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ORIGINAL PAPER

Increased gibberellin contents contribute to accelerated growth and development of transgenic tobacco overexpressing a wheat ubiquitin gene

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Abstract
Key message **Overexpressing *TaUb2* promoted stem growth and resulted in early flowering in transgenic tobacco plants. Ubiquitin are involved in the production, metabolism and proper function of gibberellin.**
Abstract The ubiquitin–26S proteasome system (UPS), in which ubiquitin (Ub) functions as a marker, is a post-translational regulatory system that plays a prominent role in various biological processes. To investigate the impact of different Ub levels on plant growth and development, transgenic tobacco (*Nicotiana tabacum* L.) plants were engineered to express an Ub gene (*TaUb2*) from wheat (*Triticum aestivum* L.) under the control of cauliflower mosaic virus 35S promoter. Transgenic tobacco plants overexpressing *TaUb2* demonstrated an accelerated growth rate at early stage and an early flowering phenotype in development. The preceding expression of MADS-box genes also corresponded to the accelerated developmental phenotypes of the transgenic tobacco plants compared to that of wild-type (WT). Total gibberellin (GA) and active GA contents in transgenic tobacco plants were higher than those in WT at the corresponding developmental stages, and some GA metabolism genes were upregulated. Treatment with GA_3 conferred a similarly accelerated grown rate in WT plants to that of transgenic tobacco plants, while growth was inhibited when transgenic tobacco plants were treated with a GA biosynthesis inhibitor. Thus, the results suggest that Ub are involved in the production, metabolism and proper function of GA, which is important in the regulation of plant growth and development.

Keywords Gibberellin metabolism · Growth and development · Transgenic tobacco · Ubiquitin · Wheat

Abbreviations

ELISA	Enzyme-linked immunosorbent assay
FW	Fresh weight
GA	Gibberellin
Ub	Ubiquitin
UPS	Ubiquitin–26S proteasome system
WT	Wild type

Introduction

Ubiquitin (Ub) is an evolutionarily conserved 76-amino-acid polypeptide (Ciechanover et al. 1980) which is attached to target proteins either for degradation by the multi-subunit 26S proteasome or regulation of their function in a proteasome-independent manner (Mukhopadhyay and Riezman 2007; van Nocker and Vierstra 1993). Ubiquitination is initiated by ubiquitin-activating enzymes (E1), which form a thioester bond with the carboxy-terminal Gly of Ub in an ATP-dependent manner. The activated Ub is then donated to ubtiquitin-conjugating enzyme (E2) by transesterification. In the final step, conjugation of target proteins with Ub is mediated or directly catalyzed by ubiquitin ligases (E3) (Hershko and Ciechanover 1998; Wilkinson 2000; Doherty et al. 2002). It is E3 that confers

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substrate specificity to the ubiquitination process. Ub contains seven lysines that can be used to form poly-Ub chains. This character and E2/E3 complex contribute to Ub chains of various lengths, type and different functional consequences (Smalle and Vierstra 2004).

In the last few decades, there has been a dramatic increase in our knowledge of the significant functions of ubiquitin–26S proteasome system (UPS) in plant development. UPS impacts a wide range of processes, including embryogenesis, hormone signaling and senescence (Dharmasiri et al. 2005; Doelling et al. 2001; Miao et al. 2012). AtUBP14 is a ubiquitin-specific protease, one of a large class of deubiquitylating enzymes (DUBs), which can disassemble the attached multi-ubiquitin chains during or following target degradation by the 26S proteasome. Mutations in the single gene that encodes AtUBP14 cause an embryonic lethal phenotype, with the homozygous embryo arresting at globular stage (Doelling et al. 2001). UPL-5, an E3 ligase containing a HECT domain, regulates leaf senescence in *Arabidoposis* through the degradation of WRKY53, thus ensuring that senescence occurs at the correct time (Miao et al. 2012). Overexpression of *CaPUB1* in *Arabidopsis*, which encodes a hot pepper U-box E3, results in a rapid growth rate and early bolting phenotype (Cho et al. 2006).

It was well-known that MADS-box genes were involved in plant growth and development. MADS-box genes contain floral meristem or floral organ-specific genes and nonfloral MADS-box genes, and these genes play roles in regulating gene expression that leads to diverse events during plant growth and development (Wu et al. 2000). Ectopic expression of *NtMADS-4*, whose transcript was detectable only in reproductive organs, caused phenotypes of extremely early flowering as well as dwarfism. In addition, its interacting partner, *NtMADS-11*′s transcript can be seen in both reproductive and vegetative organs (Jang et al. 2002). *NtMADS-11* and *NtMADS-4* have been proved that they can be used as developmental marker genes (Gallego-Giraldo et al. 2007). However, the involvement of MADS-box genes in UPS-regulated plant growth and development is unclear.

UPS plays a prominent regulatory role in hormone biology (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Melotto et al. 2008). In particular, E3 actively participates in hormone perception and response. For instance, through the action of SCF^{TIR1}, one multi-subunit Ub ligase, auxin de-represses transcription by promoting ubiquitination of Aux/IAA proteins, thus targeting them for degradation by the 26S proteasome (Kepinski and Leyser 2005; Ruegger et al. 1998; Gray et al. 2001). Gibberellins (GAs) are tetracyclic diterpenoid phytohormones that regulate diverse aspects of plant growth and development, including seed germination, stem elongation, leaf expansion, flowering and flower development (Hooley 1994; Thomas et al. 2005). GA responses are repressed, in the case of GA signaling, by DELLA proteins (named for a conserved DELLA amino acid motif). $SCF^{SLY/GID2}$ directly interacts with DELLAs, leading to ubiquitination and degradation of the DELLA repressor, thus promoting GA-mediated transcription (McGinnis et al. 2003; Sasaki et al. 2003).

Although previous studies have proved that UPS significantly contributes to growth and development in plants, most of them focused on E3 and DUBs. The role of Ub in plant development remains elusive. Here, we demonstrate that overexpression of wheat *TaUb2* in tobacco plants resulted in accelerated growth rate during the seedling stage and early flowering phenotype in the late development stage. The expression of MADS-box genes was changed in transgenic plants. We also found that the total GA and active GA contents and transcript level of some GA metabolism genes were altered in transgenic plants. A possible mechanism is proposed in which Ub may alter plant growth and developmental patterns by regulating GA metabolism.

Materials and methods

Plant materials

The transgenic tobacco plants were produced as previously described by Guo et al. (2008). Tobacco (*Nicotiana tabacum* L.) plants were grown in a chamber at 25 °C with a 16/8 h light/dark cycle (300–400 μmol photons m^{-2} s^{-1}) and a relative humidity of 75–80 %.

To detect the effect of overexpression of *TaUb2* on plant growth, two independent transgenic lines (T-2 and T-11) were selected randomly for analysis. WT and transgenic plants were cultured under standard conditions as above. Plant heights of the above ground portion and leaf numbers were analyzed statistically.

Confirmation of transgenic tobacco transformants by PCR

To confirm putative transformants, genomic DNA was isolated from leaves of putative transgenic and WT plants using the CTAB method (Dellaporta et al. 1983). PCR was performed using a set of primers with forward: 5′-GATA TCTCCACTGACGTAAG-3′ and reverse: 5′-CAAGAC CTGTGAACCCAACC-3′. Forward and reverse primers were specific to CaMV 35S promoter and *TaUb2* cDNA, respectively. PCR conditions were as follows: an initial denaturation at 94 °C for 5 min; 35 cycles of 50 s at 94 °C, 50 s at 58 °C and 50 s at 72 °C; a final extension at 72 °C for 10 min. PCR products were separated electrophoretically on a 1 % agarose gel.

