



Over-expression of the *AtGA2ox8* gene decreases the biomass accumulation and lignification in rapeseed (*Brassica napus* L.)^{*}

Xiao-ying ZHAO^{§1}, Deng-feng ZHU^{§1,2}, Bo ZHOU¹, Wu-sheng PENG³, Jian-zhong LIN¹, Xing-qun HUANG¹,
 Re-qing HE¹, Yu-hong ZHUO³, Dan PENG¹, Dong-ying TANG¹, Ming-fang LI², Xuan-ming LIU^{†‡1}

⁽¹⁾Bioenergy and Biomaterial Research Center, Institute of Life Science and Technology,

State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, Changsha 410082, China)

⁽²⁾Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China)

⁽³⁾Academy of Seed Industry of Hunan Yuhua, Changsha 410001, China)

[†]E-mail: sw_xml@hnu.cn

Received Apr. 29, 2010; Revision accepted June 11, 2010; Crosschecked June 13, 2010

Abstract: Gibberellin 2-oxidase (GA 2-oxidase) plays very important roles in plant growth and development. In this study, the *AtGA2ox8* gene, derived from *Arabidopsis* (*Arabidopsis thaliana*), was transformed and over-expressed in rapeseed (*Brassica napus* L.) to assess the role of *AtGA2ox8* in biomass accumulation and lignification in plants. The transgenic plants, identified by resistant selection, polymerase chain reaction (PCR) and reverse-transcription PCR (RT-PCR) analyses, and green fluorescence examination, showed growth retardation, flowering delay, and dwarf stature. The fresh weight and dry weight in transgenic lines were about 21% and 29% lower than those in wild type (WT), respectively, and the fresh to dry weight ratios were higher than that of WT. Quantitative measurements demonstrated that the lignin content in transgenic lines decreased by 10%–20%, and histochemical staining results also showed reduced lignification in transgenic lines. Quantitative real-time PCR analysis indicated that the transcript levels of lignin biosynthetic genes in transgenic lines were markedly decreased and were consistent with the reduced lignification. These results suggest that the reduced biomass accumulation and lignification in the *AtGA2ox8* over-expression rapeseed might be due to altered lignin biosynthetic gene expression.

Key words: Rapeseed, *AtGA2ox8*, Biomass, Lignification, Gibberellins

doi:10.1631/jzus.B1000161

Document code: A

CLC number: S565.4; Q812

1 Introduction

Gibberellins (GAs) are endogenous plant hormones playing very important roles in plant growth and regulating many aspects of plant development, including seed germination, shoot growth, flower induction, stimulation of cell elongation and cell division, hypocotyl elongation, fruit maturation, and

leaf expansion (Kende and Zeevaart, 1997; Harberd *et al.*, 1998; Hedden and Proebsting, 1999). Only a few of the 126 presently known GAs, such as GA₁, GA₃, GA₄, and GA₇, have been shown to have biological activity (Hedden and Phillips, 2000).

GA metabolism takes place in three different cellular compartments, i.e., plastids, endoplasmic reticulum (ER), and cytosol (Hedden and Phillips, 2000; Olszewski *et al.*, 2002; Sun and Gubler, 2004). Multiple enzymes including *ent*-copalyl diphosphate synthase, *ent*-kaurene synthase, P450 monooxygenases, and dioxygenases are involved in GA metabolism and catabolism. Two dioxygenases, GA 20-oxidase and GA 3β-hydroxygenase, catalyze the last

[‡] Corresponding author

[§] The two authors contributed equally to this work

^{*} Project supported by the National High-Tech R & D Program (863) of China (No. 2007AA10Z127) and the National Natural Science Foundation of China (No. 30800080)

© Zhejiang University and Springer-Verlag Berlin Heidelberg 2010

few steps in the synthesis of bioactive GA. Another dioxygenase, GA 2-oxidase, catalyzes GA catabolism of bioactive GA or their precursors (Thomas *et al.*, 1999; Hedden and Phillips, 2000; Helliwell *et al.*, 2001; Olszewski *et al.*, 2002; Zhao *et al.*, 2007b).

The genes encoding the multi-functional GA 2-oxidase have been cloned from various species, such as runner bean (*Phaseolus coccineus*) (Thomas *et al.*, 1999; Appleford *et al.*, 2007), Arabidopsis (*Arabidopsis thaliana*) (Thomas *et al.*, 1999; Schomburg *et al.*, 2003), pea (*Pisum sativum*) (Lester *et al.*, 1999; Martin *et al.*, 1999), rice (*Oryza sativa*) (Sakamoto *et al.*, 2001; 2004), poplar (*Populus tremula*) (Busov *et al.*, 2003), spinach (*Spinacia oleracea*) (Lee and Zeevaart, 2002; 2005), and lettuce (*Lactuca sativa*) (Nakaminami *et al.*, 2003). Phylogenetic analysis divides the GA 2-oxidase family into three classes according to their amino acid sequences (Lee and Zeevaart, 2005). C₁₉-GAs (GA₁ and GA₄) and C₁₉-GA precursors (GA₂₀ and GA₉) can be converted to inactive GAs by members of classes I and II, respectively (Sakamoto *et al.*, 2004; Lee and Zeevaart, 2005; Lo *et al.*, 2008), and C₂₀-GAs (GA₁₂ and GA₅₃) can be 2 β -hydroxylated by members of class III, including SoGA2ox3, AtGA2ox7, and AtGA2ox8 (Schomburg *et al.*, 2003; Lee and Zeevaart, 2005; Lo *et al.*, 2008). The 2 β -hydroxylated C₂₀-GA precursors cannot be converted to active GAs, resulting in a decrease in active GA levels.

The over-expression of the genes encoding AtGA2-oxidase causes a dwarf phenotype and delayed flowering in Arabidopsis (Schomburg *et al.*, 2003; Wang *et al.*, 2004; Zhao *et al.*, 2007a; Rieu *et al.*, 2008). The heterologous expression of AtGA2-oxidases also resulted in growth retardation in wheat (Hedden and Phillips, 2000), rice (Sakamoto *et al.*, 2001), tobacco (Schomburg *et al.*, 2003), bahiagrass (Agharkar *et al.*, 2007), and *Solanum* species (Dijkstra *et al.*, 2008). Apart from the effect of expression of AtGA2-oxidases on plant elongation, Biemelt *et al.* (2004) reported that biomass production was decreased in *AtGA2ox1* plants, and that the transgenic plants have altered lignification. Moreover, they showed that GA have different effects on xylem and pitch cell formation.

Both the rapeseed (*Brassica napus* L.) and Arabidopsis are cruciferous plants. Zhao *et al.* (2007a) and Schomburg *et al.* (2003) reported that over-

expression of *AtGA2ox8* causes reduced bioactive GA levels in Arabidopsis. To investigate the correlation of *AtGA2ox8* gene to biomass characteristics and lignin biosynthesis, we introduced it into rapeseed. Here, we show that heterologous expression of *AtGA2ox8* gene in rapeseed causes growth retardation, flowering delay, dwarf stature, and reduced biomass accumulation and lignification.

