Planta (2013) 237:991–999
DOI 10.1007/s00425-012-1818-4

ORIGINAL ARTICLE

Overexpression of glucosyltransferase UGT85A1 influences *trans*-zeatin homeostasis and *trans*-zeatin responses likely through *O*-glucosylation

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Received: 12 October 2012 / Accepted: 16 November 2012 / Published online: 28 November 2012

Abstract *Trans*-zeatin is a kind of cytokinins that plays a crucial role in plant growth and development. The master *trans*-zeatin *O*-glucosyltransferase of *Arabidopsis thaliana*, UGT85A1, has been previously identified through biochemical approach. To determine the in planta role of *UGT85A1* gene, the characterization of transgenic *Arabidopsis* plants overexpressing *UGT85A1* was carried out. Under normal conditions, transgenic *Arabidopsis* did not display clearly altered phenotypes. A remarkable alteration is that the accumulation level of the *trans*-zeatin *O*-glucosides was significantly increased in *UGT85A1* overexpressing transgenic *Arabidopsis*, while other forms of cytokinins kept the similar concentrations compared to the wild type. When treated with exogenously applied *trans*-zeatin, *UGT85A1* overexpressing *Arabidopsis* showed much less sensitivity to *trans*-zeatin in primary root elongation and lateral root formation. Meanwhile, the chlorophyll content of detached leaves of transgenic *Arabidopsis* was much lower than wild type. Studies of spatial–temporal expression patterns showed that *UGT85A1* was mainly expressed in the early seedlings and developing seeds. Analysis of subcellular localization suggested that UGT85A1 was localized to cytoplasm and nucleus. Taken together, our data suggest that overexpression of *Arabidopsis* glucosyltransferase UGT85A1 influences *trans*-zeatin homeostasis and *trans*-zeatin responses likely through *O*-glucosylation in planta.

Keywords *Arabidopsis* · Cytokinin · Glucosyltransferase · *Trans*-zeatin · Transgenic plant

Abbreviations

tZ	*Trans*-zeatin
tZR	tZ riboside
tZRPs	tZR phosphates
cZ	*cis*-zeatin
cZR	cZ riboside
cZRPs	cZR phosphates
iP	N^6-(Δ^2-isopentenyl) adenine
iPR	iP riboside
iPRPs	iPR phosphates
tZOG	tZ-*O*-glucoside
tZROG	tZR-*O*-glucoside
cZROG	cZR-*O*-glucoside
tZRPsOG	tZRPs-*O*-glucoside
tZ7G	tZ-7-*N*-glucoside
tZ9G	tZ-9-*N*-glucoside
iP7G	iP-7-*N*-glucoside
iP9G	iP-9-*N*–glucoside
UGT	Uridine diphosphate glycosyltransferase

Introduction

Cytokinins are a class of phytohormones that play an important role at all phases of plant development from seed

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germination to senescence (Mok and Mok 1994; Sakakibara 2006; Hirose et al. 2008; Argueso et al. 2009). Naturally occurring cytokinins are adenine derivatives and can be classified by their N^6-side chain as isoprenoid or aromatic cytokinins. *Trans*-zeatin (tZ) is considered to be an essential isoprenoid cytokinin in higher plants due to its ubiquitous nature and high activity. Other isoprenoid cytokinins such as N^6-(Δ^2-isopentenyl) adenine (iP), dihydrozeatin (DHZ) and *cis*-zeatin (cZ) are also present in most plant tissues (Mok and Mok 2001). *O*-glucosylation, in which a glucose molecule is attached to the hydroxyl group of isoprenoid N^6-side chain, is thought to be one of the main modifications for *trans*-zeatin and other zeatin-type cytokinins (DHZ and cZ). This modification is found to occur in about all plant species examined (Jameson 1994).

The biological activities of cytokinin *O*-glucosides (the product of *O*-glucosylation) have drawn a great deal of attention as a starting point to understanding their possible physiological significance and the regulation of cytokinin metabolism in planta. Until recently, however, the true activities of the cytokinin *O*-glucosides were difficult to assess. Traditionally, the activities of cytokinins have been determined by bioassays through supplying plant tissues with exogenous cytokinins (Murashige and Skoog 1962; Letham et al. 1983; Mok et al. 1978). However, apparent cytokinin activities may not necessarily reflect their true activities in planta due to rapid metabolism or catabolism. For example, tZ-*O*-glucoside (tZOG) was biologically active in *Arabidopsis* P_{ARR5}*::GUS* reporter gene assay, while tZOG was not able to activate cytokinin receptors AHK3 and AHK4 itself (Spíchal et al. 2004; Romanov et al. 2006). Later, this was also confirmed for cytokinin receptor AHK2 (Stolz et al. 2011). The activity of tZOG in bioassay is believed most probably due to the liberation of the free base from *trans*-zeatin *O*-glucosides by endogenous hydrolases. It was shown that a β-glucosidase isolated from maize and encoded by *Zm-p60.1* gene cleaved the biologically inactive hormone conjugates such as *O*-β-glucosylzeatin and 3-β-glucosylkinetin, releasing active cytokinins. Tobacco protoplasts that transiently expressed *Zm-p60.1* gene could use the inactive cytokinin glucosides to initiate cell division (Brzobohaty et al. 1993).