Detecting TaUb2 mRNA levels with semi-quantitative RT-PCR

Plant tissue samples were stored at −80 °C until used for RNA isolation. Total RNA was isolated from tobacco leaves using Trizol reagent (INVITROGEN) according to the manufacturer's protocol and treated with DNaseI (RNase-free; Promega) to remove genomic DNA. Reverse transcription was performed using the primer oligo $(dT)_{15}$ and Moloney murine leukemia virus reverse transcriptase (Promega) at 42 °C for 60 min. The amplification conditions were as follows: 1 min at 94 °C, 45 s at 55 °C, and 1 min at 72 °C. The cycle was repeated 28 times. The *TaUb2* gene-specific primers prepared from the 3′ non-coding region, namely, TaUb2-F (5′-CAATGTGAA GGCGAAAATCC-3′) and TaUb2-R (5′-CAAGACCTGT GAACCCAACC-3′) were used for *TaUb2* amplification. To screen transgenic tobacco plants, the tobacco actin transcript was used as an internal positive control for equal cDNA amounts. PCR was carried out using the primers NtACT2a (5′-CTATTCTCCGCTTTGGACTTGGCA-3′) and NtACT2b (5′-ACCTG CTGGAAGGTGCTGAGGGA A-3′) (Yang et al. 2006). The experiments were independently repeated three times under identical conditions.

Analyses of Ub protein levels by SDS-PAGE and immunological blotting

Total protein was extracted from the apical leaves of tobacco plants. Protein content was determined by the dye-binding assay according to Bradford (1976). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Saint-Quentin, France). Ub proteins were routinely detected with the Ub antibody (Sigma) by immunological blot. Ub monomer (Sigma) was used as a positive control.

Quantification of total gibberellin contents

Expanding apical leaves and stems of tobacco plants were harvested for total GA assays. The extraction and purification of the total GA were performed as previously described (Kusaba et al. 1998; Vidal et al. 2001), with some modifications. Tobacco leaves after freeze drying (0.5 ± 0.01 g, dry weight) were homogenized in cold 100 % acetonitrile extraction medium containing 30 μg/mL sodium diethyldithiocarbamate as an antioxidant. The homogenate was further extracted in the dark at 4 °C for 16 h. The extracts were centrifuged at 5,000*g* (4 °C) for 10 min, and the supernatant dried under low pressure and re-dissolved in 8.0 mL 0.4 mol/L phosphate buffer (pH 8.0). Then, 4.0 mL trichloromethane was added to remove pigment and 150 mg polyvinylpolypyrrolidone (PVPP) was used to remove hydroxybenzene. The aqueous phase after extraction was extracted by two ethyl acetate treatments. The ethyl acetate phase was dried under low pressure and re-dissolved in 1.0 mL mobile phase buffer. Finally, the hormone fraction was purified through microfiltration and subjected to high-performance liquid chromatography (HPLC) analysis.

Analyses of total GA were carried out using a HPLC system according to previously described methods (Tanaka-Ueguchi et al. 1998; Gallego-Giraldo et al. 2008), with modifications. LC-10ATvp separation module equipped with SPD-10Avp detector (SHIMADZU) was applied for determining hormone. An aliquot (15 μL) of the filtered samples was injected into a Waters symmetry C_{18} column (4.6 mm × 150 mm, 5 μm) with isocratic elution at a flow rate of 0.8 mL/min at 25 °C, using methanol and double distilled water (0.5 % acetic acid) as mobile phase (45:55). Detection of different hormones was carried out at 254 nm by SPD-10Avp detector (SHIMADZU) and co-chromatography with authentic standards (Sigma). The retention times and wavelengths of characteristic absorbances of hormones were determined with authentic standard compounds, and samples were identified by comparing the eluting peaks to these authentic standard compounds. Routine sample calculations were based on the comparison of peak areas with external standard peak areas using Shimadzu Class-VP software. All values were corrected against internal standards with known concentrations of hormone.

Active gibberellin extraction, purification and quantification

The methods for extraction and purification of GA_3 and GA_4 were as described previously (Yang et al. 2001). Apical leaves and stems of tobacco plants were ground in an ice-cooled mortar in 4 mL 80 % (v/v) aqueous methanol containing 1 mM butylated hydroxytoluence as an antioxidant. The extract was incubated at 4 °C for 4 h and centrifuged at 3,500*g* (4 °C) for 8 min. The supernatant was passed through C18 Sep-Pak cartridges (Waters) prewashed with 10 mL 100 % (v/v) and 5 mL (v/v) methanol, respectively. The hormone fractions eluted with 10 mL 100 % (v/v) methanol and 5 mL ether from the columns were dried by evaporation with N_2 and dissolved in 2 mL phosphate buffer saline (PBS) containing 0.1 % (v/v) Tween 20 and 0.1 % (v/v) gelatin (pH 7.5) for analysis by an enzyme-linked immunosorbent assay (ELISA).

GA_3 and GA_4 were determined in indirect competitive ELISA following methods described in previous publications (Yang et al. 2001; Zhu et al. 2005). The GA_3 and GA_4 standard, monoclonal antibodies against GA_3 and GA_4, 96-well microtitration plate coated with synthetic GA_3 and

GA_4, and IgG-horseradish peroxidase used in ELISA were purchased from the Phytohormones Research Institute (China Agricultural University). Standard GA_3 and GA_4, samples and antibodies were added and incubated for 30 min at 37 °C. After washing four times with PBS + Tween 20 (0.1 % [v/v]) buffer (pH 7.4), IgG-horseradish peroxidase was added to each well and incubated for 30 min at 37 °C. After washing as above, 100 μL color-appearing solution containing 1.5 mg/mL ortho-phenylenediamino (OPD) substrate and 0.008 % (v/v) H_2O_2 was added to each well. The reaction progress was stopped by adding of 50 μL 2 M H_2SO_4 per well when the 200 ng/mL GA_3 and 50 ng/mL GA_4 standard had a pale color, and the 0 ng/mL standard had a deep color in the wells. Color development in each well was detected using an ELISA Reader (Thermo Multiskan MK3) at optical density *A*490. GA_3 and GA_4 contents were calculated following Weiler et al. (1981).

PCR primer design and real-time RT-PCR analysis

Total RNA extracted from apical leaves and apical stems was treated with DNaseI (RNase-free; Promega) to remove genomic DNA. Reverse transcription was performed using the primer oligo $(dT)_{15}$ and Moloney murine leukemia virus reverse transcriptase (Promega) at 42 °C for 60 min.

The cDNA reaction was diluted to 100 μL with sterile water. Real-time PCR was performed using the first-strand cDNA generated above, and selected primer sets listed in Table 1 were either described previously (Gallego-Giraldo et al. 2008) or designed using the Primer Premier 5.0 software. The primer concentration for real-time PCR was optimized for each primer gene pair, and the best primer concentration was determined by calculating PCR efficiency and CT values. Gene transcription levels of *NtMADS-11* (AF385746), *NtMADS-4* (AF068723), *Nt20ox1* (Ntc12, AB012856.1), *Nt20ox2* (NTc16, AB016084), *Nt3ox1* (Nty, AB032198), *NtGA2ox3* (EF471117) were analyzed by real-time PCR. *NtActin* (U60489) was amplified as a loading control. The analyses were carried out using two biological replicates of apical shoots and apical young leaves at different stages of development. Each sample consisted of material from five plants.