2 Materials and methods

2.1 Plant materials and genetic transformation

The cultivar of rapeseed (*Brassica napus* L.) in this study was N529, provided by the Academy of Seed Industry of Hunan Yahua, Changsha, China. *AtGA2ox8* transgenic plants were prepared by the first amplifying *AtGA2ox8* (GenBank accession No. AL021960, accession number in The Arabidopsis Information Resource (TAIR) website is At4g21200) gene from Arabidopsis cDNA using a reverse-transcription polymerase chain reaction (RT-PCR) method with primers *AtGA2ox8*-F (5'-TCCCCCGGATGGATCCACCATTCAACGAAATAT-3') and *AtGA2ox8*-R (5'-CCGCTCGAGTTAGTAGACGTGTTAAGGAACCAGGAA-3') (restriction sites are underlined), and then subcloning it into the *Sma*I and *Xho*I sites of pEGAD vector downstream of the CaMV35S promoter. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 by the electroporation method, as described in our previous study (Lin *et al.*, 2009). When the rapeseed plants begin to form boll and produce flora inflorescences, the healthy flora inflorescences grown at the top of rapeseed plants were selected and immersed into a plastic bag with *Agrobacterium* inoculum for 30 s, and then covered with an agricultural parchment bag to maintain high humidity. One week later, the agricultural parchment bags were uncovered and the flower buds at the top of dipped inflorescences were removed. When the siliques became brown and dry, the seeds from the dry siliques were collected and stored at -20 °C for screening.

2.2 Transgenic line screening

Transgenic plants were first selected using herbicide Basta (1:800, v/v). Total genomic DNA was

extracted from Basta-resistant transgenic lines using hexadecyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The detection of *Basta^R* gene was carried out by PCR analysis with primers *Bar-F* (5'-CTACATCGAGACAAGCACG GT-3') and *Bar-R* (5'-CTGAAGTCCAGCTGCCAG AA-3'). The PCR program was as follows: 94 °C for 5 min, and then 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 5 min. The PCR products were analyzed using 1.0% (w/v) agarose gel electrophoresis.

To investigate whether the transgene is expressed in the transgenic plants, some lateral roots were excised from the Basta-resistant transgenic lines. The green fluorescent protein (GFP) fusion proteins were examined using inverted fluorescence microscope (Nikon TE2000-U, Japan).

2.3 GA₃ treatment

To investigate the response of hypocotyl elongation to exogenous GA₃, about 100 seeds were sown on filter paper saturated with 100 μmol/L GA₃ (Shanghai Solvent) in a plate and grown under continuous white light in a temperature-controlled growth chamber at 22 °C. Hypocotyl lengths of more than twenty 6-d-old seedlings were measured manually following the method of Lin *et al.* (1998), and the standard deviations (SDs) of the measurements were calculated from three independent experiments with 20 samples each.

2.4 Greenhouse evaluation of growth and biomass characteristics

Transgenic plants and wild-type plants were grown in a greenhouse. The stem heights of the plants were evaluated in the flowering and maturation periods. Leaves were counted in the maturation period. Fresh weight and dry weight of the whole plant, including the roots, were determined after harvest according to the method of Biemelt *et al.* (2004).

The seed oil content and the glucosinolate, erucic acid, and oleic acid contents in seed oil of rapeseed were determined by near-infrared reflectance (NIR) spectroscopy after harvest (Panford and de Man, 1990).

Each value of all the experiments represents the mean±SD of 10 to 15 samples each.

2.5 Histochemical detection and quantitative determination of lignin

Histochemical detection was carried out on the hand-cut cross sections of stems from the second, eighth, and seventeenth internodes (counting from the bottom up to the top) of the plants grown in greenhouse during the flowering period. The sections were stained using phloroglucinol-HCl reagent, and then analyzed on an inverted microscope (Nikon TE2000-U, Japan) according to the methods described previously (Campbell and Ellis, 1992; Biemelt *et al.*, 2004). Each experiment was repeated three times with at least three samples each.

The lignin contents in about 100 mg fresh weight of the apical, middle, and basal parts of the stems of both the *AtGA2ox8* transgenic plants and the wild-type plants grown in the greenhouse during the flowering period were determined by acetyl bromide ultraviolet spectrophotometry (280 nm) according to the methods described previously (Fukushima and Dehority, 2000; Fukushima and Hatfield, 2001; Biemelt *et al.*, 2004). Each value was obtained from two independent experiments with three samples each.

2.6 Semi-quantitative RT-PCR analysis

To investigate whether the *AtGA2ox8* gene was over-expressed in the transgenic rapeseed plant, about 100 seeds were sown on filter paper saturated with Murashige and Skoog (MS) salt liquid medium in a plate and grown under continuous white light in a temperature-controlled growth chamber at 22 °C. Total RNA was isolated from 6-d-old seedlings using Puprep RNAeasy mini kit (Ambiogen Life Tech Ltd., China). The transcript level of *AtGA2ox8* was analyzed using semi-quantitative RT-PCR as described previously (Zhao *et al.*, 2007a). The rapeseed *Actin* (*ACT*) (European Molecular Biology Laboratory (EMBL) accession No. AF111812) gene was used as an internal control. The PCR started with a denaturation stage at 95 °C for 5 min, which was then followed by 26 (for the *ACT*) or 35 (for the *AtGA2ox8*) cycles with each cycle composed of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The RT-PCR reactions for each experiment were repeated at least three times in three independent trials. The sequences of the primers

were: *AtGA2ox8*-F (5'-CGGAATCAGAGGCATTA GC-3'), *AtGA2ox8*-R (5'-CCACCTTTGGGTTTCGTC AT-3'), *ACT*-F (5'-TCCCTCAGCACTTTCCAACA G-3'), and *ACT*-R (5'-AAGGACCAGAGCATCATC ACAAG-3').

2.7 Quantitative real-time PCR analysis

To detect the mRNA level of lignin biosynthetic gene, total RNA was isolated from apical parts of stems of rapeseed during the flowering period using Puprep RNAeasy mini kit (Ambiogen Life Tech Ltd.). The amount of mRNA was analyzed using quantitative real-time PCR as described previously (Wang et al., 2009). The PCR was performed in Mx3000P (Stratagene), and started with a denaturation stage at 95 °C for 10 min, which was then followed by 40 cycles with each cycle composed of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 20 s. The PCR reactions were repeated three times from three independent experiments. The *ACT* gene of rapeseed was used as an internal control. The sequences of primers used in this study are listed in Table 1.

3 Results

3.1 Transformation and molecular characterization

The *AtGA2ox8* gene was introduced into rapeseed by *Agrobacterium*-mediated floral dip transformation as stated in Section 2.1. The expression cassette contains the *GFP* report gene, the target gene (*AtGA2ox8*), and the herbicide Basta-resistant (*Basta^R*) select maker gene. *AtGA2ox8* and *GFP* were driven by the CaMV35S promoter and would express fusion protein in the transgenic plants (Fig. 1a in p.478). Ten Basta-resistant independent lines were obtained after the first screening with herbicide Basta (Fig. 1b).

