N-glucosylation of cytokinins by glycosyltransferases of Arabidopsis thaliana

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ABSTRACT

Cytokinins are plant hormones that can be glucosylated to form O-glucosides and N-glucosides. The glycoconjugates are inactive and are thought to play a role in homeostasis of the hormones. Whilst O-glucosyltransferases have been identified that recognise cytokinins, the enzymes involved in N-glucosylation have not been identified even though the process has been recognised for many years. This study utilises a screening strategy in which 105 recombinant glycosyltransferases (UGTs) of Arabidopsis have been analysed for catalytic activity towards the classical cytokinins: trans-zeatin, dihydrozeatin, N⁶-benzyladenine, N⁶-isopentenyladenine and kinetin. Five UGTs were identified in the screen. UGT76C1 and UGT76C2 recognised all cytokinins and glucosylated the hormones at the N⁷ and N⁹ positions. UGT85A1, UGT73C5 and UGT73C1 recognised trans-zeatin and dihydrozeatin, which have an available hydroxyl group for glucosylation and formed the O-glucosides. The biochemical characteristics of the N-glucosyltransferases were analysed and highly effective inhibitors of their activities were identified. Constitutive over-expression of UGT76C1 in transgenic Arabidopsis confirmed that the recombinant enzyme functioned in vivo to glucosylate cytokinin applied to the plant. The role of the N-glucosyltransferases in cytokinin metabolism is discussed.
INTRODUCTION

The family of plant hormones, known as cytokinins, were discovered by their ability to induce cell division in cell cultures (1, 2). Naturally occurring cytokinins are adenine derivatives and, dependent on the nature of the N^6-side chain, are classified into isoprenoid or aromatic cytokinins (3). In the plant, cytokinins are known to be involved in many different metabolic and developmental processes (4–7) that are often also influenced by other stimuli including environmental factors and additional hormones (3, 8–10).

The metabolism of cytokinins is highly complex and little is known of the mechanisms that regulate homeostasis. Cytokinins can be present in plants as the free base and the corresponding nucleosides and nucleotides. Inter-conversions amongst these three forms are most probably carried out by enzymes involved in general purine metabolism and their impact on cytokinin activity, transport and homeostasis remain unknown (3, 11). In contrast, modifications of the N^6-side chain are known to have profound effects on cytokinin activity and extensive studies over many years have analysed structure-activity relationships of the side chain (12). Genes encoding enzymes involved in cytokinin biosynthesis have been identified (3, 13–16) and processes leading to irreversible degradation by cytokinin oxidases have also been well characterised (17, 18). The physiological significance of the biosynthetic and degradative pathways has been confirmed in transgenic studies (15, 19–22).

Conjugation of cytokinins also occurs and can involve O-xylosylation, O-glucosylation and N-glucosylation (23–30). Whilst all of these cytokinin glycosides are known to be inactive, their role in homeostasis is uncertain (3, 13). However, O-glucosylation is reversible and the O-glucosides are resistant to cleavage of the N^6-side chain by cytokinin oxidases (17, 18). Since these conjugates can be converted back into active cytokinins by β-glucosidases (31, 32), it has been suggested that they represent inactive, stable storage forms of the hormone. When plants are subjected to high levels of cytokinin application, the major
conjugate that forms is the 7-N-glucoside (33, 34). In contrast to the O-glucosides, the 7-N- and 9-N-glucosides are resistant to glucosidases (32, 35) and this feature, together with their accumulation in the plant has led to the suggestion that N-glucosylation is involved in detoxification (35). The N-glucosylation of cytokinins has thus attracted considerable interest, since understanding of the enzyme(s) and catalytic mechanism(s) could aid the design of novel long-lasting cytokinin analogues for use in field applications.

We now present data to show we have identified Arabidopsis genes encoding enzymes capable of cytokinin N-glucosylation. Glycosyltransferases have been classified into 69 multigene families (36, 37, http://afmb.cnrs-mrs.fr/CAZY/), of which Family 1 includes enzymes involved in small molecule glucosylation, such as conjugation of plant hormones (38). In the Arabidopsis genome, open-reading frames corresponding to these glycosyltransferases (UGTs) number 107 and can be further classified into 14 subgroups (39, 40). Through in vitro screening >100 recombinant UGTs from this multigene Family 1, two UGTs capable of N-glucosylation and three UGTs capable of O-glucosylation have now been identified. This study focuses on the biochemical characterisation of the N-glucosyltransferases and shows that over-expression of one of these UGTs in planta leads to an enhanced accumulation of the 7-N-glucoside on spraying with cytokinin.
EXPERIMENTAL PROCEDURES