All PCRs were performed in triplicate and a template-free control was included for gene amplification. PCRs were performed in an optical 96-well plate with Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad), using SYBR®Green to monitor double-stranded DNA synthesis. The reactions contained 9 μL of 2.5× RealMasterMix/20× SYBR solution (Tiangen, China), the optimal concentration of primers for each gene, RNase-free water and 1 μL of cDNA solution, in a final volume of 20 μL. The thermal profile conditions for all PCRs were: 95 °C for 3 min; followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s, and 68 °C for 30 s. A melting curve program was used to confirm the presence of only one amplicon according to the Tm expected for each gene amplification. Data were analyzed using the CFX Manager Software, version 1.6 (Bio-Rad) and relative transcript expression levels of each target were normalized with respect to the tobacco actin gene.

Treatment of plants with GA_3 or GA biosynthesis inhibitor

The sensitivity of plants to GA_3 or paclobutrazol (the GA biosynthesis inhibitor) was tested by irrigation and spray with 10^{-5} M corresponding solutions. Treatment was started 40 days after sowing, and repeated every 5 days.

Statistical analysis

The data were presented as the mean ± standard error (SE) of three independent experiments. Statistical analysis was conducted using the procedures of data processing system (DPS; Zhejiang University, China). Significant differences were detected using the two-way analysis of variance. The

Table 1 Primers pair used in a real-time RT-PCR analysis

Gene	Accession no.[a]	Amplicon (bp)	Sequence	
			Forward	Reverse
NtGA20ox1	AB012856.1	106	5′-TGTAGCACGAGAACTTCC-3′	5′-ACGGCATGCTTCACCAACA-3′
NtGA20ox2	AB016084	146	5′-TGAGGTTCCTTCTTCACAGCA-3′	5′-CTCCCCTAAAAGCTCCATTACC-3′
NtGA3ox1	AB032198	76	5′-CGGCTTTGT CCCCTCTA-3′	5′-CTTATCGAGT TTAGCCAACTTGCA-3′
NtGA2ox3	EF471117	94	5′-AAGTCCCACCTTGTTACTTG-3′	5′-GCTATCTATAGGAAATCCAATG-3′
NtMADS-11	AF385746	160	5′-TGGGAGCAACAGAGC-3′	5′-ATGGCGGCATTACAG-3′
NtMADS-4	AF068723	249	5′-CGTCAACTCCCTCCACAAA-3′	5′-AATGCGGATTAGCCACAGC-3′
NtActin	U60489	69	5′-CATTGGCGCTGAGAGATTCC-3′	5′-GCAGCTTCCATTCCGATCA-3′

[a] Sequence identifiers are indicated from GenBank

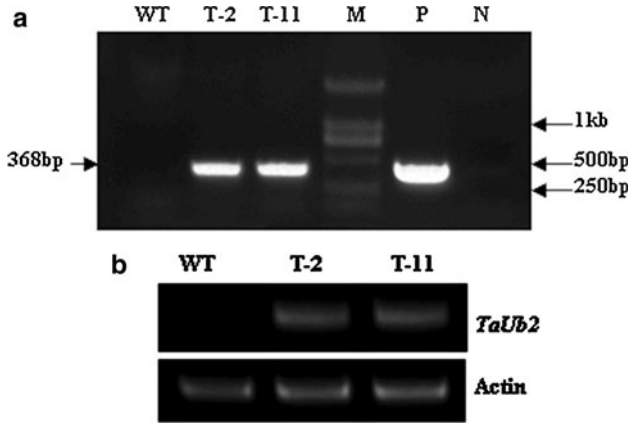

Fig. 1 Molecular identification of transgenic tobacco lines. **a** Genomic PCR with forward primer and reverse primer specific to the CaMV 35S promoter and *TaUb2* cDNA, respectively. The expected fragment was 368 bp in size. *WT* DNA from wild-type tobacco, *T-2*, *T-11* DNA from independent transgenic tobacco lines, *M* DNA marker, *P* recombinant pBI121 vector was used as a positive control, *N* negative control (double-distilled water). **b** *TaUb2* expression level in wild-type (WT) and transgenic tobacco plants (T-2, T-11). Total RNAs from leaves of WT, T-2 and T-11 plants were isolated and subjected to RT-PCR using gene-specific primers and actin as an internal controls

means were compared using Duncan's multiple range tests at $P < 0.05$.

Results

Overexpressing TaUb2 accelerated growth and development of transgenic tobacco

The transgenic tobacco plants were produced by Guo et al. (2008) in our laboratory as previously described. Several independently transformed lines were randomly chosen, and two homozygous T2 lines were selected for investigation in this study. Genomic PCR (Fig. 1a) and *TaUb2* expression level PCR (Fig. 1b) were used to identify the transgenic tobacco plants.

We compared the phenotype of transgenic and WT tobacco plants. As shown in Fig. 2, transgenic tobacco plants demonstrated obvious alterations in growth and developmental patterns compared to WT. These changes were characterized by faster growth during the early stage, earlier bolting and flowering at middle stage and earlier senescence at late stage than in WT (Fig. 2a–d). However, they exhibited more slender stems (data not shown) and smaller mature leaves than WT (Fig. 2e).

The leaf numbers and plant height were also investigated during growth and development. As shown in Fig. 2f and g, the transgenic plant grew faster than WT at early and middle stage, indicated by more leaves and higher plant height in T-2 and T-11 than WT, concomitant with early anthesis because of the precocious growth and development pattern (Fig. 2f, g). The anthesis of the transgenic tobacco plants was about 30 days earlier than that of WT. These results suggest that constitutive overexpression of wheat *TaUb2* altered the course of growth and development, resulting in a shorter life cycle in transgenic plants than that of WT.

The effects of overexpressing TaUb2 on expression of MADS-box genes

To confirm the change in the developmental course of transgenic tobacco plants and the involvement of MADS-box gene in UPS-regulated plant growth and development, we tested the expression levels of some MADS-box genes by real-time quantitative RT-PCR.

NtMADS-4 was only expressed in the apical stem of 150-day-old plants, and its expression level was significantly higher in transgenic plants than WT (Fig. 3f), but no expression was observed in plants of other ages. *NtMADS-11* expressed in all the samples detected. When we compared the expression levels of *NtMADS-11* and *NtMADS-4* in transgenic tobacco plants and WT plants of the same age, we found that expression was significantly higher in transgenic plants (Fig. 3a, c, e, f). Whereas, when we compared expression between transgenic tobacco plants and WT of the similar developmental stages, that is, a 90-day-old transgenic plant versus 120-day-old WT plant; a 120-day-old transgenic tobacco plants versus a 150-day-old WT (transgenic tobacco plants appear to develop about 30 days earlier than WT, according to Fig. 2f, g), almost the same expression level was observed (Fig. 3b, d). These results showed again that overexpression of wheat *TaUb2* accelerated growth and developmental patterns in transgenic tobacco plants, suggesting that the floral transition was about 30 days earlier in transgenic tobacco plants than WT.