The identification of enzymes and genes involved in zeatin *O*-glucosylation opens the new door for investigating the biological activities of cytokinin *O*-glucosides in planta. The first gene encoding *O*-glucosyltransferase with a preference for *trans*-zeatin (*ZOG1*) was isolated from *P. lunatus* by Martin et al. (1999). Later, two maize genes (*cis-ZOG1* and *cis-ZOG2*) encoding *O*-glucosyltransferases with a preference for *cis*-zeatin were isolated (Martin et al. 2001; Veach et al. 2003). More recently, *cis*-zeatin-specific *O*-glucosyltransferases (*cZOGT1*, *cZOGT2*, and *cZOGT3*) were identified from rice (Kudo et al. 2012). When inducing the expression of *ZOG1* in transgenic tobacco leaf disks, a tenfold higher zeatin level was required for the formation of shoots and callus compared with the controls (Martin et al. 2001), suggesting the *O*-glucosylation could lower the activity of zeatin. However, the observed axillary shoot growth appears to indicate a rise in cytokinin activity. Overexpression of *ZOG1* in maize led to cytokinin deficient vegetative characteristics including shorter stature, thinner stems, narrower leaves, smaller meristems, and increased root mass and branching, but transformant had higher chlorophyll content and increased levels of active cytokinins in leaves compared with those of non-transformed sibs, as well as a tasselseed phenotype (Rodo et al. 2008). Transgenic rice lines ectopically overexpressing the *cZOGT1* and *cZOGT2* genes exhibited short shoot phenotypes, delay of leaf senescence, and decrease in crown root number (Kudo et al. 2012). Those observations likely reflect the subtle and complex effects of *O*-glucosylation on the regulation of active cytokinin levels and on the plant growth and development. It may need more genetic investigations using distinct cytokinin *O*-glucosyltransferase genes to further determine the biological activities of cytokinin *O*-glucosides and the biological effects of cytokinin *O*-glucosylation.

UGT85A1 is another zeatin *O*-glucosyltransferase with a preference for *trans*-zeatin, which was first isolated from *Arabidopsis thaliana* (Hou et al. 2004). Although there are two additional zeatin *O*-glucosyltransferases in *Arabidopsis*, i.e., UGT73C5 and UGT73C1, they have only trace enzyme activities. Moreover, UGT73C5 has been characterized as brassinosteroid-specific glucosyltransferase (Poppenberger et al. 2005). Therefore, UGT85A1 is so far the master *O*-glucosyltransferase identified from *Arabidopsis* with a responsibility for the *O*-glucosylation and *O*-glucoside formation of *trans*-zeatin. Compared to *Phaseolus trans*-zeatin glucosyltransferase ZOG1, UGT85A1 has only low sequence similarity (<30 %). On the phylogenetic tree containing 107 uridine diphosphate glycosyltransferases (UGTs) of *Arabidopsis* and 4 UGTs of *Phaseolus* and *Zea*, it was observed that UGT85A1 and ZOG1 landed on distinct branches. We speculate that the *Arabidopsis* UGT85A1 might evolve from a distinct ancestor from the *Phaseolus* ZOG1 (Hou et al. 2004).

We are interested in determining the regulatory properties of overexpressing *UGT85A1* genes on cytokinin metabolism and cytokinin responses. The phenotypical and physiological analyses of transgenic *Arabidopsis* plants overexpressing *UGT85A1* are reported here. Our data strongly suggest that *Arabidopsis* glucosyltransferase gene *UGT85A1* is involved in *trans*-zeatin homeostasis and *trans*-zeatin responses likely through *O*-glucosylation in planta.

Materials and methods

Plant materials and growth conditions

Plants of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0; seeds obtained from Nottingham Arabidopsis Stock Centre, UK) were used in this study. The plants were grown in greenhouse in flowerpots of Nutrition Soil (Shangdao Biotech Co. Ltd., Shandong, China) with vermiculite (Nutrition Soil:vermiculite = 2:1) at 22 ± 2 °C under a 16/8 h light/dark cycle. Light intensity in greenhouse was ~100 μmol m^{-2} s^{-1}. Tissue cultures and regenerated plantlets were maintained in a culture room on MS medium (Murashige and Skoog 1962) supplemented with 3 % sucrose and 0.7 % agar unless otherwise specified. Growth conditions in culture room are 22 ± 2 °C, 14 h light/10 h dark photoperiod and a light intensity of ~60 μmol $m^{-2}s^{-1}$.

Overexpression plasmid construction and plant transformation

Full-length cDNA of *UGT85A1* was isolated from 2-week-old *Arabidopsis* seedlings by RT-PCR. Total RNA was extracted from seedlings with the TRIzol method and treated with RNase-free DNase I (TaKaRa). RNA (0.5 μg) was used as a template for first-strand cDNA synthesis using the PrimeScript RT reagent kit (DRR037, TaKaRa). The 1,470-bp full-length DNA sequence of *UGT85A1* was amplified using gene-specific primers: forward, 5′-CGG GATCCATGGGATCTCAGATCATTCAT-3′; and reverse, 5′-CGGATATCTTAATCCTGTGATTTTTGTCCCA-3′. The PCR product was cloned into the pBluescript II SK (+) vector and subcloned into the binary vector pBI121 to replace the *GUS* and generate the CaMV35S_{pro}::*UGT85A1* recombinant plasmid. *Agrobacterium* strain GV3101 harboring the recombinant plasmid was used for *Arabidopsis* transformation through floral dip method (Clough and Bent 1998). Kanamycin-resistant and PCR-positive transgenic plants were transferred to greenhouse and maintained up to T2 generation. At least two independent homozygous lines were then selected and used for further analysis.

Molecular analysis of *UGT85A1* overexpression lines

For RT-PCR, total RNA from *Arabidopsis* leaves was isolated and cDNA synthesized as mentioned above. *UGT85A1* transcripts were detected by semi-quantitative RT-PCR using gene-specific primers as listed above. PCR amplification of *UGT85A1* was performed as follows: 94 °C for 50 s, 50 °C for 50 s and 72 °C for 2 min, with an initial denaturation at 94 °C for 5 min. *Tubulin* PCR product was used as a loading control for cDNA amplification of each genotypes.

Quantification of cytokinins and their glucosides

For the quantification of cytokinins in *Arabidopsis* seedlings, seeds were surface sterilized and planted on MS plates containing 3 % sucrose and 0.7 % agar, incubated in darkness at 4 °C for 3 days, then transferred to a growth chamber at 22 °C under a 16/8 h light/dark cycle for vertical culture. After 2 weeks, seedlings were harvested and weighed; ~100 mg of seedlings per sample was pooled and three independent biological samples analyzed for each genotype. The procedure used for cytokinin analysis was previously described by Kojima et al. (2009).