The *Basta^R* gene was detected by PCR analysis in all Basta-resistant plants but not in the wild-type plants (partly in Fig. 1c), indicating that the transgene was indeed integrated into the genomic DNA. To determine whether the transgene is normally expressed in the transgenic plants, the GFP fusion proteins were examined using inverted fluorescence microscope, and strong green fluorescence was detected in the root of transgenic lines (partly in Fig. 1d). No green fluorescence was observed in root of the wild-type plant (Fig. 1e). These data confirmed that transgenic plants were obtained. Two independent transgenic lines, *35S::GFP-AtGA2ox8-2* and *35S::GFP-AtGA2ox8-3*, were then randomly selected for the further detection of the target gene expression using semi-quantitative RT-PCR analysis. The results showed that the *AtGA2ox8* signals of the two independent transgenic lines were quite strong, whereas the corresponding signal of the wild type was hardly detectable (Fig. 2). These results indicated that the *AtGA2ox8* gene was normally over-expressed in the two independent transgenic lines.

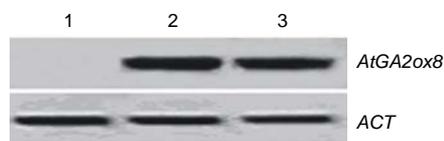


Fig. 2 Semi-quantitative RT-PCR analysis of *AtGA2ox8* gene in the transgenic rapeseed

Total RNA was isolated from 6-d-old seedlings of the wild type (WT) and the two independent transgenic lines grown on filter paper saturated with MS salt liquid medium in a plate under continuous white light in a temperature-controlled growth chamber at 22 °C. The transcript levels are shown as gel images, and the *ACT* is used as an internal control. Each experiment was repeated at least three times in three independent trials and similar results were obtained. Lane 1: WT; Lane 2: *35S::GFP-AtGA2ox8-2*; Lane 3: *35S::GFP-AtGA2ox8-3*

Table 1 Primer sequences used for quantitative real-time PCR analysis

Gene name	EMBL accession No.	Forward and reverse (5'-3')
<i>PAL1</i>	DQ341309	Forward: CAAAGCGATTACGGAGGTAA; reverse: CGCTCCTTTGAAACCGTAGTC
<i>PAL2</i>	AY795080	Forward: TTGGATTACGGATTCAAAGGA; reverse: CGAGATGAGTCCCAAAGAGTT
<i>C4H</i>	DQ485129	Forward: TGACTTTAAGTATGTGCCGTTT; reverse: GGACCTTGGCTTCATTACGAT
<i>4CL</i>	CX190902	Forward: TAATCCGAATCTTTACTTCCACAG; reverse: GCAACCGTCACTTTACACCTCT
<i>HCT</i>	CD832138	Forward: CTCAAGGCTAAAGCCAAGGAG; reverse: TGACGTTGCCAAAGTAACCAG
<i>CCR</i>	CD844319	Forward: GGTGGAAGTTTAGGTCATTAGAAGA; reverse: CCAATAGTAGACTTGAGGAGGTGAA
<i>CAD</i>	CD814354	Forward: TTATGTCCTGGTTGGTTTCCC; reverse: CACCCTTTCAATAGCTTCGTTT
<i>ACT</i>	AF111812	Forward: TCCCTCAGCACTTTCCAACAG; reverse: AACTCACCACCACGAACCAG

3.2 Effect of over-expression of *AtGA2ox8* in rapeseed on plant growth

Transgenic seeds were sown in plates to investigate the hypocotyl phenotype. Compared with wild type, the over-expression of *AtGA2ox8* in rapeseed inhibited the hypocotyl elongation and led to shorter hypocotyls (Figs. 3a and 3b). Furthermore, the phenotype of *35S::GFP-AtGA2ox8* could be rescued by exogenous GA_3 (Figs. 3c and 3d). These results are consistent with our previous reports that the over-expression of *AtGA2ox8* in *Arabidopsis* resulted in short hypocotyl (Zhao et al., 2007a), and the *gal* mutant impaired in the *ent-copalyl* diphosphate synthase gene caused short hypocotyl (Sun et al., 1992; Alabadi et al., 2004). Therefore, these observations strongly suggested that *AtGA2ox8* could induce a GA-deficient phenotype in different species.

To evaluate the growth characteristics, the rapeseed seedlings were planted in a greenhouse. The phenotypes were investigated during different growth periods. The *AtGA2ox8* transgenic lines exhibited growth retardation, flowering delay, and dwarf phenotype. The stem heights of the *AtGA2ox8* over-expression lines were only about 70% of those of the wild-type plants (Fig. 4). Moreover, the *AtGA2ox8*

over-expression lines have shorter leaves (data not shown). Compared with the wild-type plants, the flowering time in the *AtGA2ox8* over-expression lines was delayed for approximately one week (Fig. 4a).

3.3 Evaluation of biomass characteristics of transgenic lines

The biomass characteristics of the transgenic plants during maturation period were also analyzed (Table 2). As far as the number of leaves is concerned, there was almost no difference between the transgenic plants and the wild type. In order to assess the impact of the transgenic plants on biomass production, the fresh weight and dry weight of the whole plants were determined. As shown in Table 2, significant differences in biomass production between the wild type and the transgenic lines were observed. Compared with the wild type, the fresh weight and dry weight in the transgenic lines decreased by about 21% and 29%, respectively. The fresh to dry weight ratios for wild type plants, *35S::GFP-AtGA2ox8-2*, and *35S::GFP-AtGA2ox8-3* were 10.1, 11.2, and 11.3, respectively. Moreover, the 1000-seed weight in the transgenic plants was significantly increased, though the seed yield per plant was only slightly increased.

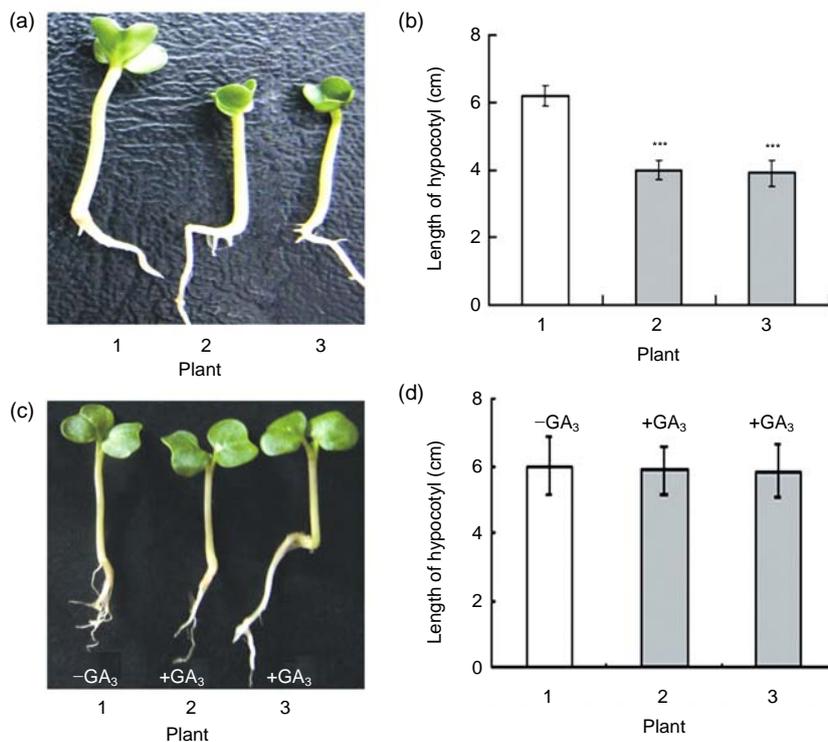


Fig. 3 Response of *AtGA2ox8* transgenic plants to GA_3

(a) & (b) Six-day-old seedlings of both the wild type (WT) and the two independent transgenic lines and their hypocotyl lengths are shown, respectively; (c) & (d) GA_3 -rescueable phenotype of 6-d-old seedlings of the two independent transgenic lines and their hypocotyl lengths are shown, respectively. The data represent the mean values obtained from three independent experiments with 20 samples each. The error bars represent the SDs. *** $P < 0.001$, significant difference between the WT and transgenic plants. Plant: 1. WT; 2. *35S::GFP-AtGA2ox8-2*; 3. *35S::GFP-AtGA2ox8-3*