The effects of overexpressing Ub on GA levels in apical leaves and apical stems of transgenic plants

Plant hormones are closely related to growth and development of the plant. To investigate the mechanism underlying the altered growth and developmental pattern in *TaUb2*-overexpressing tobacco plants, we analyzed the changes in the levels of gibberellin (GA). Figure 4 shows total GA content in apical leaves and apical stems of the transgenic and WT plants at different stages during development. The results, shown in Fig. 4, indicated that total GA content in apical leaves is higher than that found in WT in 60-day-old, 90-day-old and 120-day-old transgenic plants. Similarly, the GA content in the apical stems of 120-day-old transgenic tobacco plants was also higher than that of WT. Furthermore, it was also higher than

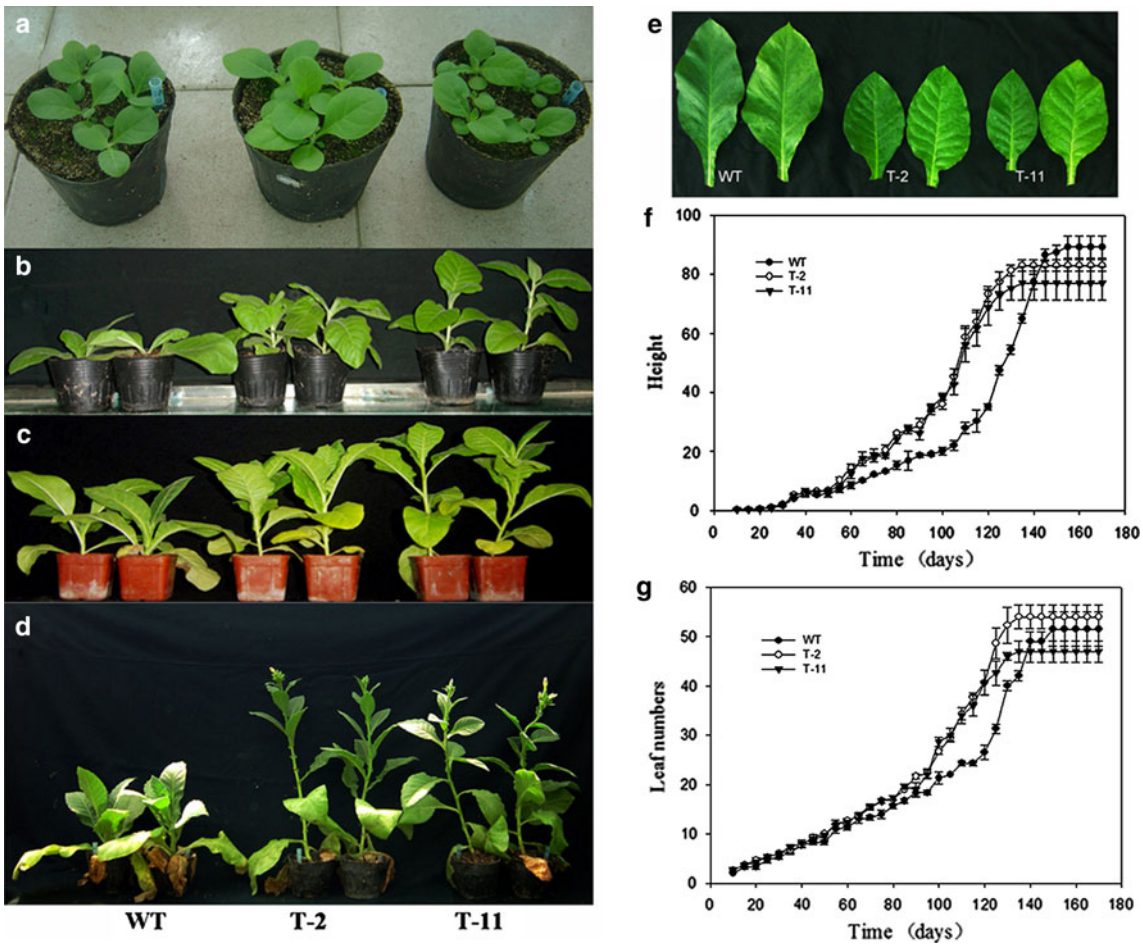

Fig. 2 Phenotype and growth curves of transgenic (*T-2*, *T-11*) and wild-type (*WT*) tobacco plants. Phenotype of plants at different developmental stages is shown. Photos were taken at 30 (**a**), 60 (**b**), 90 (**c**) and 120 days (**d**) after sowing. Overexpression of *TaUb2* resulted in accelerated growth and development at early stage (**a**–**c**), earlier bolting and flowering (**b**–**d**), and smaller mature leaves (**e**) compared to WT. Shoot height (**f**) and leaf numbers (**g**) of WT and transgenic tobacco plants were investigated during growth and development. The anthesis of transgenic and WT tobacco plants are day 120 and 150, respectively. The values are the average of 10 plants ± SE

150-day-old WT plants, which are at a similar developmental stage to 120-day-old transgenic tobacco plants (there was no apical stem in 90-day-old WT).

It may be unconvincing of the role of increase in total GA content since total GA contain GA precursors, active GAs, and GA catabolites. So we detected the active GA levels (GA_3 and GA_4) in apical leaves and apical stems of tobacco plants at different stages using ELISA. The specificity of the monoclonal antibodies and the other possible nonspecific immunoreactive interference have been checked (Yang et al. 2001) and proved reliable. As shown in Fig. 5, GA_3 and GA_4 contents in apical leaves and apical stems were significantly higher in transgenic tobacco plants compared to WT tobacco plants of the same age or similar developmental age. These results suggest that the increased active GA levels contribute to the accelerated growth in tobacco plants overexpressing *TaUb2*.

The effects of GA or paclobutrazol application on the growth and bolting of WT and transgenic tobacco plants

Furthermore, exogenous GA or paclobutrazol (GA biosynthesis inhibitor) was applied to 40-day-old tobacco seedlings. As shown in Fig. 6, the addition of paclobutrazol reduced the height of WT and transgenic tobacco plants. However, when treated with 10^{-5} M GA_3, WT exhibited an earlier bolting pattern, similar to that of transgenic plants (Fig. 6a, b, d), while transgenic tobacco plants under treatment with 10^{-5} M GA_3 were also higher than WT tobacco plants (Fig. 6e). In contrast, exogenous paclobutrazol inhibited the growth and resulted in a similar pattern in transgenic and WT tobacco plants (Fig. 6c, f). This result correlates with that in Figs. 4 and 5, which suggest the involvement of GA in the changes of growth and bolting in transgenic tobacco plants.