Assays for root elongation and lateral root number

Surface-sterilized wild type and *Arabidopsis* overexpressor seeds were planted in a line on vertical 1/2 MS plates containing 3 % sucrose and 0.7 % agar. When roots had grown to a length of 1.0 cm after ~3 days, 10 healthy seedlings of wild type and overexpressors with approximately equal root lengths were transferred to new vertical 1/2 MS plates with different concentrations of *trans*-zeatin. The positions of the root tips were indicated using a fine-tipped marker pen. After 7 days, images were captured and the advancement of the root tips was measured by NIH-Image. After 14 days, lateral root number for each main root was counted with the aid of a magnifier. All data were quantitatively analyzed in Microsoft Excel. There were at least three replicates per experiment.

Chlorophyll content measurement

Arabidopsis plants subjected to chlorophyll content measurement were grown in soil in the same conditions. The seventh leaves of 4-week-old *Arabidopsis* wild type and transgenic plants were collected and weighed. The chlorophyll measurement was performed essentially as described by Chory et al. (1991). Briefly, the leaves were frozen in liquid nitrogen, ground to a fine powder, total chlorophyll was extracted into 80 % acetone, and chlorophyll *a* and *b* contents calculated using MacKinney's-specific absorption coefficients in which chlorophyll $a = 12.7\,(A663) - 2.69\,(A645)$ and chlorophyll $b = 22.9\,(A645) - 4.68\,(A663)$. The total specific chlorophyll content was expressed as mg of chlorophyll per gram of leaves. Chlorophyll contents were determined at the zero time point or after 1 week of treatment with different concentrations of *trans*-zeatin in darkness. There were at least three replicates per experiment.

GUS fusion construction and GUS staining assay

A 2-kb DNA fragment upstream of the start codon of *UGT85A1* (AT1g22400), which was used as *UGT85A1* promoter, was amplified by PCR using high fidelity Taq enzyme (DR010, TaKaRa) and the pair of primers: forward, 5′-CGCAAGCTTTGGGAAATAAGATTGAATAA AAACTC-3′; and reverse, 5′-CGCGGATCCTTTTCTTT CTCTTGCGCGAAATCAG-3′. The PCR product was cloned into the pBluescript II SK (+) vector and subcloned into the binary vector pBI121 to replace the CaMV35S promoter and generate the *UGT85A1pro::GUS* recombinant plasmid. The genetic transformation of *Arabidopsis* is performed according to the method mentioned above. At least two independent homozygous transgenic lines of $UGT85A1_{pro}$*::GUS* were selected and used for GUS staining. Seedlings of 1–14 days after germination grown on MS plates, and roots, leaves, inflorescence and siliques from plants grown on soil were collected for GUS staining assay. For each transgenic line, 5–10 independent transformants were used for this experiment. Samples were placed in 90 % acetone on ice for 15 min, then washed twice with staining buffer [50 mM sodium phosphate, pH 7.2, 0.2 mM $K_3Fe(CN)_6$, 0.2 mM $K_4Fe(CN)_6$ and 0.2 % Triton X-100] without X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) before adding staining buffer with X-Gluc (to a final concentration of 2 mM), and incubating overnight at 37 °C. The samples were washed in 70 % ethanol for 30 min before observation under a dissecting microscope (Olympus). Only representative staining patterns were photographed.

Subcellular localization analysis of UGT85A1

The cDNA sequence of *UGT85A1* without stop codon was amplified with the primers: forward, 5′-CGTCTAGAA TGGGATCTCAGATCATTCAT-3′; and reverse, 5′-CGG GATCCATCCTGTGATTTTTGTCCCA-3′. The PCR products were digested with *Xba*I and *Bam*HI, and then ligated into p326-SGFP vector, in which the coding sequence of the UGT85A1 gene was fused to the 5′ terminus of the GFP gene in frame, driven by the CaMV35S promoter. The fusion construct as well as the control vector with GFP alone were transformed into the onion epidermis cells using a particle gun (PDS-1000; Bio-Rad). After 24 h of incubation, GFP fluorescence in transformed onion cells was observed under a Nikon fluorescence microscope. Pictures were taken with more than 10 cells for each plant material, in which GFP fluorescence was observed, and all cells showed nearly the identical pattern representing the localization of the fusion protein.

Statistical analyses

All data were collected from at least three independent experiments with at least 15 plant samples per genotype. The experimental data were statistically analyzed by Student's *t* test. Data presented are expressed as mean values ± SD. Significant difference between transformant and control is indicated by asterisk above the bars in the figures ($*P < 0.05$, $**P < 0.01$).

Results

Characteristics of UGT85A1 overexpression lines

The gene *UGT85A1* was cloned into the binary vector pBI121 under the control of CaMV35S promoter (Fig. 1a) and used to transform *Arabidopsis* (Col-0). Kanamycin-resistant and PCR-positive transgenic plants constitutively expressing *35S::UGT85A1* were selected. Independent T2 transgenic lines grown in greenhouse conditions were screened by PCR analysis (data not shown). Semi-quantitative RT-PCR analysis demonstrated that the levels of *UGT85A1* mRNA were increased in multiple homozygous lines of transgenic *Arabidopsis* (Fig. 1b). It was noticed that the growth and appearance of transgenic *Arabidopsis* were almost the same as those of wild-type plants throughout the growth period under normal conditions (data not shown). This result suggests that the overexpression of UGT85A1 could not alter *Arabidopsis* phenotypes.