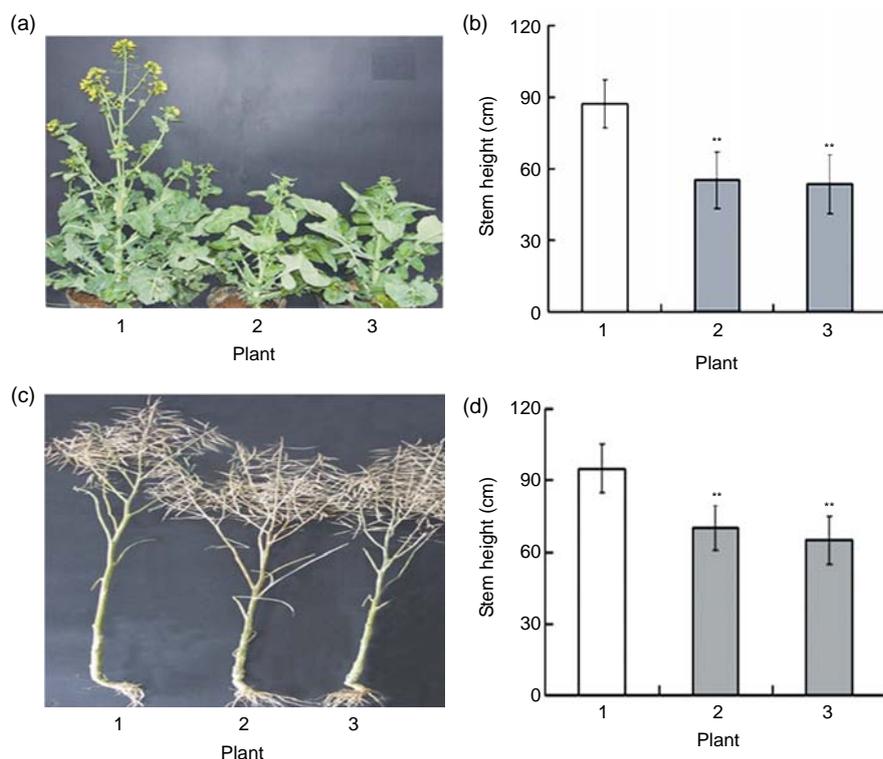


Fig. 4 Comparison of the stem heights of both the *AtGA2ox8* transgenic plants and the wild type (WT) grown in a greenhouse during different growth periods (a) & (c) Plants of the WT and the two independent transgenic lines during the flowering (a) and maturation (c) periods, respectively; (b) & (d) Stem heights of plants during the flowering (b) and maturation (d) periods, respectively. The error bars represent the SDs of 10 to 15 samples each. ** $P < 0.01$, significant difference between the WT and transgenic plants. Plant: 1. WT; 2. *35S::GFP-AtGA2ox8-2*; 3. *35S::GFP-AtGA2ox8-3*

Table 2 Effects of over-expression of *AtGA2ox8* in rapeseed on biomass accumulation

Plant	Number of leaves	FW (g)	DW (g)	FW/DW	Seed yield per plant (g)	1000-seed weight (g)	Seed oil content (%)	Erucic acid content (%)	Glucosinolate content ($\mu\text{mol/g}$)	Oleic acid content (%)
1	30.0 \pm 1.3	620.4 \pm 20.5	61.7 \pm 5.6	10.1	17.7 \pm 1.8	2.36 \pm 0.09	32.5 \pm 0.6	0.86 \pm 0.35	40.6 \pm 2.7	62.2 \pm 0.17
2	29.5 \pm 1.2	495.5 \pm 14.5***	44.2 \pm 7.6**	11.2	18.8 \pm 1.8	2.85 \pm 0.10***	33.5 \pm 0.5*	1.30 \pm 0.50	33.5 \pm 2.7*	62.5 \pm 0.20
3	30.0 \pm 1.0	488.7 \pm 13.2***	43.5 \pm 7.3**	11.3	17.8 \pm 1.4	2.92 \pm 0.09***	34.4 \pm 0.4**	1.35 \pm 0.49	30.8 \pm 2.8*	63.3 \pm 0.28

Results are given for two independent lines and represent the mean \pm SD of 10 to 15 samples each. FW: fresh weight; DW: dry weight. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant differences between the WT and the two mutant lines. Plant: 1. WT; 2. *35S::GFP-AtGA2ox8-2*; 3. *35S::GFP-AtGA2ox8-3*

The determination of the seed oil content and the glucosinolate, erucic acid, and oleic acid contents in seed oil revealed that the two transgenic lines produced 3% or 6% more seed oil and 15%–25% less glucosinolate in seed oil than the wild type. The erucic acid content in seed oil was higher in the transgenic lines as compared to the wild type. However, there was almost no difference in the oleic acid content between the transgenic lines and the wild type. These results demonstrated that *AtGA2ox8* reduced the biomass accumulation and also affected the yield and quality of seed oil in rapeseed.

3.4 Effect of over-expression of *AtGA2ox8* in rapeseed on lignification

It has been reported that the change in fresh to dry weight ratio might result from the altered composition

or deposition of the structure-forming substances like cellulose and/or lignin, and that higher fresh to dry weight ratio of plants usually led to a relatively low lignin content in plants (Biemelt *et al.*, 2004). Lignification is also a characteristic feature of secondary growth and increases with maturity (Israelsson *et al.*, 2005). To determine the different levels of lignification in the transgenic lines and the wild type, different parts of stems, i.e., apical, middle, and basal parts, were collected and their lignin contents were measured. For both the transgenic lines and the wild type, the lignin content increased gradually from the apical parts to the basal parts (Fig. 5). Lignin content comparison revealed that the two independent transgenic lines, *35S::GFP-AtGA2ox8-2* and *35S::GFP-AtGA2ox8-3*, produced about 10% and 20% lower lignin contents in stems than the wild-type plants, respectively.