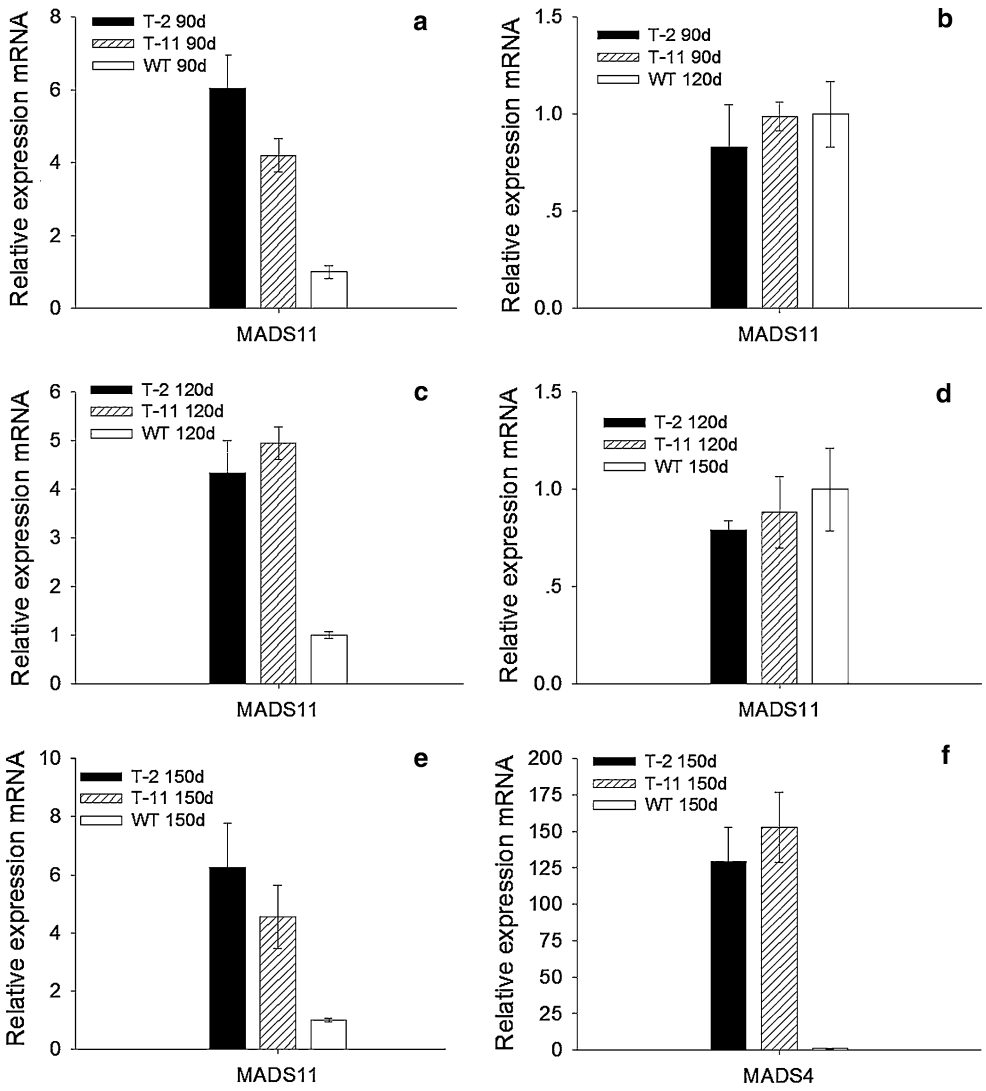

Fig. 3 Transcript levels of *NtMADS-11* and *NtMADS-4* in apical leaves and apical stems of transgenic (T-2, T-11) and wild-type (WT) tobacco plants. **a** Apical leaves, **b–f** apical stems. Gene expression was quantified by real-time RT-PCR. Relative expression of mRNA was normalized relative to the NtActin gene and the transgenic values reflect fold change expression compared to wild type. Results are means of two biological replicates SE. Each PCR was run three times. *d* Days old

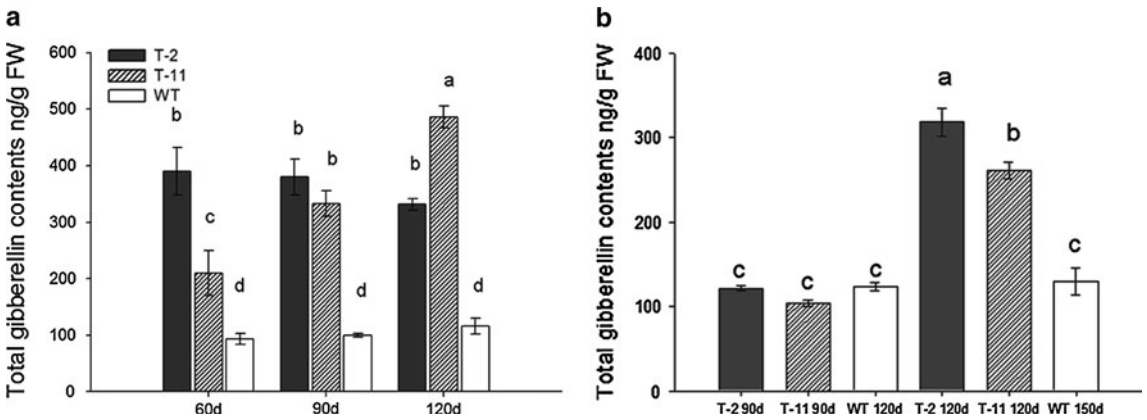

Fig. 4 Total gibberellin contents in apical leaves and apical stems of transgenic (T-2, T-11) and wild-type (WT) tobacco plants at different ages. **a** The leaf samples were from 30-, 60-, 90- and 120-day-old tobacco plants. **b** Total gibberellin contents in apical stems are presented here. Data represent the mean ± SE of three independent experiments. *Bars* with the *same letter* were not significantly different at $P < 0.05$. *d* Days old

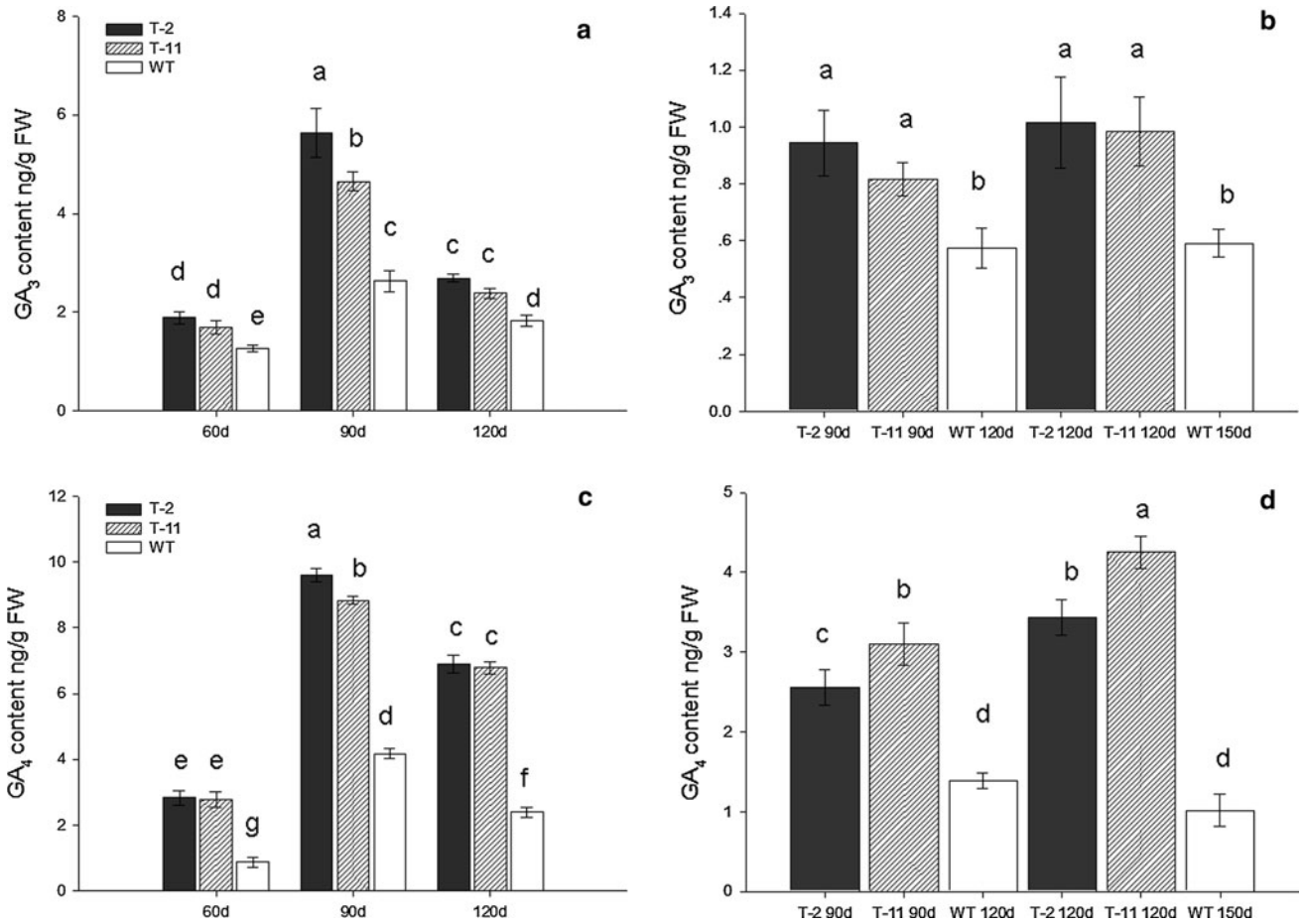

Fig. 5 Active gibberellin contents in apical stems of transgenic (T-2, T-11) and wild-type (WT) tobacco plants at different ages. GA_3 (**a**) and GA_4 (**c**) in apical leaves and GA_3 (**b**) and GA_4 (**d**) in apical stems are presented here. Data represent the mean ± SE of three independent experiments. *Bars* with *the same letter* were not significantly different at $P < 0.05$. *d* Days old