Contents of cytokinins and their glucosides in *Arabidopsis* overexpressors

To determine the in vivo activity of UGT85A1, the levels of endogenous cytokinins and their respective glucosides were determined from the overexpressors and wild type grown under normal conditions. The iP and zeatin-type cytokinins—including their free forms, *N*-glucosides, *O*-glucosides, ribosides and nucleotides—were chosen for determination. It was found that the concentrations of the most active free forms of cytokinins (i.e., iP, tZ and cZ) and other forms of cytokinins including ribosides, nucleotides and *N*-glucosides were comparable to those of the wild type (Fig. 2). However, the content of *trans*-zeatin-*O*-glucoside (tZOG) was substantially increased in both *Arabidopsis* overexpressors (Fig. 2). These data indicated that the overexpression of glucosyltransferase UGT85A1 resulted in intensive *O*-glucosylation of *trans*-zeatin in planta. On the other hand, however, the almost unchanged level of

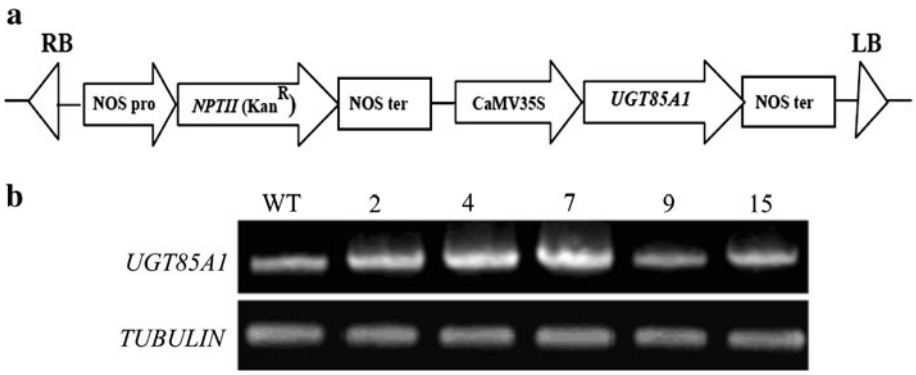

Fig. 1 Analysis of *UGT85A1* overexpression lines. **a** Construct design of 35S::*UGT85A1* that was inserted into the *Arabidopsis* genome to constitutively express *UGT85A1* in transgenic lines. **b** Semi-quantitative RT-PCR analyses of wild-type and transgenic *Arabidopsis* lines harboring *UGT85A1*. Expression of *UGT85A1* mRNA in different transgenic lines is shown. *Tubulin* was used as a loading control

cytokinin free forms in *Arabidopsis* overexpressors may be a consequence of regulation in whole network of cytokinin metabolism.

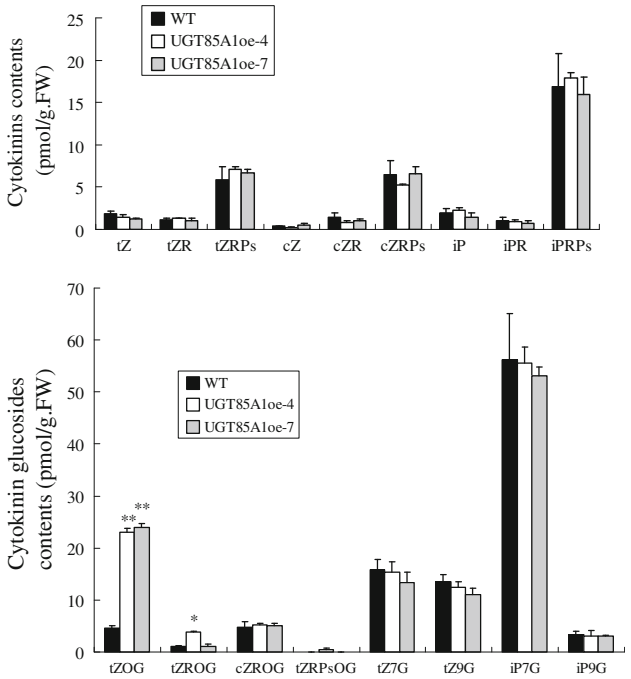

Fig. 2 Contents of cytokinins and their glucosides of *UGT85A1* overexpression lines and wild type in *Arabidopsis*. Seedlings were grown on vertical MS plates for 2 weeks and then used for hormone determination: ~100 mg of seedlings per sample was pooled and three independent biological samples were analyzed for each genotype. Values are mean ± SD ($n = 3$). Significant difference between transformant and control is indicated by *asterisks* above the *error bars* (*$P < 0.05$, **$P < 0.01$). tZ, *trans*-zeatin; tZR, tZ riboside; tZRPs, tZR phosphates; cZ, *cis*-zeatin; cZR, cZ riboside; cZRPs, cZR phosphates; iP, N^6-(Δ^2-isopentenyl) adenine; iPR, iP riboside; iPRPs, iPR phosphates; tZOG, tZ-*O*-glucoside; tZROG, tZR-*O*-glucoside; cZROG, cZR-*O*-glucoside; tZRPsOG, tZRPs-*O*-glucoside; tZ7G, tZ-7-*N*-glucoside; tZ9G, tZ-9-*N*-glucoside; iP7G, iP-7-*N*-glucoside; iP9G, iP-9-*N*-glucoside

Effects of exogenously applied cytokinin on primary root elongation and lateral root growth

Since cytokinins have strong inhibitory effects on root growth (Cary et al. 1995; Beemster and Baskin 2000; Werner et al. 2003; Ioio et al. 2007; Bishopp et al. 2009), root elongation can be used as a marker to analyze the responses of a specific plant genotype to cytokinins. We analyzed the effect of exogenously applied *trans*-zeatin on root development of the *Arabidopsis* overexpressors. We found that the main roots of *UGT85A1* overexpressors were longer than those of wild type on media containing 0.05–1.0 μM *trans*-zeatin (Fig. 3a). Several reports described the inhibitory effect of cytokinins on lateral root formation (Li et al. 2006; Laplaze et al. 2007). We further investigated lateral root number of 17-day-old overexpression lines and wild type on 1/2 MS media with or without 0.01–0.5 μM *trans*-zeatin. Experiments showed that the lateral root number of the *UGT85A1* overexpressors was much more than that of wild type on media containing 0.05–0.1 μM *trans*-zeatin (Fig. 3b).