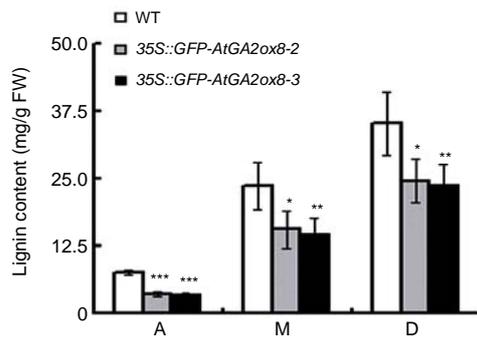


Fig. 5 Lignin content in different parts of stems of both the *AtGA2ox8* transgenic plants and the wild-type (WT) plants grown in a greenhouse during the flowering period

A, M, and B symbolize apical, middle, and basal parts, respectively. The error bars represent the SDs of two independent experiments with three samples each. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant difference between the WT and transgenic plants. FW: fresh weight

The cell walls of lignification display red or purple-red color in acidic phloroglucinol. For visually investigating the differences in lignification between the transgenic lines and the wild type, different parts of stems (the seventeenth internode, the eighth internode, and the second internode) were analyzed by histochemical detection. The biomass characteristics and lignin contents of the two transgenic lines were essentially the same. Hence, only one of the two transgenic lines, *35S::GFP-AtGA2ox8-2*, was selected for the histochemical detection, and its staining pattern is shown in Fig. 6. In general, the number of lignified vessels and the amount of lignin deposition were gradually increased from the apex to the base of the stem. The *AtGA2ox8* over-expression line had fewer lignified vessels than the wild-type controls, which was in good accordance with the lignin contents shown in Fig. 5. These results demonstrate that the heterologous expression of *AtGA2ox8* in rapeseed can result in lignin deposition reduction.

3.5 Effect of over-expression of *AtGA2ox8* in rapeseed on lignin biosynthetic gene expression

Lignin is a complex phenylpropanoid polymer, and lignin monolignols are synthesized through the phenylpropanoid pathway (Boerjan *et al.*, 2003). To determine whether the changes of lignin content in transgenic lines could be a result of altered genes expression, a search for the cDNA coding or the expressed sequence tag (EST) sites for the enzymes in

the lignin pathway was performed on the Brassica website (<http://www.brassica.info/>). The cDNA coding sequences of seven key enzymes in lignin synthesis, i.e., phenylalanine ammonialyase 1 (PAL1), phenylalanine ammonialyase 2 (PAL2), 4-coumarate: CoA ligase (4CL), cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD), were found. The relative expression of these genes in apical parts of the stems of the *AtGA2ox8* over-expression lines and the wild-type plants was analyzed by quantitative real-time PCR. The transcript levels of all the seven genes were found to have a significant difference between the wild type and the transgenic plants, and were markedly reduced in the *AtGA2ox8* over-expression lines (Fig. 7). These transcript reductions coincided with the reduced lignification in the apical parts of stems of the transgenic plants (Figs. 5 and 6). All these results indicate that the changes in lignification in transgenic plants might be associated with the altered expression of lignin biosynthetic genes.

4 Discussion

This study investigated the over-expression of *AtGA2ox8* in rapeseed by genetic engineering technology. It was found that the over-expression of *AtGA2ox8* could inhibit hypocotyl elongation in rapeseed. This inhibition effect can be removed by the application of GA₃ to the *AtGA2ox8* transgenic lines. *AtGA2ox8* acts as GA 2-oxidases that hydroxylate carbon-2 of C₂₀-GA precursors (e.g., GA₁₂), but not active GAs (Schomburg *et al.*, 2003; Lee and Zeevaart, 2005; Lo *et al.*, 2008). Therefore, active C₁₉-GAs such as GA₃ can be employed to rescue *AtGA2ox8*-derived short hypocotyl phenotype.

The over-expression of *AtGA2ox8* in rapeseed also resulted in dwarf phenotype with delayed flowering, shorter stems, and shorter leaves. Nevertheless, viable seeds were obtained from the transgenic lines. Moreover, the 1000-seed weight in the transgenic plants was significantly increased, though the seed yield per plant was only slightly increased. Similarly, heterologous over-expression of C₂₀-GA2ox, including Arabidopsis GA2ox8 (Schomburg *et al.*, 2003), spinach GA2ox3 (Lee and Zeevaart, 2005), rice

GA2ox5, and GA2ox6 (Lo *et al.*, 2008) in transgenic tobacco, resulted in reduced plant height, while allowing the production of viable seeds. The same results were also obtained from homologous over-expression of C₂₀-GA2ox, including rice GA2ox5 and GA2ox6, and Arabidopsis GA2ox8 in transgenic plants. In contrast, the rice over-expressing C₁₉-GA2ox, including

GA2ox1, completely suppressed the formation of inflorescences (Sakamoto *et al.*, 2001). These seemingly contradictory results may be explained as follows based on the findings in previous studies: there are so many more C₂₀-GA precursors than the C₁₉-GA precursors and active C₁₉-GAs in plants that some of the C₂₀-GA precursors could escape from inactivation

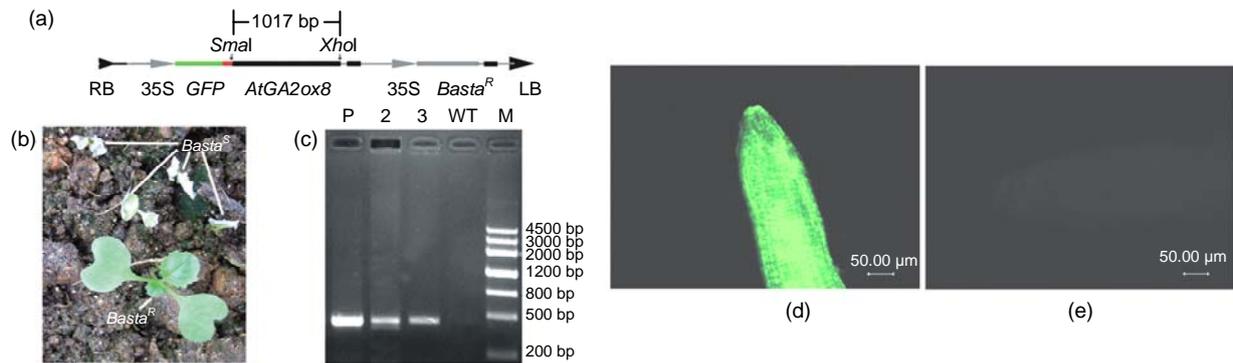


Fig. 1 Transformation and transgenic rapeseed plant screening

(a) Transgene expression cassette contains the CaMV35S promoter (35S), *AtGA2ox8* gene, green fluorescent protein (*GFP*) reporter gene, and herbicide Basta-resistant (*Basta^R*) select marker gene, and the positions of enzyme sites and the T-DNA left border (LB) and right border (RB) are shown; (b) Transgenic rapeseed plants were screened by herbicide Basta. *Basta^S* or *Basta^R* symbolizes Basta-susceptible or Basta-resistance, respectively; (c) PCR analysis of *Basta^R* gene in the transgenic rapeseed. DNA was extracted from the wild type (WT) and the Basta-resistant lines, respectively. P, 2, 3, WT, and M symbolize pEGAD plasmid, 35S::*GFP-AtGA2ox8-2*, 35S::*GFP-AtGA2ox8-3*, wild type, and marker, respectively; (d) & (e) GFP fluorescence imaging of transgenic plants with inverted fluorescence microscope. The images of the lateral root cap of the transgenic plant and WT are shown in (d) and (e), respectively