Effects of overexpressing Ub on the expression of GA metabolism genes in apical leaves and stems of transgenic plants

Considering that both total GA and active GA were enhanced in transgenic tobacco plants, we then focused on GA metabolism. To investigate whether overexpression of *TaUb2* modifies GA metabolism, and resulted in the changes of active GA contents in apical leaves and stems, we evaluated the accumulation of the mRNA of GA metabolism genes, including GA 20-oxidases (*NtGA20ox1* and -2, GA biosynthesis genes), GA 3β-hydroxylase (*NtGA3ox1*, GA biosynthesis genes) and GA 2-oxidase (*NtGA2ox3*, GA catabolism genes) in WT and transgenic tobacco plants by real-time quantitative RT-PCR. As shown in Fig. 7a, c and e, GA metabolism genes were modified in the transgenic plants compared to WT plants of the same age (90-, 120- and 150-day-old plants). This was characterized by an increase in *NtGA2ox3* expression. This increase is caused by the response to the elevated active GA (Fig. 5) in transgenic tobacco plants. And also, the varied developmental stages may be responsible for these changes in expression levels (Fig. 3; Gallego-Giraldo et al. 2007).

Thus, we compared their expression in transgenic and WT tobacco plants at similar developmental stages. The results in Fig. 7b and d show that the expression levels of these GA metabolism genes were higher in transgenic tobacco plants than WT. The increase in *NtGA2ox3* expression results from the response to the active GA of higher level in transgenic tobacco plants. The higher transcript levels of *NtGA20ox1*, *NtGA20ox2*, *and NtGA3ox1* provide more active GA in transgenic tobacco plants compared to WT, which mainly leads to the faster growth of *TaUb2*-overexpressing tobacco plants compared to the wild-type tobacco plants at the similar developmental stages.

By integrating all the results shown in Fig. 7, we can conclude the following: when tobacco plants started bolting, transcript levels of *NtGA20ox1*, *NtGA20ox2*, *NtGA3ox1* and *NtGA2ox3* were higher in transgenic

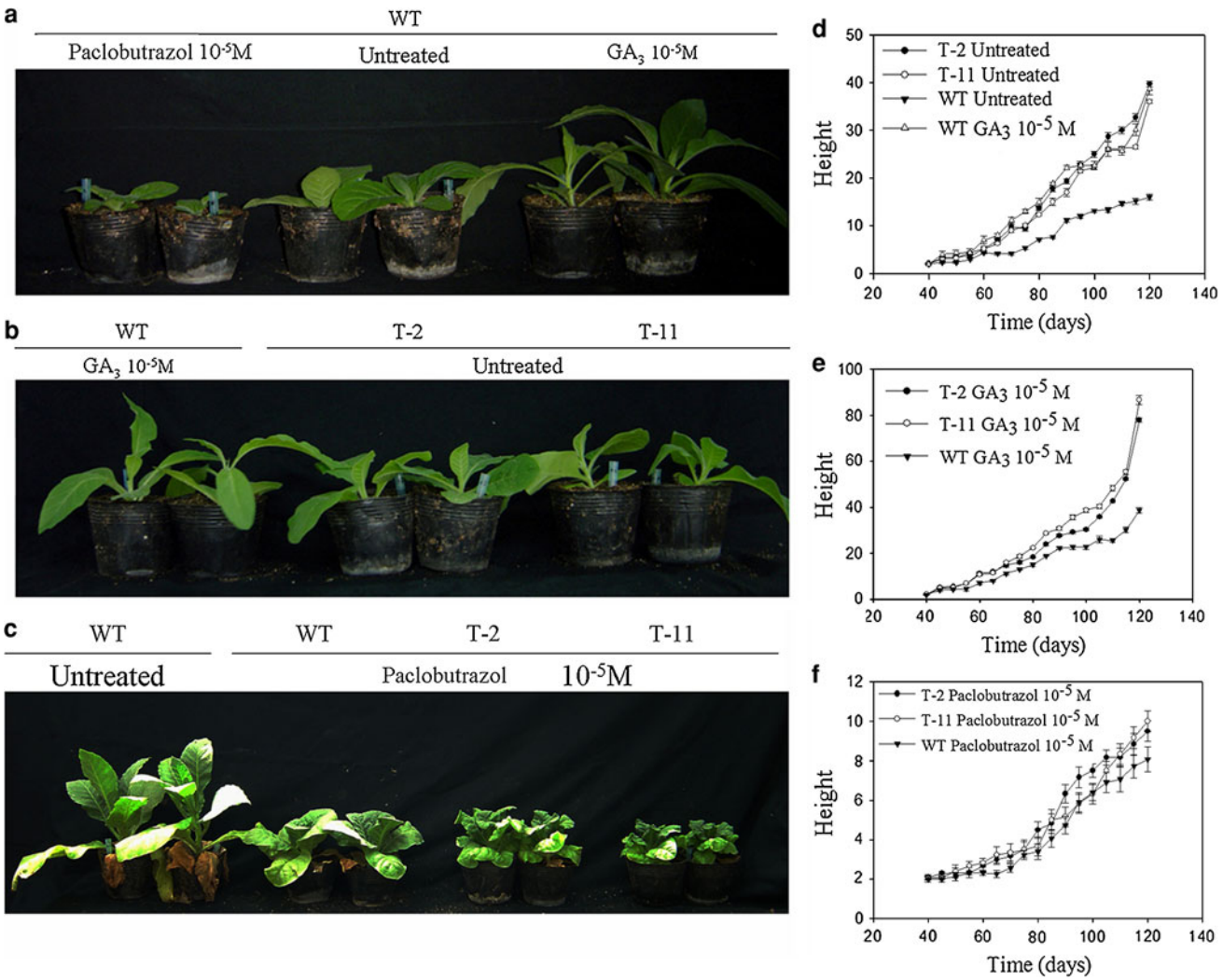

Fig. 6 The effects of exogenous GA or paclobutrazol on the growth and bolting of transgenic (T-2, T-11) and wild-type (WT) tobacco seedlings. Plants were treated or untreated with GA_3 (10^{-5} M), paclobutrazol (10^{-5} M), by irrigation and spray every 5 days. Treatment was started 40 days after sowing. Plants were evaluated at 25 days (**a**, **b**) and 80 days (**c**) after treatments. Growth curves of tobacco plants after treatments with GA_3 (10^{-5} M) (**d**, **e**) and paclobutrazol (10^{-5} M) (**f**) are presented here

tobacco plants; during flowering, tobacco stems stopped elongating, but expression levels of *NtGA20ox1*, *NtGA20ox2*, *NtGA3ox1* and *NtGA2ox3* were still higher in transgenic plants compared to WT. Thus, at the same developmental stages, transgenic tobacco plants exhibited accelerated GA metabolism rates compared to WT (Fig. 7b, d). The higher total GA content (Fig. 4) and enhanced GA metabolism (Fig. 7) provides more bioactive GA (Fig. 5), which results in accelerated bolting and early flowering in transgenic tobacco plants.

