig. 2b). This fragment was ligated with the vector pCAMBIA1301 after digestion with restriction enzymes, such that sense and antisense fragments were inserted together. Finally, two dsRNA vectors were identified by sequencing, named LBA3 and LBA11, respectively, which were transformed into *A. tumefaciens* strain LBA4404.

3.2. Selection and identification of tobacco transformants

After a hygromycin selection, 45 and 39 putative transformant lines were obtained from the LBA3 and LBA11 transformation systems, respectively. PCR results showed that the insertion of the foreign gene was very complicated. Most of the putative transformants had only one fragment integrated into their genome, and only eight lines had the sense and antisense fragments integrated together (data not shown). These eight lines were used for the gene expression experiments.

3.3. γ-Tubulin expression at the RNA and protein levels in tobacco transformants

Further analysis of γ-tubulin expression by semi-quantitative RT-PCR on the eight lines showed that the 454 bp band could be identified from all the eight lines, but line

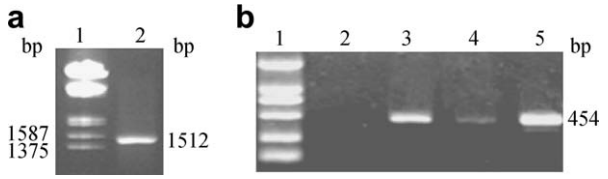

Fig. 2. The amplification of γ-tubulin cDNA fragments. (a) RT-PCR result for γ-tubulin cDNA fragment (lane 2). (b) PCR result of inserted γ-tubulin cDNA fragment used for vector construction (lanes 3–5). Lane 1 is DNA markers.

LBA3_21 had a slightly lower expression level when compared to the other seven lines (Fig. 3a). Western blotting of γ-tubulin on this line also demonstrated a weaker 48 kDa band than that of the control (Fig. 3b). The 53 kDa band of α-tubulin also showed the same phenomenon (Fig. 3b). Therefore, γ-tubulin gene silencing not only decreased γ-tubulin expression at both the transcriptional and translational levels, but also clearly disturbed α-tubulin expression, suggesting that the whole microtubular cytoskeleton network was disordered.

3.4. Phenotypes of tobacco transformants

There were big differences in the phenotypes between line LBA3_21 and the control plants (transformed with an empty vector). The roots of the control plants were swollen, and their taproots were not easily distinguished from lateral roots (Fig. 4a); roots of wild-type plants were thin with undeveloped lateral roots (Fig. 4b), which became elongated as the plant developed. However, taproots of the LBA3_21 transformant line were swollen in the initial stage, with lateral roots distributed in parallel and of a similar length to lateral roots (Fig. 4c). As the plants grew, the taproots ceased to elongate and they developed circular tips; the lateral roots continued to develop (Fig. 4d). Observation of the microstructure of the plants showed that root tip cells of control plants grew well, and the root cap developed with abundant cells. Cells in the meristematic, elongation and maturation zones could be distinguished easily and formed tight and regular arrays (Fig. 4e); however, there was no detectable root cap in the root tips from the LBA3 line, and cell arrays in other plant parts were irregular with large interspaces (Fig. 4f).

Shoot development was also abnormal in 80% of the LBA3_21 plants. The plants developed more than one meristem and lateral branches appeared on the plants (Fig. 4g and h). Leaf shape was also disordered, with a variety of conjoined leaves and trumpet-shaped leaves appearing (Fig. 4i and k). Many leaves had no obvious dorso-ventral polarity (Fig. 4i). Compared with the control plants, the plants of line LBA3_21 grew relatively weakly with short internodes. Importantly, all these phenotypes appeared continuously on subsequent subculture, which proved that the phenotypes were stably inherited and caused by silencing of the γ-tubulin gene.