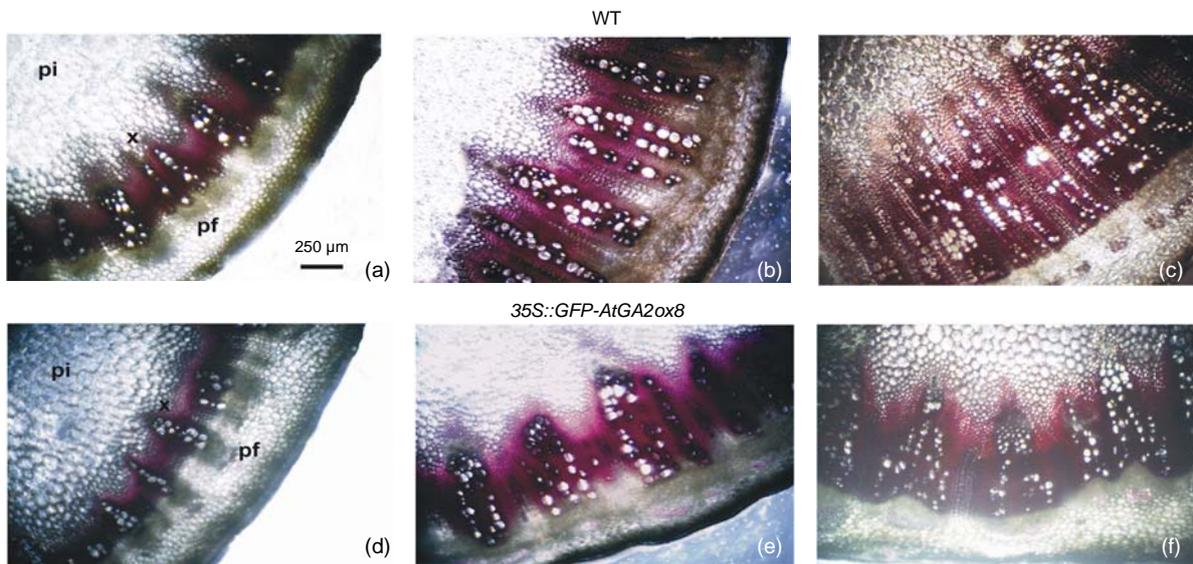
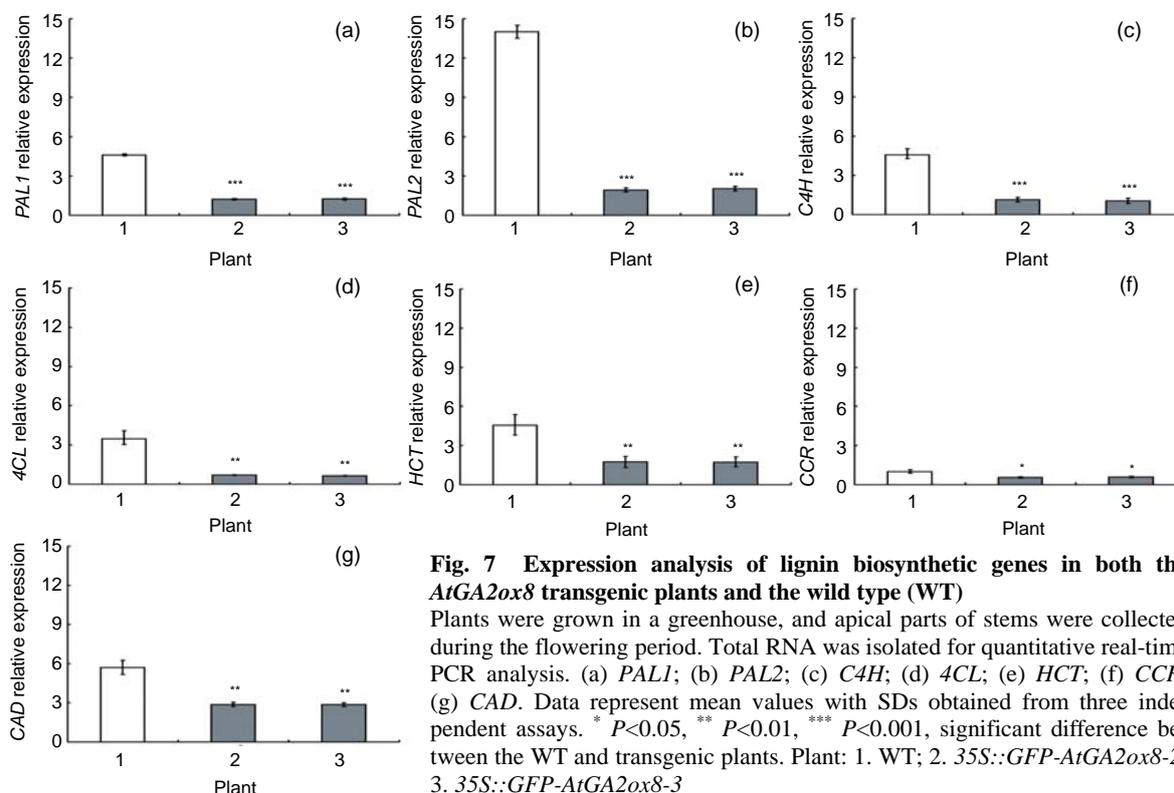


Fig. 6 Histochemical detection of lignin in the stem cross sections of both the *AtGA2ox8* transgenic plants and the wild type (WT)

Different internodes were collected from plants during the flowering period for histochemical detection. (a), (b) & (c) Phloroglucinol-HCl stained second, eighth, and seventeenth internodes of the WT, respectively; (d), (e) & (f) Phloroglucinol-HCl stained second, eighth, and seventeenth internodes of the *AtGA2ox8* transgenic plants, respectively. pf, pi, and x symbolize phloem fiber, pith, and xylem, respectively. Each experiment was repeated three times using at least three samples each with similar results



by C_{20} -GA2oxs (Sakamoto *et al.*, 2001; Schomburg *et al.*, 2003; Lee and Zeevaart, 2005). Hence, they can be converted to active C_{19} -GAs by GA 20-oxidase and GA 3-oxidase (Lo *et al.*, 2008).

To date, there has been no report about whether altered GA metabolism might change the seed oil content of rapeseed, glucosinolate and erucic acid contents in seed oil. Experimental results obtained in this work revealed that, compared to the wild-type plants, the *AtGA2ox8* over-expression lines could produce significantly more seed oil content. The erucic acid content also increased in the seed oil of the transgenic plants, but agreed with the criteria for double-low rapeseed species (Olsen and Soerensen, 1980; Sivaraman *et al.*, 2004). However, the glucosinolate content in the seed oil of the *AtGA2ox8* over-expression lines was obviously lower than that of the wild type. These preliminary results suggested that *AtGA2ox8* might be useful in rapeseed breeding without affecting the yield and quality of seed oil. More detailed studies are needed, however, to fully elucidate the effect of GA on the formation and quality of seed oil.