Changes in Ub content in tobacco leaves during flowering

Previous studies have showed that UPS plays a prominent role in flower development (Jang et al. 2008). In order to determine the involvement of Ub in flowering, the apical leaves of WT tobacco plants were selected at different developmental stages to detect the level and different forms of Ub protein present (including conjugated Ub, multiubiquitin and Ub moiety), using protein immunoblot analysis. As shown in Fig. 8, the conjugated Ub increased slightly during flowering, while Ub monomer content decreased during this process, indicating the possible involvement of Ub in tobacco flowering. The Ub protein content of transgenic tobacco plants has already reported by Zhang et al. (2011), and they found that transgenic tobacco plants overexpressing *TaUb2* exhibited a slight increase in free Ub (8.6 kDa), and higher levels of Ub–protein conjugates than WT. These results proved that changes in Ub levels are involved in tobacco flowering.

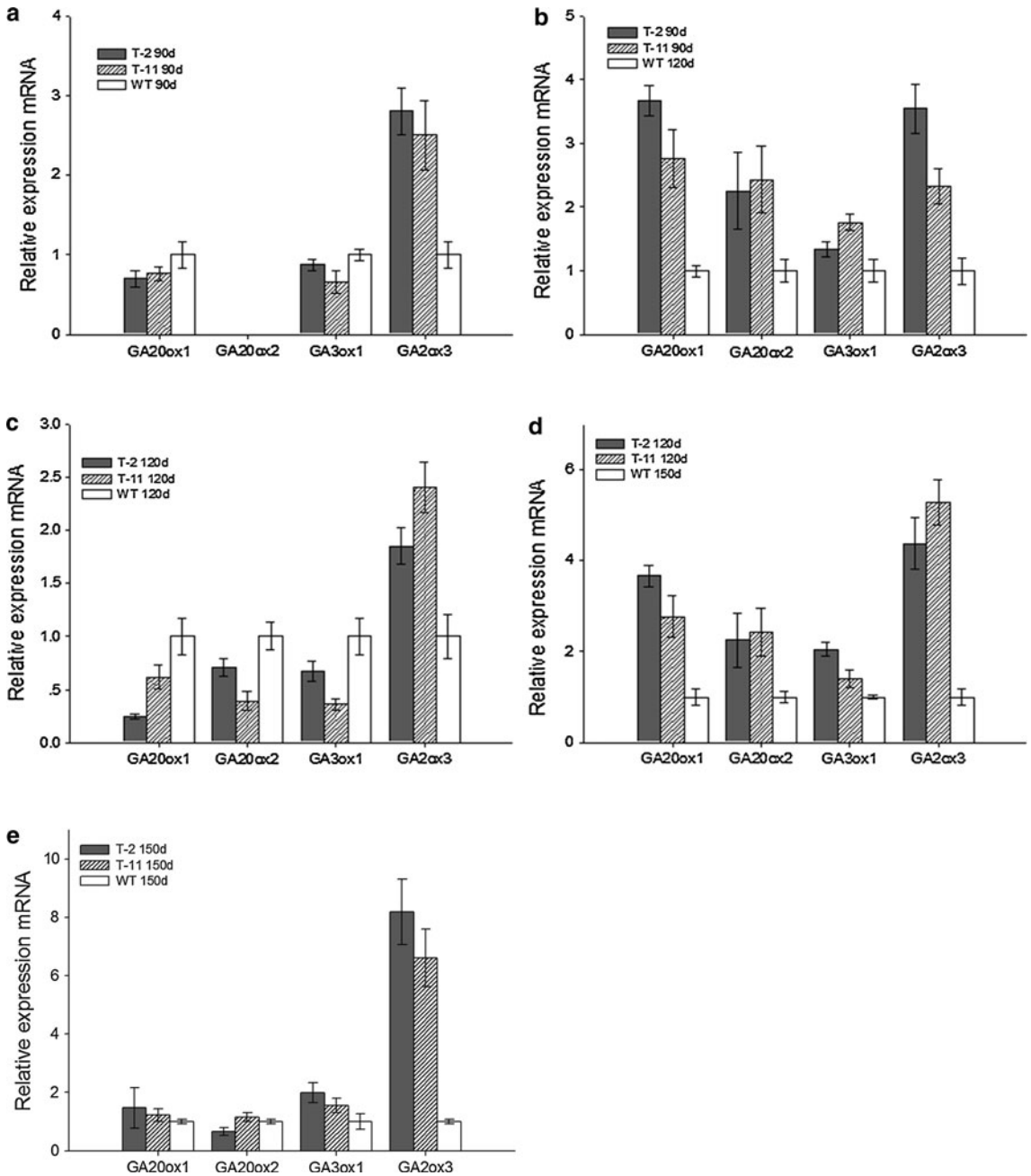

Fig. 7 Transcript levels of gibberellin metabolism related genes in leaves and apical stems of transgenic (T-2, T-11) and wild-type (WT) tobacco plants. **a** Apical leaves, **b**–**e** apical stems. **a**, **c**, **e** Comparison between transgenic and WT tobacco plants at the same age, **b**, **d** comparison between transgenic and WT tobacco plants at the similar developmental stage according to Fig. 3. Gene expression was quantified by real-time RT-PCR. *NtGA20ox2* was not detected in leaves. Relative expression of mRNA was normalized relative to the *NtActin* gene and the transgenic values reflect fold change expression compared to wild type. Results are means of two biological replicates SE. Each PCR was performed three times. *d* Days old

Discussion

Overexpression of wheat TaUb2 gene promoted stem growth and floral transition of transgenic tobacco plants

Our results demonstrate that constitutive overexpression of *TaUb2* results in accelerated growth rate (especially stem growth) at early stage and promoted early flowering phenotype, though transgenic tobacco plants exhibited a slightly lower final height than WT tobacco plants (Fig. 2). The height of the transgenic plants was higher than that of WT during growth stage (60–140 days), and the anthesis of the transgenic tobacco plants was about 30 days earlier than in WT plants. However, we observed smaller leaves