Changes in chlorophyll retention of detached leaves

The influence of cytokinins on the chlorophyll content of leaves and their ability to retard leaf senescence were described soon after their discovery (Richmond and Lang 1957). We investigated the participation of *UGT85A1* in mediating chlorophyll retention of detached leaves when applying exogenous cytokinin and dark conditions. Experimental results showed that, before treatment, *Arabidopsis* overexpression lines *UGT85A1oe-4* and *UGT85A1oe-7* had the similar chlorophyll levels to wild type. But after 1 week of incubation in darkness in water or in solution containing 1 μM *trans*-zeatin, the chlorophyll contents of *UGT85A1oe-4* and *UGT85A1oe-7* were much lower than wild type (Fig. 4). These data suggested that

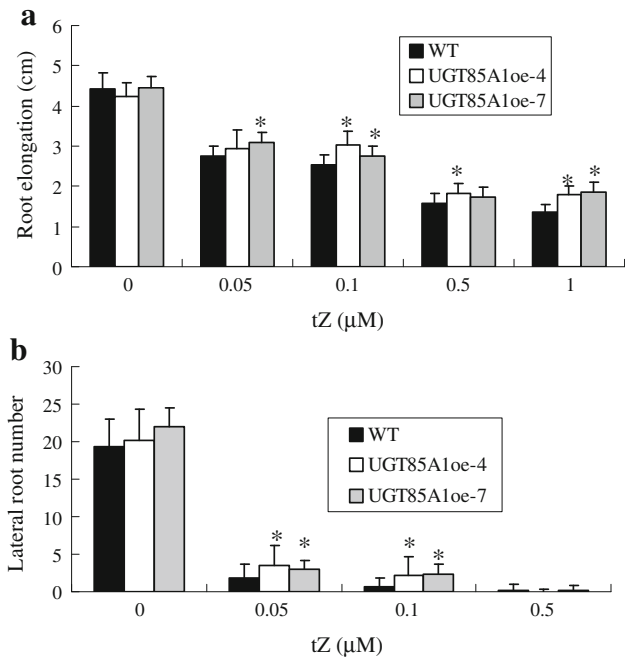

Fig. 3 Analysis of root elongation and lateral root number of *UGT85A1* overexpression lines in *Arabidopsis*. **a** Seedlings with equal root length were transferred onto vertical 1/2 MS plates supplemented with 0, 0.05, 0.1, 0.5, 1 μM *trans*-zeatin and grown for 1 week. Then root elongation was measured. **b** Seedlings with equal root length were transferred onto vertical 1/2 MS plates supplemented with 0, 0.05, 0.1, 0.5 μM *trans*-zeatin and grown for 2 weeks. Then lateral root number was counted. Three independent biological replicates for each genotype were performed. Data are the mean ± SD ($n = 30$). Significant difference between transformant and control is indicated by *asterisks* above the *error bars* ($*P < 0.05$)

UGT85A1 contributed to regulating the effects of exogenously applied cytokinins on chlorophyll retention in leaves.

In their entirety, the combined results from our abovementioned experiments indicate that the enhancement of UGT85A1 activity decreased the responses of plants to *trans*-zeatin, resulting in less sensitive phenotypes. Therefore, the tentative conclusion drawn from the experiments is that UGT85A1 is involved in *trans*-zeatin homeostasis and *trans*-zeatin responses likely through catalyzing *O*-glucosylation of *trans*-zeatin in planta.

Expression pattern of *UGT85A1* gene

To investigate the expression patterns of *UGT85A1*, the vector harboring *UGT85A1pro::GUS* construct was introduced into *Arabidopsis* and the spatial and temporal expression patterns of *UGT85A1* gene in plants were studied. Our data showed that *UGT85A1* gene mainly expressed in the geminated seeds and tissues of young seedlings such as the cotyledons, hypocotyls, primary roots and root tips. In addition, *UGT85A1* also expressed in maturing embryos (Fig. 5). These results indicate that the expression of *UGT85A1* gene is in the temporal–spatial specific manner.

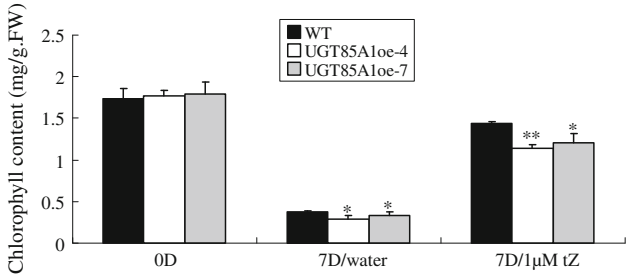

Fig. 4 Chlorophyll contents analysis of *UGT85A1* overexpression lines of *Arabidopsis*. Plant leaves for each genotype were collected, weighed and chlorophyll contents determined at zero time point (without treatment, 0D) or after 1 week of treatment with water (7D/water) or 1 μM tZ (7D/1 μM tZ) in darkness. Three independent biological replicates for each genotype were performed. Data are the mean ± SD ($n = 3$). Significant difference between transformant and control is indicated by *asterisks* above the *error bars* ($*P < 0.05$, $**P < 0.01$)

Subcellular localization of UGT85A1 protein

To know the subcellular localization of UGT85A1 protein, the vector *35Spro::UGT85A1-GFP* was constructed and transformed into onion epidermis by particle bombardment. As shown in Fig. 6, the diffuse fluorescence pattern attributable to transiently expressed UGT85A1–GFP was similar to the fluorescence of transiently expressed GFP where a characteristic pattern can be found in both cytoplasm and nucleus.

Discussion

This study provides first evidence for the biological effects of *Arabidopsis UGT85A1* gene in planta. At first, our data showed much higher concentration of *trans*-zeatin *O*-glucosides in *UGT85A1* overexpressors, indicating that biochemical function of UGT85A1 is in the *O*-glucosylation of zeatin-type cytokinins, at least the *trans*-zeatin. This in vivo observation is consistent with what reported previously through in vitro biochemical analysis (Hou et al. 2004). Secondly, although the *UGT85A1* overexpressors of *Arabidopsis* have no obvious phenotypic change in comparison with wild type under normal growth condition, several phenotypic and physiological changes in primary root length, lateral root number, chlorophyll retention can be clearly observed when exogenously applied with *trans*-zeatin. These findings indicate that *Arabidopsis* UGT85A1 has effects on *trans*-zeatin homeostasis and *trans*-zeatin responses. Thirdly, all of abovementioned phenotypic and