Biomass measurements demonstrated that there were some significant differences between the wild

type and the transgenic lines. The fresh to dry weight ratio was higher in the transgenic plants than in the wild-type plant, which might be due to the altered deposition of lignin. The *AtGA2ox8* over-expression lines produced lower lignin content than the wild-type plants. The histochemical staining results also showed that the number of lignified vessels was less in the *AtGA2ox8* over-expression lines, compared to the wild-type controls. The explanations of these results are as follows: GAs stimulate xylem fiber elongation (Eriksson *et al.*, 2000; Israelsson *et al.*, 2005) and lignin accumulation (Biemelt *et al.*, 2004). The *AtGA2ox8* could 2β -hydroxylate the C_{20} -GA precursors and render them unable to be converted to active GAs. The decrease in the levels of active GAs resulted in the decrease in lignin accumulation (Schomburg *et al.*, 2003; Biemelt *et al.*, 2004). Lignin is a complex phenylpropanoid polymer, and lignin monolignols are synthesized through the phenylpropanoid pathway (Boerjan *et al.*, 2003). To elucidate the cause for the effect of GA on lignin accumulation, we analyzed the transcript levels of seven key enzymes in the lignin pathway and found that their transcripts were lower in the apical parts of the stems of the transgenic plants than in those of the wild type.

The results demonstrate that changes in lignification in *AtGA2ox8* over-expression lines might be due to the altered expression of biosynthetic genes. Similar results were obtained in *AtGA2ox1* over-expression tobacco (Biemelt et al., 2004). Thus, our data suggest that GAs might affect plant lignification by regulating transcript levels of lignin biosynthetic genes.

The stems of the transgenic plants with low lignin content are conducive to rational use of resources and environment protection in the paper industry, and also more susceptible to fiber digestion by livestock (Fukushima and Dehority, 2000; Fukushima and Hatfield, 2004). Therefore, one of the major targets for intervention by genetic manipulation in our future work may be to inhibit GA biosynthesis and obtain plants with decreased lignin content. A focus will be placed on the molecular mechanism of gibberellins-regulated lignin biosynthesis.

References

- Agharkar, M., Lomba, P., Altpeter, F., Zhang, H., Kenworthy, K., Lange, T., 2007. Stable expression of *AtGA2ox1* in a low-input turfgrass (*Paspalum notatum* Flugge) reduces bioactive gibberellin levels and improves turf quality under field conditions. *Plant Biotechnology Journal*, **5**(6): 791-801. [doi:10.1111/j.1467-7652.2007.00284.x]
- Alabadi, D., Gil, J., Blazquez, M.A., Garcia-Martinez, J.L., 2004. Gibberellins repress photomorphogenesis in darkness. *Plant Physiology*, **134**(3):1050-1057. [doi:10.1104/pp.103.035451]
- Appleford, N.E.J., Wilkinson, M.D., Ma, Q., Evans, D.J., Stone, M.C., Pearce, S.P., Powers, S.J., Thomas, S.G., Jones, H.D., Phillips, A.L., et al., 2007. Decreased shoot stature and grain α -amylase activity following ectopic expression of a gibberellin 2-oxidase gene in transgenic wheat. *Journal of Experimental Botany*, **58**(12):3213-3226. [doi:10.1093/jxb/erm166]
- Biemelt, S., Tschiersch, H., Sonnewald, U., 2004. Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. *Plant Physiology*, **135**(1):254-265. [doi:10.1104/pp.103.036988]
- Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. *Annual Review of Plant Biology*, **54**(1):519-546. [doi:10.1146/annurev.arplant.54.031902.134938]
- Busov, V.B., Meilan, R., Pearce, D.W., Ma, C.P., Rood, S.B., Strauss, S.H., 2003. Activation tagging of a dominant gibberellin catabolism gene (GA 2-oxidase) from poplar that regulates tree stature. *Plant Physiology*, **132**(3):1283-1291. [doi:10.1104/pp.103.020354]
- Campbell, M.M., Ellis, B.E., 1992. Fungal elicitor-mediated responses in pine cell cultures I. Induction of phenylpropanoid metabolism. *Planta*, **186**(3):409-417. [doi:10.1007/BF00195322]
- Dijkstra, C., Adams, E., Bhattacharya, A., Page, A.F., Anthony, P., Kourmpetli, S., Power, J.B., Lowe, K.C., Thomas, S.G., Hedden, P., et al., 2008. Over-expression of a gibberellin 2-oxidase gene from *Phaseolus coccineus* L. enhances gibberellin inactivation and induces dwarfism in *Solanum* species. *Plant Cell Reports*, **27**(3):463-470. [doi:10.1007/s00299-007-0471-z]
- Eriksson, M.E., Israelsson, M., Olsson, O., Moritz, T., 2000. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnology*, **18**(7):784-788. [doi:10.038/77355]
- Fukushima, R.S., Dehority, B.A., 2000. Feasibility of using lignin isolated from forages by solubilization in acetyl bromide as a standard for lignin analyses. *Journal of Animal Science*, **78**(12):3135-3143.
- Fukushima, R.S., Hatfield, R.D., 2001. Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. *Journal of Agricultural and Food Chemistry*, **49**(7):3133-3139. [doi:10.1021/jf010449r]
- Fukushima, R.S., Hatfield, R.D., 2004. Comparison of the acetyl bromide spectrophotometric method with other analytical lignin methods for determining lignin concentration in forage samples. *Journal of Agricultural and Food Chemistry*, **52**(12):3713-3720. [doi:10.1021/jf035497f]
- Harberd, N.P., King, K.E., Carol, P., Cowling, R.J., Peng, J., Richards, D.E., 1998. Gibberellin: inhibitor of an inhibitor of ...? *Bioessays*, **20**(12):1001-1008. [doi:10.1002/SICI)1521-1878(199812)20:12<1001::AID-BIES6>3.0.CO;2-O]
- Hedden, P., Proebsting, W.M., 1999. Genetic analysis of gibberellin biosynthesis. *Plant Physiology*, **119**(2):365-370. [doi:10.1104/pp.119.2.365]
- Hedden, P., Phillips, A.L., 2000. Gibberellin metabolism: new insights revealed by the genes. *Trends in Plant Science*, **5**(12):523-530. [doi:10.1016/S1360-1385(00)01790-8]
- Helliwell, C.A., Chandler, P.M., Poole, A., Dennis, E.S., Peacock, W.J., 2001. The CYP88A cytochrome P450, *ent*-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. *Proceedings of the National Academy of Sciences of the United States of America*, **98**(4):2065-2070. [doi:10.1073/pnas.041588998]
- Israelsson, M., Sundberg, B., Moritz, T., 2005. Tissue-specific localization of gibberellins and expression of gibberellin biosynthetic and signalling genes in wood-forming tissues in aspen. *The Plant Journal*, **44**(3):494-504. [doi:10.1111/j.1365-313X.2005.02547.x]
- Kende, H., Zeevaart, J.A.D., 1997. The five "classical" plant hormones. *Plant Cell*, **9**(7):1197-1210. [doi:10.1105/tpc.9.7.1197]
- Lee, D.J., Zeevaart, J.A.D., 2002. Differential regulation of RNA levels of gibberellin dioxygenases by photoperiod in spinach. *Plant Physiology*, **130**(4):2085-2094. [doi:10.1104/pp.008581]
- Lee, D.J., Zeevaart, J.A.D., 2005. Molecular cloning of *GA 2-oxidase3* from spinach and its ectopic expression in *Nicotiana glauca*. *Plant Physiology*, **138**(1):243-254. [doi:10.1104/pp.104.056499]
- Lester, D.R., Ross, J.J., Smith, J.J., Elliott, R.C., Reid, J.B., 1999. Gibberellin 2-oxidation and the SLN gene of *Pisum sativum*. *The Plant Journal*, **19**(1):65-73. [doi:10.1046/j.1365-313X.1999.00501.x]