3.5. Endogenous IAA contents and PGP1 expression changes

According to peak determinations, the IAA standard sample retention time was 6.9 min, and the biggest absorption was at 254 nm. Comparing the endogenous IAA content in different plants, the results showed that the IAA absorption value was only 0.5 mAU in the control plants (Fig. 5a), but reached 4.0 mAU in the plants of line LBA3_21 (Fig. 5b). Thus the IAA content was much higher in line LBA3_21 than that in the control.

The PGP1 gene expression at the apical tissue of plants was also detected. The 1141 bp target band could be identified from all four control plant specimens, but there was almost no signal appearing in the same region for the LBA3_21 plants (Fig. 5c). This showed that PGP1 was expressed at a relatively low level in LBA3_21 plants.

4. Discussion

4.1. γ-Tubulin gene silencing disturbed plant polar growth

In this study, we silenced the γ-tubulin gene using a dsRNA-induced RNA interference technique, and obtained γ-tubulin down-regulated tobacco plant (line LBA3_21). The gene was not silenced at high frequency, but the plant phenotype was observed to be stable and this proved that γ-tubulin gene silencing had disrupted plant growth and development. We observed multiple meristems and/or lateral buds, large amounts of conjoined or trumped-shaped leaves, loss of leaf dorso-ventral polarity

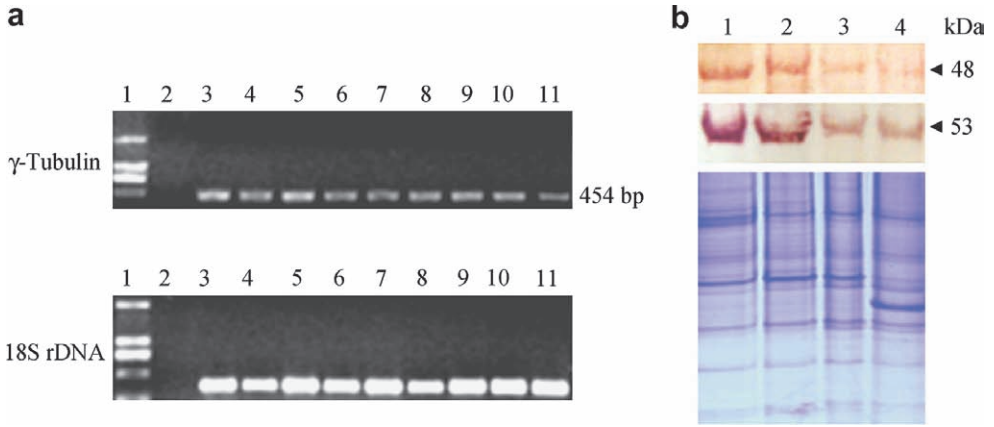

Fig. 3. Detection of γ-tubulin expression in different transformants. (a) The expression of γ-tubulin and 18S rDNA RNA. Lane 1, DL2000 marker; 2, negative control; 3, positive control; 4–10, the result of different LBA11 transformant lines; 11, the result of line LBA3_21. (b) Western blotting results of γ-tubulin (48 kDa) and α-tubulin (53 kDa). The bottom panel represents the total protein. 1 and 2, control plants; 3 and 4, line LBA3_21.