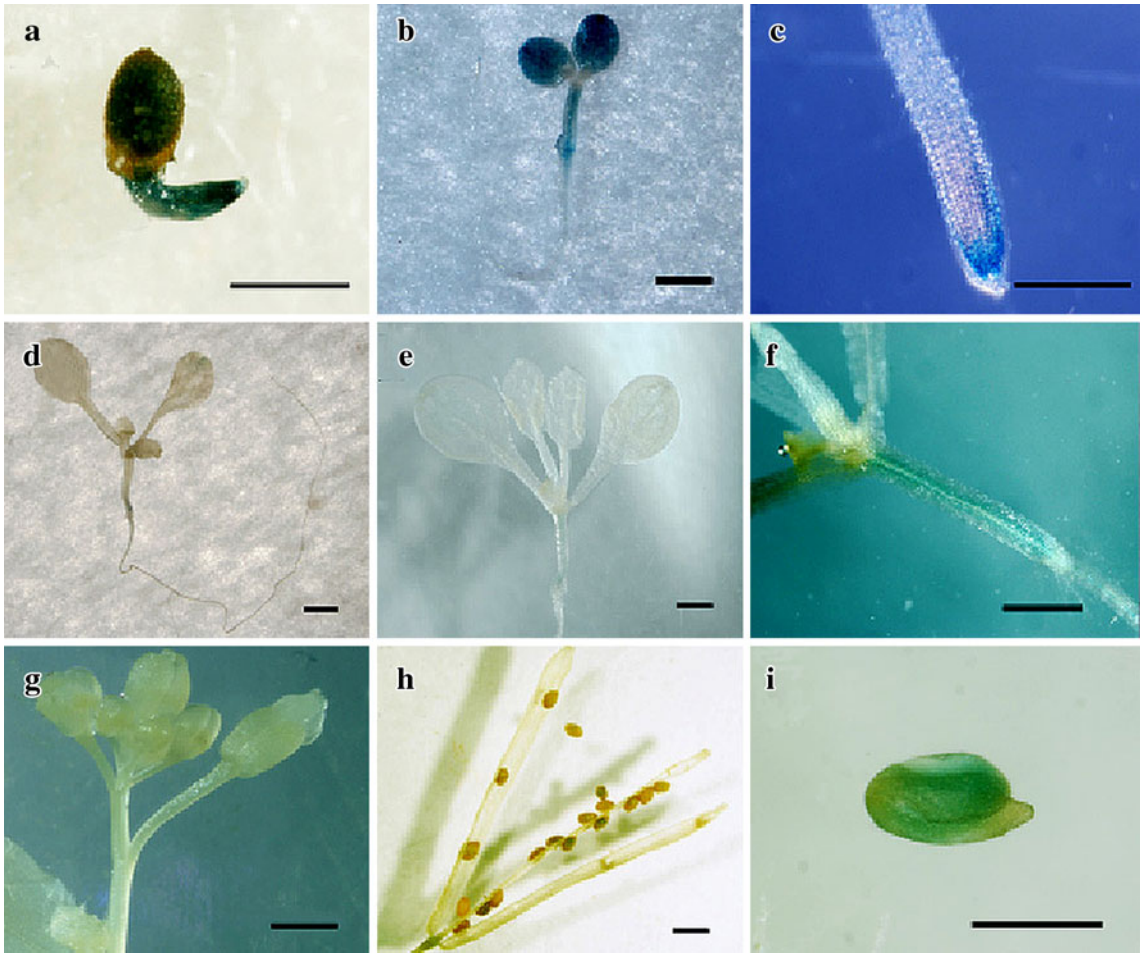

Fig. 5 GUS expression analysis of *UGT85A1* promoters in *Arabidopsis*. **a** Seed at 1 DAG. **b** Seedling at 4 DAG. **c** Root at 4 DAG. **d** Seedling at 7 DAG. **e** Seedling at 8 DAG. **f** Hypocotyl at 10 DAG. **g** Inflorescence. **h** Maturing pod. **i** Maturing embryo. *Bars* 500 μm (**a**, **i**), 1 mm (**b**, **d**–**h**), 200 μm (**c**)

physiological changes in *UGT85A1* overexpressors are the results of reduced sensitivity to applied *trans*-zeatin. It makes us propose that *Arabidopsis* UGT85A1 could deactivate *trans*-zeatin through *O*-glucosylation, thus resulting in less sensitive phenotypic changes in UGT85A1 overexpressors.

Martin and co-workers reported that the overexpression of the cytokinin *O*-glucosyltransferase (ZOG1) of *Phaseolus* in tobacco resulted in adventitious root formation, shorter stature, more shoot branches and thinner stem (Martin et al. 2001). Ectopically overexpressing the *cZOGT1* and *cZOGT2* genes in rice caused short shoot phenotypes, delay of leaf senescence, and decrease in crown root number (Kudo et al. 2012). In our transgenic *Arabidopsis* plants, however, we did not find those phenotypic alterations when overexpressing UGT85A1. These findings indicate that, although *Arabidopsis* UGT85A1, *Phaseolus* ZOG1, and rice cZOGT1 and cZOGT2 have similar biochemical activity toward zeatin-type cytokinins, their in planta function might have diverged but overlapped since they have a low sequence similarity evolved from different ancestors in evolution.

Serving as cytokinin *O*-glucosyltransferase gene, *UGT85A1* is mainly expressed in young seedlings and maturing embryos. This temporal–spatial expression pattern may reflect the biological role for UGT85A1 to play in growth and development. Generally, germinating seeds and young seedlings are very active in cell division and produce high levels of cytokinins. High expression level of UGT85A1 in these developmental stages likely plays a role in deactivating and storing the excessive zeatin-type cytokinins through catalyzing the formation of *O*-glucosides, thus making plants maintain a normal growth and development. In addition, because the storage forms of cytokinin *O*-glucosides might be converted into their active forms (Brzobohaty et al. 1993; Mok and Mok 2001), the stored cytokinin *O*-glucosides could be used as cytokinin bank for the later vegetative growth. It may be important to