- Lin, C., Yang, H., Guo, H., Mockler, T., Chen, J., Cashmore, A.R., 1998. Enhancement of blue-light sensitivity of Arabidopsis seedlings by a blue light receptor cryptochrome 2. *Proceedings of the National Academy of Sciences of the United States of America*, **95**(5):2686-2690. [doi:10.1073/pnas.95.5.2686]
- Lin, J., Zhou, B., Yang, Y., Mei, J., Zhao, X., Guo, X., Huang, X., Tang, D., Liu, X., 2009. Piercing and vacuum infiltration of the mature embryo: a simplified method for *Agrobacterium*-mediated transformation of indica rice. *Plant Cell Reports*, **28**(7):1065-1074. [doi:10.1007/s00299-009-0706-2]
- Lo, S.F., Yang, S.Y., Chen, K.T., Hsing, Y.I., Zeevaart, J.A., Chen, L.J., Yu, S.M., 2008. A novel class of gibberellin 2-oxidases control semidwarfism, tillering, and root development in rice. *Plant Cell*, **20**(10):2603-2618. [doi:10.1105/tpc.108.060913]
- Martin, D.N., Proebsting, W.M., Hedden, P., 1999. The SLENDER gene of pea encodes a gibberellin 2-oxidase. *Plant Physiology*, **121**(3):775-781. [doi:10.1104/pp.121.3.775]
- Murray, M.G., Thompson, W.F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, **8**(19):4321-4325. [doi:10.1093/nar/8.19.4321]
- Nakaminami, K., Sawada, Y., Suzuki, M., Kenmoku, H., Kawaide, H., Mitsushashi, W., Sassa, T., Inoue, Y., Kamiya, Y., Toyomasu, T., 2003. Deactivation of gibberellin by 2-oxidation during germination of photoblastic lettuce seeds. *Bioscience, Biotechnology, and Biochemistry*, **67**(7):1551-1558. [doi:10.1271/bbb.67.1551]
- Olsen, O., Soerensen, H., 1980. Sinalbin and other glucosinolates in seeds of double low rape species and *Brassica napus* cv. Bronowski. *Journal of Agricultural and Food Chemistry*, **28**(1):43-48. [doi:10.1021/jf60227a023]
- Olszewski, N., Sun, T.P., Gubler, F., 2002. Gibberellin signaling: biosynthesis, catabolism, and response pathways. *Plant Cell*, **14**(Suppl. 1):S61-S80.
- Panford, J.A., de Man, J.M., 1990. Determination of oil content of seeds by NIR: influence of fatty acid composition on wavelength selection. *Journal of the American Oil Chemists' Society*, **67**(8):473-482. [doi:10.1007/BF02540751]
- Rieu, I., Eriksson, S., Powers, S.J., Gong, F., Griffiths, J., Woodley, L., Benlloch, R., Nilsson, O., Thomas, S.G., Hedden, P., et al., 2008. Genetic analysis reveals that C₁₉-GA 2 oxidation is a major gibberellin inactivation pathway in *Arabidopsis*. *Plant Cell*, **20**(9):2420-2436. [doi:10.1105/tpc.108.058818]
- Sakamoto, T., Kobayashi, M., Itoh, H., Tagiri, A., Kayano, T., Tanaka, H., Iwahori, S., Matsuoka, M., 2001. Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. *Plant Physiology*, **125**(3):1508-1516. [doi:10.1104/pp.125.3.1508]
- Sakamoto, T., Miura, K., Ihoh, H., Tatsumi, T., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Agrawal, G.K., Takeda, S., Abe, K., et al., 2004. An overview of gibberellin metabolism enzyme genes and their related mutants in rice. *Plant Physiology*, **134**(4):1642-1653. [doi:10.1104/pp.103.033696]
- Schomburg, F.M., Bizzell, C.M., Lee, D.J., Zeevaart, J.A.D., Amasino, R.M., 2003. Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. *Plant Cell*, **15**(1):151-163. [doi:10.1105/tpc.005975]
- Sivaraman, I., Arumugam, N., Sodhi, Y.S., Gupta, V., Mukhopadhyay, A., Pradhan, A.K., Burma1, P.K., Pental, D., 2004. Development of high oleic and low linoleic acid transgenics in a zero erucic acid *Brassica juncea* L. (Indian mustard) line by antisense suppression of the *fad2* gene. *Molecular Breeding*, **13**(4):365-375. [doi:10.1023/B:MOLB.0000034092.47934.d6]
- Sun, T., Goodman, H.M., Ausubel, F.M., 1992. Cloning the *Arabidopsis* GA1 locus by genomic subtraction. *Plant Cell*, **4**(2):119-128. [doi:10.1105/tpc.4.2.119]
- Sun, T.P., Gubler, F., 2004. Molecular mechanism of gibberellin signaling in plants. *Annual Review of Plant Biology*, **55**(1):197-223. [doi:10.1146/annurev.arplant.55.031903.141753]
- Thomas, S.G., Phillips, A.L., Hedden, P., 1999. Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. *Proceedings of the National Academy of Science of the United States of America*, **96**(8):4698-4703. [doi:10.1073/pnas.96.8.4698]
- Wang, H., Caruso, L.V., Downie, A.B., Perry, S.E., 2004. The embryo MADS domain protein AGAMOUS-Like 15 directly regulates expression of a gene encoding an enzyme involved in gibberellin metabolism. *Plant Cell*, **16**(5):1206-1219. [doi:10.1105/tpc.021261]
- Wang, Q.M., Tu, X.J., Deng, K.Q., Zeng, J.X., Zhao, X.Y., Tang, D.Y., Liu, X.M., 2009. A defect in zinc finger protein double B-box 1a (DBB1a) causes abnormal floral development in *Arabidopsis*. *Journal of Plant Biology*, **52**(6):543-549. [doi:10.1007/s12374-009-9070-6]
- Zhao, X.Y., Yu, X.H., Foo, E., Symons, G.M., Lopez, J., Bendehakkalu, K.T., Xiang, J., Weller, J.L., Liu, X.M., Reid, J.B., et al., 2007a. A study of gibberellin homeostasis and cryptochrome-mediated blue light inhibition of hypocotyl elongation. *Plant Physiology*, **145**(1):106-118. [doi:10.1104/pp.107.099838]
- Zhao, X.Y., Yu, X.H., Liu, X.M., Lin, C.T., 2007b. Light regulation of gibberellins metabolism in seedling development. *Journal of Integrative Plant Biology*, **49**(1):21-27. [doi:10.1111/j.1744-7909.2006.00407.x]

Introducing editorial board member:

Prof. Xuan-ming LIU, the corresponding author of this article, is the academic leader of a research group on plant molecular biology in Hunan University. He gained his PhD in 1995. Shortly after, he obtained a position of Professor in 1999. His study is focused on functional genomics of plant and on the molecular mechanism of phytohormone metabolism. In addition, he is interested in biochemical analysis of plant. In recent years, He dedicates to studying the function of transcription factors and receptor-like kinases (RLKs) in plant development, which was supported by different kinds of foundation, such as National High-Tech R & D Program (863), National Natural Science Foundation, National Science and Technology Major Project, and International Coordination.



Prof. Xuan-ming LIU