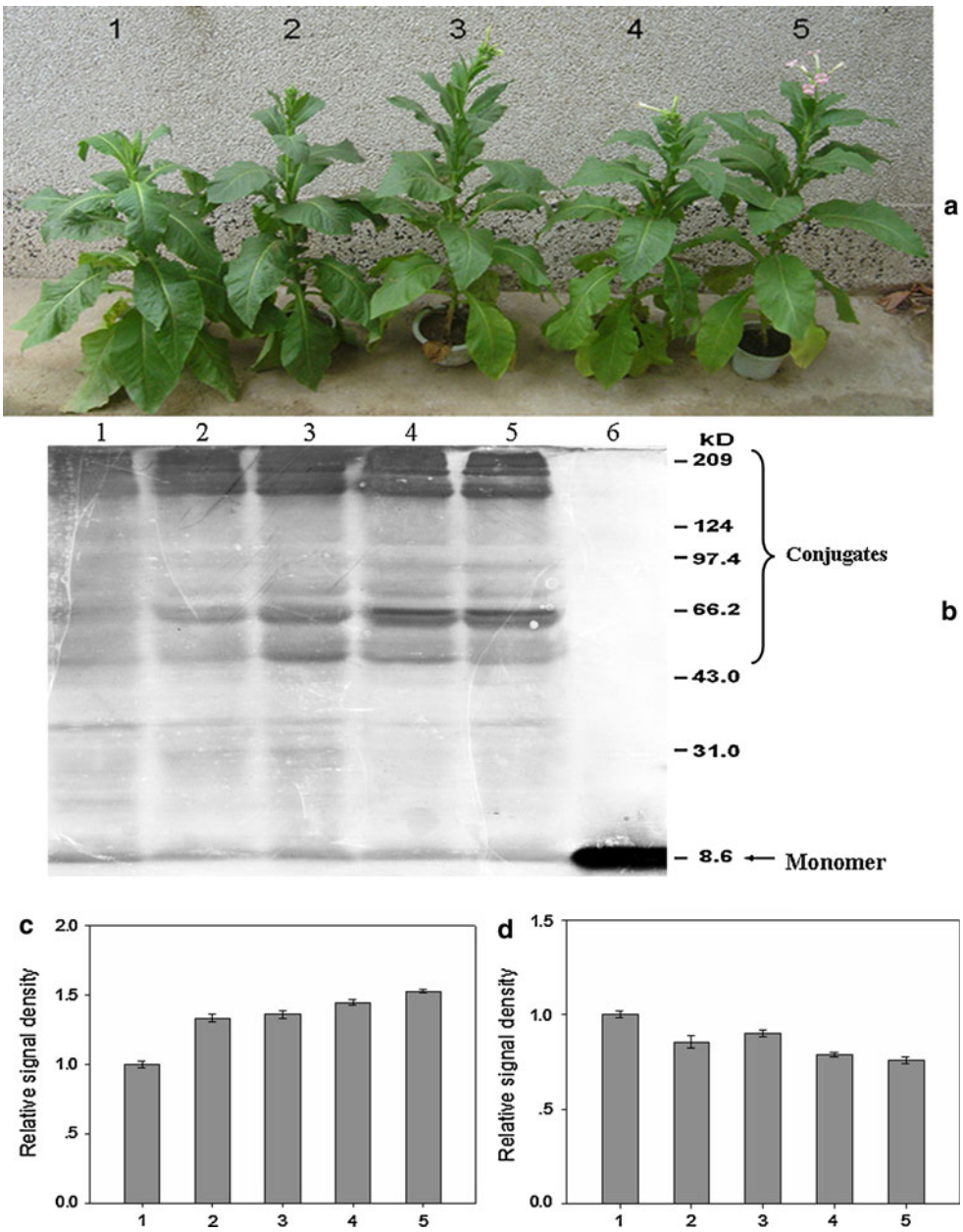

Fig. 8 The changes of Ub protein contents in tobacco apical leaves during development. **a** WT tobacco plants at different ages during floral transition, **b** Ub protein contents. *1–5* the Ub levels at the different ages coincide with **a** *6* Ub monomer marker, **c** relative signal density of Ub conjugates, **d** relative signal density of free Ub (8.6 kDa)

and more slender stems in transgenic plants compared to WT.

MADS-box genes encode regulatory factors that are involved in plant development at various stages (Jang et al. 2002). *NtMADS-11* and *NtMADS-4* can be used as developmental markers (Gallego-Giraldo et al. 2007). The transcripts of these two genes were upregulated in *TaUb2*-overexpressing tobacco plants, compared to WT of the same age (Fig. 3). However, when comparing the expression of *NtMADS-11* at a similar developmental stage (in the transgenic tobacco plants that appears to be about 30 days earlier than in WT plants, according to Fig. 2f, g), the obvious difference in gene expression levels disappeared (Fig. 3b, d). These results suggest that Ub plays a significant role in plant growth and development. The changes in Ub protein shown in Fig. 8 also suggest Ub is involved in floral transition.

Differences in GA production and metabolism are responsible for the modified growth and development phenotype in transgenic tobacco plants

Biologically active gibberellins (bioactive GAs) control diverse aspects of plant growth and development, including leaf expansion, stem elongation and flower development (Fleet and Sun 2005; Yamaguchi 2008). In spinach (*Spinacia oleracea* L.), bolting in response to long days is associated with increased GA levels (Gilmour et al. 1986;

Wu et al. 1996). In this study, the earlier bolting in transgenic tobacco plants was the typical phenotype and may be responsible for the accelerated growth pattern (Fig. 2). Figures 4 and 5 show the difference in total GA content and active GA in leaves and stems of plants with different Ub levels, at specific stages. Treatment with GA or GA biosynthesis inhibitors (Fig. 6) confirmed the contribution of GA to the altered development patterns of tobacco seedlings. Thus, our results suggested the involvement of an increase in GA (especially the increase in active GA content) in the accelerated growth and development in transgenic tobacco plants overexpressing wheat *TaUb2*.

GA20oxs and GA3oxs are key biosynthetic enzymes in the pathways to creating bioactive GAs (Tanaka-Ueguchi et al. 1998) and are regulated by negative feedback, mainly due to high GA levels (Gallego-Giraldo et al. 2008). Meanwhile, bioactive GA is metabolically deactivated in several different ways. The best-characterized deactivation reaction is 2β-hydroxylation, which is catalyzed by a class of GA2oxs (Yamaguchi 2008). The results in tobacco plants of the same age (Fig. 7a–e) showed that the expression of GA metabolism genes (GA2ox3 in particular) were different between transgenic and WT tobacco plants. The difference results from the feedback regulation of active GA levels and may arise from variations in the developmental stage. Furthermore, the results in tobacco plants at similar stages of development (Fig. 7b, e) showed all the GA metabolism genes detected were upregulated. There was no alteration in total GA content of apical stems between 90-day-old transgenic tobacco plants and 120-day-old WT (Fig. 4), but increase of active GA in transgenic tobacco plants (Fig. 5), thus, these results suggest that transgenic plants have an enhanced GA metabolism rate, which provided an elevated level of active GA (Fig. 5) in transgenic tobacco plants. The active GA of high level may result in accelerated bolting and early flowering in transgenic tobacco plants.

The hypothetical mechanism underlying Ub-mediated GA signaling

DELLA proteins restrain the cell proliferation and enlargement that characterizes plant growth (Harberd et al. 1998). GA stimulates growth via phosphorylation and 26S proteasome-dependent destruction of DELLAs, thus relieving plants of DELLA-mediated growth restraint (Fu et al. 2002). DELLAs are known to be one of the key intracellular repressors of GA responses, however, DELLAs also upregulate GA synthesis genes and downregulate GA deactivation (2-oxidation) genes (Zentella et al. 2007; Weston et al. 2008). A previous study found that the overexpression of *CaPUB1*, which encodes hot pepper U-box E3, a key enzyme in UPS, resulted in rapid growth rate and early bolting phenotype (Cho et al. 2006). Ub is another key factor in UPS, and our results showed that overexpression of *TaUb2* accelerated stem growth, promoted early flowering phenotype, while simultaneously altering the expression of GA metabolism genes. Therefore, we speculate that overexpressed Ub may regulate tobacco growth and development via UPS, in a system involving Ub-regulated GA metabolism genes and DELLA-mediated GA signaling. However, further research is needed to confirm the mechanism.

In conclusion, the present study suggests that overexpressing *TaUb2* promoted stem growth at early stage and resulted in early flowering in transgenic tobacco plants, and the altered expression of MADS-box genes also corresponded to the changed phenotypes of the transgenic tobacco plants. The alteration in the phenotype of the tobacco plant may result from increased total GA and active GA contents. Ub was involved in production of total GA, and overexpression of *TaUb2* affected the level of bioactive GA by regulating GA metabolism genes.

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