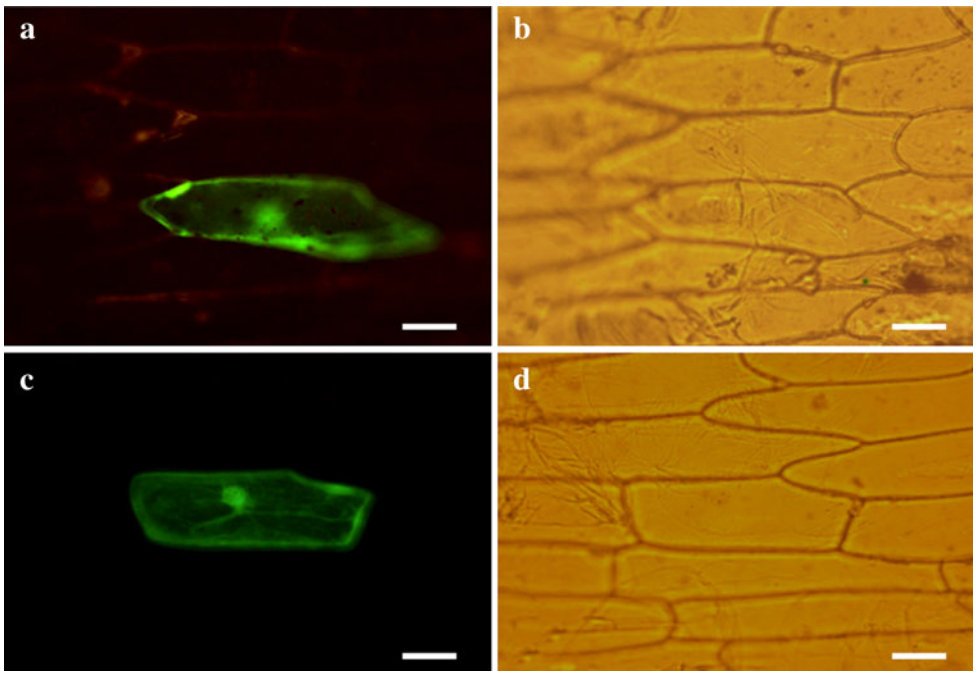

Fig. 6 Subcellular localization analysis of UGT85A1 protein. Green fluorescent protein alone (**a**) or UGT85A1:GFP (**c**) in onion epidermal cells. Corresponding images in bright field (**b**) and (**d**), respectively. *Bars* 50 μm

an exactly the same degree for UGT85A1 to strongly express in maturing embryos. We speculate that the stored cytokinin *O*-glucosides in maturing embryos, possibly serving as a reservoir of cytokinins, could rapidly supply the early seed germination with active cytokinins through the hydrolysis action of glucosidase as the case in maize (Brzobohaty et al. 1993).

A widely held belief is that plant UGTs for secondary products are cytoplasmic enzymes (Ross et al. 2001). However, exceptions were observed for two UGTs, UGT73C6 and UGT87A2, which were demonstrated to localize in cytoplasm and nucleus (Husar et al. 2011; Wang et al. 2012). Because cytokinin *O*-glycosides are mainly localized in plant vacuoles, cytokinin glycosyltransferases are speculated to localize mainly in vacuoles (Meek et al. 2008; Rodo et al. 2008; Bajguz and Piotrowska 2009). There are also different speculations on the localization of cytokinin *O*-glycosyltransferases. For example, cytokinin *O*-glucosyltransferase of *Phaseolus*, ZOG1, is expected to be localized in the cytosol and cytokinin *O*-glucoside is likely delivered to both the chloroplast and the vacuole in the conjugated form (Kiran et al. 2012). Our data on subcellular distribution showed that UGT85A1 protein was localized both in cytoplasm and nucleus, an observation similar with the cases in UGT73C6 and UGT87A2. UGT85A1 localized in cytoplasm may be involved in zeatin-type cytokinin glucosylation and cytokinin homeostasis, as its counterpart, the β-glucosidase Zm-p60.1, was also demonstrated to localize in cytoplasm, particularly in plastids/chloroplasts (Kristoffersen et al. 2000). Redirection of Zm-p60.1 to vacuole was also important in maintaining cytokinin homeostasis (Kiran et al. 2012). Therefore, cytoplasm localized cytokinin *O*-glucoslytransferase and β-glucosidase might coordinately regulate the whole network of zeatin conversions and active cytokinin pool. On the other hand, the fluorescence of UGT85A1-GFP fusion was also found in nucleus. This result makes us hypothesize that, besides its involvement in zeatin-type cytokinin glucosylation in cytoplasm, UGT85A1 protein may have unknown function in nucleus, which remains to be determined. Certainly, the possibility of the partial bidirectional diffusion of larger proteins back to the nuclei cannot be excluded (von Arnim et al. 1998; Smehilova et al. 2009).

Acknowledgments This work was supported by National Natural Science Foundation of China (No. 30770214 and No. 90917006) to B.K.H.

Conflict of interest None.

References

Argueso CT, Ferreira FJ, Kieber JJ (2009) Environmental perception avenues: the interaction of cytokinin and environmental response pathways. Plant Cell Environ 32:1147–1160

Bajguz A, Piotrowska A (2009) Conjugates of auxin and cytokinin. Phytochemistry 70:957–969

Beemster GTS, Baskin TI (2000) *STUNTED PLANT 1* mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of Arabidopsis. Plant Physiol 124:1718–1727

Bishopp A, Help H, Helariutta Y (2009) Cytokinin signaling during root development. Int Rev Cell Mol Biol 276:1–48

Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palmet K (1993) Release of active cytokinin by a β-glucosidase localized to the maize root meristem. Science 262:1051–1054

Cary AJ, Liu W, Howell SH (1995) Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyls elongation in *Arabidopsis thaliana* seedlings. Plant Physiol 107:1075–1082

Chory J, Nagpal P, Peto CA (1991) Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in *Arabidopsis*. Plant Cell 3:445–459

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H (2008) Regulation of cytokinin biosynthesis, compartmentalization and translocation. J Exp Bot 59:75–83

Hou B, Lim EK, Higgins G, Bowles DJ (2004) *N*-Glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. J Biol Chem 279:47822–47832