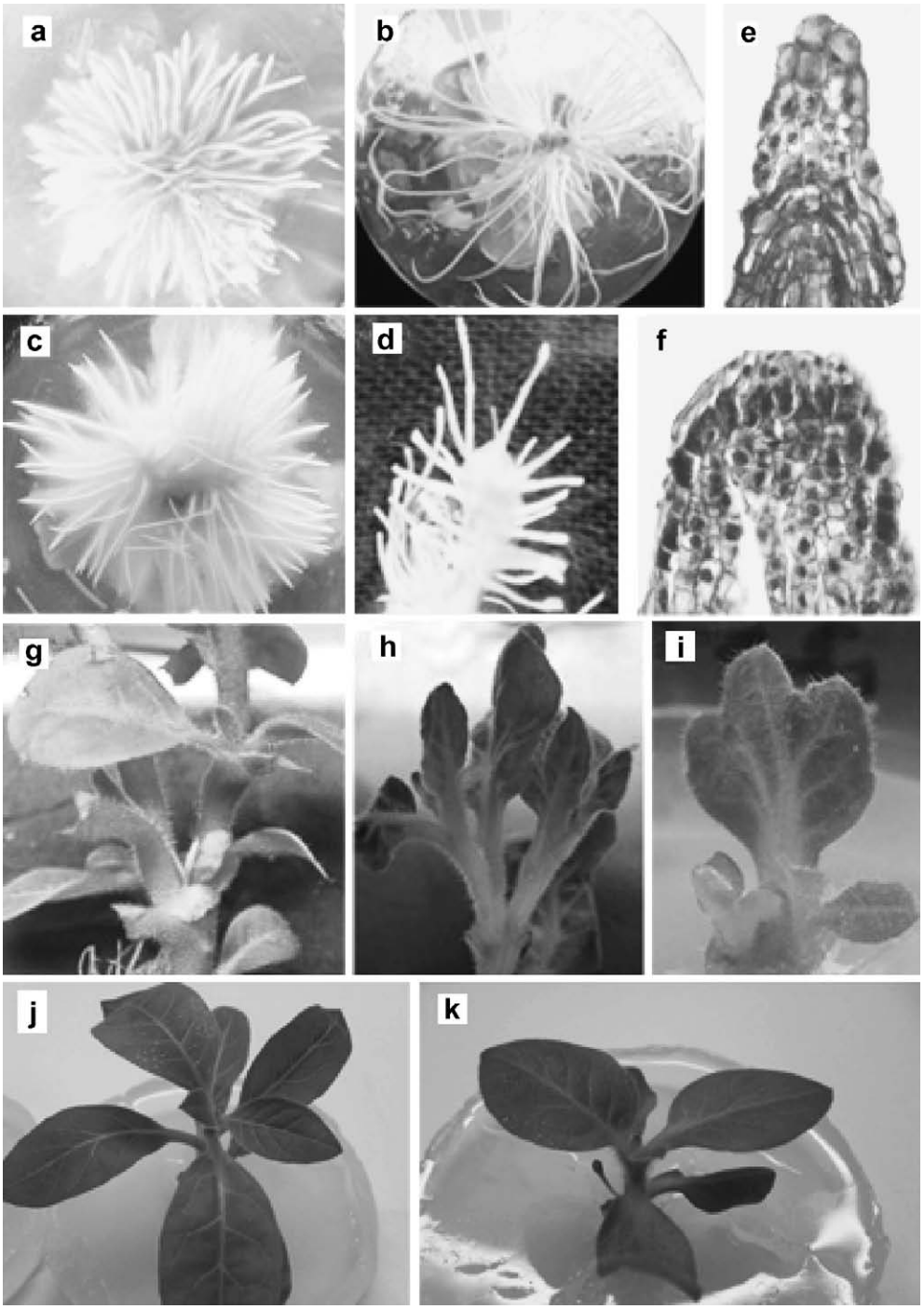

Fig. 4. Phenotypes of transgenic plants. (a) and (b), Roots of control and wild-type plants grown for 25 days, respectively; (c) and (d), roots of targeted lines grown for 25 and 35 days, respectively; (e) and (f), straight roots of control and target line, respectively; (g) and (h), line LBA3_21 showing multiple branches; (i) conjoined leaf developed in line LBA3_21; (j) and (k), plants of control and line LBA3_21 grown for 20 days, respectively.

and lateral roots growing on undeveloped taproots. All these observations indicated that plant polar growth had been inhibited.

Previous studies have observed that the depletion of γ-tubulin could cause loss of leaf dorso-ventrality [8,9], but phenotypes showing multiple meristems or lateral branches together with large amounts of conjoined or trumped-shaped leaves have not been previously described. The multiple meristems and lateral branches phenomena also occurred in the PVX-induced γ-tubulin gene-silenced tobacco plants that were previously grown in our laboratory. Thus, we found that the γ-tubulin gene was essential for tobacco normal polar growth; its gene silencing disturbed plant polarity.