Husar S, Berthiller F, Fujioka S, Rozhon W, Khan M, Kalaivanan F, Elias L, Higgins GS, Li Y, Schuhmacher R, Krska R, Seto H, Vaistij FE, Bowles D, Poppenberger B (2011) Overexpression of the UGT73C6 alters brassinosteroid glucoside formation in *Arabidopsis thaliana*. BMC Plant Biol 11:51

Ioio RD, Linhares FS, Scacchi E, Casamitjana-Martinez E, Heidstra R, Costantino P, Sabatini S (2007) Cytokinins determine *Arabidopsis* root-meristem size by controlling cell differentiation. Curr Biol 17:678–682

Jameson PE (1994) Cytokinin metabolism and compartmentation. In: Mok DWS, Mok MC (eds) Cytokinins: chemistry, activity and function. CRC Press, Boca Raton, pp 113–128

Kiran NS, Eva Benková, Reková A, Dubová J, Malbeck J, Palme K, Brzobohaty B (2012) Retargeting a maize β-glucosidase to the vacuole—evidence from intact plants that zeatin-*O*-glucoside is stored in the vacuole. Phytochemistry 79:67–77

Kojima M, Kamada-Nobusada T, Komatsu H, Takei K, Kuroha T, Mizutani M, Ashikari M, Ueguchi-Tanaka M, Matsuoka M, Suzuki K, Sakakibara H (2009) Highly-sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatography-tandem mass spectrometry: an application for hormone profiling in *Oryza sativa*. Plant Cell Physiol 50:1201–1214

Kristoffersen P, Brzobohaty B, Hohfeld I, Bako L, Melkonian M, Palme K (2000) Developmental regulation of the maize *Zm-p60.1* gene encoding a β-glucosidase located to plastids. Planta 210:407–415

Kudo T, Makita N, Kojima M, Tokunaga H, Sakakibara H (2012) Cytokinin activity of *cis*-zeatin and phenotypic alterations induced by over-expression of putative *cis*-zeatin-*O*-glucosyltransferase in rice. Plant Physiol 160:319–331

Laplaze L, Benkova E, Casimiro I, Maes L, Vanneste S, Swarup R, Weijers D, Calvo V, Parizot B, Herrera-Rodriguez MB, Offringa R, Graham N, Doumas P, Friml J, Bogusz D, Beeckman T, Bennett M (2007) Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell 19:3889–3900

Letham DS, Palni LMS, Tao GQ, Gollnow BI, Bates CM (1983) Regulators of cell division in plant tissues XXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassays. J Plant Growth Regul 2:103–115

Li X, Mo X, Shou H, Wu P (2006) Cytokinin-mediated cell cycling arrest of pericycle founder cells in lateral root initiation of *Arabidopsis*. Plant Cell Physiol 47:1112–1123

Martin RC, Mok MC, Mok DWS (1999) Isolation of a cytokinin gene, *ZOG1*, encoding zeatin *O*-glucosyltransferase from *Phaseolus lunatus*. Proc Natl Acad Sci USA 96:284–289

Martin RC, Mok MC, Habben JE, Mok DWS (2001) A maize cytokinin gene encoding an *O*-glucosyltransferase specific to *cis*-zeatin. Proc Natl Acad Sci USA 98:5922–5926

Meek L, Martin RC, Shan X, Karplus PA, Mok DWS, Mok MC (2008) Isolation of legume glycosyltransferases and active site mapping of the *Phaseolus lunatus* zeatin *O*-glucosyltransferase ZOG1. J Plant Growth Regul 27:192–201

Mok DWS, Mok MC (1994) Cytokinins: chemistry, activity and function. CRC Press, Boca Raton

Mok DWS, Mok MC (2001) Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol 52:89–118

Mok MC, Mok DWS, Armstrong DJ (1978) Differential cytokinin structure–activity relationships in *Phaseolus*. Plant Physiol 61:72–75

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Poppenberger B, Fujioka S, Soeno K, George GL, Vaistij FE, Hiranuma S, Seto H, Takatsuto S, Adam G, Yoshida S, Bowles DJ (2005) The UGT73C5 of *Arabidopsis thaliana* glucosylates brassinosteroids. Proc Natl Acad Sci USA 102:15253–15258

Richmond AE, Lang A (1957) Effect of kinetin on protein content and survival of detached *Xanthium* leaves. Science 125:650–651

Rodo AP, Brugiere N, Vankova R, Malbeck J, Olson JM, Haines SC, Martin RC, Habben JE, Mok DWS, Mok MC (2008) Over-expression of a zeatin *O*-glucosylation gene in maize leads to growth retardation and tasselseed formation. J Exp Bot 59:2673–2686

Romanov GA, Lomin SN, Schmülling T (2006) Biochemical characteristics and ligand-binding properties of *Arabidopsis* cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J Exp Bot 57:4051–4058

Ross J, Li Y, Lim E, Bowles DJ (2001) Higher plant glycosyltransferases. Genome Biol 2(3004):1–6

Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol 57:431–449

Smehilova M, Galuszka P, Bilyeu KD, Jaworek P, Kowalska M, Sebela M, Sedlarova M, English JT, Frebort I (2009) Subcellular localization and biochemical comparison of cytosolic and secreted cytokinin dehydrogenase enzymes from maize. J Exp Bot 60:2701–2712

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T (2004) Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. Plant Cell Physiol 45:1299–1305

Stolz A, Riefler M, Lomin SN, Achazi K, Romanov GA, Schmülling T (2011) The specificity of cytokinin signalling in *Arabidopsis thaliana* is mediated by differing ligand affinities and expression profiles of the receptors. Plant J 67:157–168

Veach YK, Martin RC, Mok DWS, Malbeck J, Vankova R, Mok MC (2003) *O*-glucosylation of *cis*-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. Plant Physiol 131:1374–1380

von Arnim AG, Deng XW, Stacey MG (1998) Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. Gene 221:35–43

Wang B, Jin SH, Hu HQ, Sun YG, Wang YW, Han P, Hou BK (2012) *UGT87A2*, an Arabidopsis glycosyltransferase, regulates flowering time via *FLOWERING LOCUS C*. New Phytol 194:666–675

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15:2532–2550