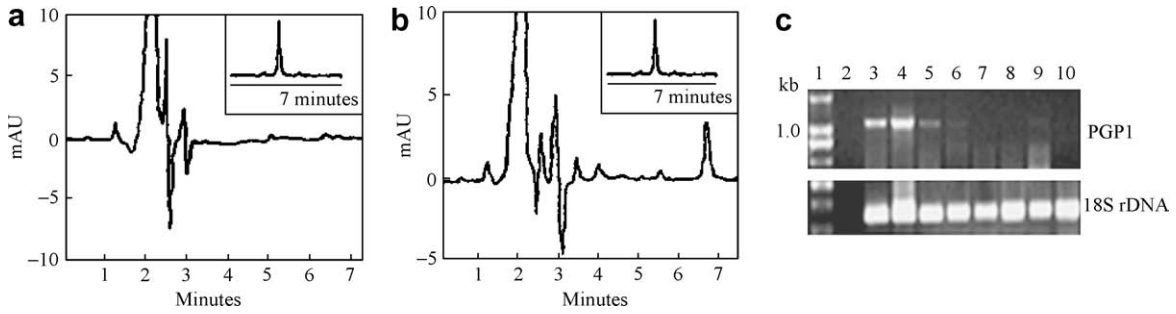

Fig. 5. Detection of endogenous IAA and PGP1 expression. (a and b) Endogenous IAA of control plants and line LBA3_21, respectively; (c), RT-PCR result of PGP1 and 18S rDNA fragments in different transformants. 1, DL2000 marker; 2, negative control; 3–6, control plants; 7–10, line LBA3_21.

4.2. γ-Tubulin gene silencing affected IAA content and transportation

It is generally accepted that auxin (including IAA) can control the orientation of cellulose microfibril indirectly, by regulating the cortical array, improving or attenuating microtubular depolymerization, and regulating cell growth [15]. This is very important for root elongation, microtubule organization and apical dominancy [16–18]. Many studies have confirmed that the ratio of auxin to cytokinin determines types of shoot branching; it can also prompt lateral bud formation when the ratio is low [19]. In this study, low levels of expression of γ-tubulin caused an increase in IAA; theoretically this should not instigate lateral bud or multiple meristem appearance as displayed by our silenced plant line. The phenotype was probably due to the depletion of γ-tubulin that resulted in other hormonal abnormalities.

IAA is also a hormone affecting polar transportation in plants [20–22], which is regulated by an auxin efflux carrier that binds with many kinds of transportation inhibition factors, such as *N*-1-naphthylphthalamic acid (NPA) [23–25]. This complicated dynamic process controls plant development [26]. Ni et al. observed a variety of trumpet-shaped leaves while adding different concentrations of NPA and 6-benzylaminopurine (BA); this provided evidence for the effects of auxin polar transportation on symmetrical leaf growth [27,28]. PGP1 is a carrier of auxin polar transportation and a member of the *P*-glycoproteins family that binds with NPA and reduces its effect. In this study, PGP1 expression was lower in line LBA3 _21 than that in the control, and therefore could not bind large amounts of NPA. This large excess of NPA inhibited auxin polar transportation. As PGP1 is located at the lower part of the cell, its reduced expression would only disturb auxin polar transportation in a lengthways direction. Eventually, the leaf lost its symmetrical development model, and developed conjoined or trumped-shaped leaves.

We conclude that our study has proved that γ-tubulin gene silencing disturbed plant polar growth, which was probably correlated with abnormal IAA synthesis and polar transportation and may even lead to the disruption of the cell microtubule network. However, plant growth and development is a very complex process involving many other signals and reactions. More work will be necessary to discover further roles for γ-tubulin in plant development.

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