Materials—Cytokinins, cytokinin glycosides (N⁶-benzyladenine-3-N-glucoside, N⁶-benzyladenine-7-N-glucoside, N⁶-benzyladenine-9-N-glucoside, dihydrozeatin-7-N-glucoside, dihydrozeatin-9-N-glucoside, dihydrozeatin-O-glucoside, N⁶-isopentenyladenine-7-N-glucoside, N⁶-isopentenyladenine-9-N-glucoside, kinetin-3-N-glucoside, trans-zeatin-7-N-glucoside, trans-zeatin-9-N-glucoside, trans-zeatin-O-glucoside, trans-zeatin-9-N-riboside, trans-zeatin-9-N-ribosyl-O-glucoside, trans-zeatin-9-N-glucosyl-O-glucoside, meta-topolin-9-N-glucoside) and cytokinin analogues used in this study were purchased from OlChemIm Ltd (Czech Republic). The plants used were grown in Levington’s seed and modular compost in a controlled environment of 16/8 h light-dark cycle (light, ~150 µmol photons m⁻²s⁻¹, 22 °C; dark, 18 °C).

Glucosyltransferase Activity Assay—The construction of the UGT expression plasmids was described in our previous report (39). The plasmids were transformed into Escherichia coli XL-1 Blue individually for recombinant protein expression. Recombinant UGTs were purified as fusion proteins with GST (glutathione-S-transferase) attached to the N-terminus of the UGTs (41). 105 UGTs out of the total 107 UGTs (40) were subjected to the initial screen (Table S1). UGTs 76E9 and 90A1 were not included due to the inability to recover recombinant proteins. The glucosyltransferase assay was carried out following the conditions described by Mok and co-workers with modifications (23, 24). In the initial screen, each assay mix (200 µl) contained 1 µg of recombinant protein, 50 mM Tris-HCl, pH 8.0, 2.5 mM UDP-glucose, 0.5 mM ATP, 50 mM MgCl₂, and 0.5 mM of a cytokinin substrate. The reaction was carried out at 30 °C for 3 h, and was stopped by the addition of 20 µl of trichloroacetic acid (240 mg/ml), quick-frozen and stored at −20 °C prior to the reverse-phase HPLC analysis. The specific enzyme activity was expressed as nanomoles of cytokinins glucosylated per second (nanokatal, nkat) by 1 mg of protein.
Identification of the Bacterial Chaperonin GroEL Co-Purifying with UGTs 76C1 and 76C2—The polyacrylamide gel containing the polypeptide which co-purified with recombinant UGTs 76C1 and 76C2 was excised for in-gel tryptic digestion. After reduction with DTT and S-carboxymethylation with iodoacetamide, the gel pieces were washed three times with 50% (v/v) aqueous acetonitrile containing 25 mM ammonium bicarbonate and dried in vacuo. The gel pieces were rehydrated in 10 µl trypsin solution (0.2 µg trypsin, 10 mM acetic acid, 20 mM ammonium bicarbonate) and were incubated overnight at 37 °C. A 0.5 µl aliquot of trypsin solution was applied directly to the Matrix-Assisted Laser Desorption/Ionization (MALDI) target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/ml solution of 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode with an accelerating voltage of 20 kV. MS spectra were acquired with a total of 1000 laser pulses over a mass range of m/z 800–4000. Final mass spectra were the summation of 20 sub-spectra, each acquired with 50 laser pulses, and internally calibrated using the tryptic peptides at m/z 842.509 and 2211.104. Monoisotopic masses were obtained from centroids of raw, unsmoothed data.

For Collision-Induced Dissociation (CID)-MS/MS, a Source 1 accelerating voltage of 8 kV, a collision energy of 1 kV, and a Source 2 accelerating voltage of 15 kV were used. Air was used as the collision gas at the instrument’s ‘medium’ pressure setting with a recharge threshold of 9.9 × 10⁻⁷ torr, which produced a Source 2 pressure of about 1 × 10⁻⁶ torr. The precursor mass window was set to +/-10 “Da”, and the metastable suppressor was enabled. The default calibration was used for MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky-Golay with three points and polynomial order 4).
Mass spectral data obtained in batch mode were submitted to database searching using the Mascot program (Matrix Science Ltd., version 1.9). Batch-acquired MS and MS/MS spectral data were submitted to a combined peptide mass fingerprint and MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 2.0) to Mascot. Search criteria included: Maximum missed cleavages, 1; Variable modifications, Oxidation (M), Carbamidomethyl; Peptide tolerance, 200 ppm; MS/MS tolerance, 0.15 Da.

Removal of the Bacterial Chaperonin GroEL from Recombinant UGTs 76C1 and 76C2—E. coli strain XL1-Blue expressing UGT76C1 or UGT76C2 was grown at 20 °C in 1.5 L 2× YT media containing 50 µg/ml ampicillin until the $A_{600}$ nm reached 1.0. The culture was then incubated with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 24 h at 20 °C to induce synthesis of the recombinant proteins. Cells were harvested (5,000 × g for 5 min), resuspended (5 ml, ice-cold PBS), disrupted by French Press (ThermoElectron), and centrifuged again (40,000 × g for 30 min). The supernatant was mixed with 100 µl of 50% (v/v) glutathione-coupled Sepharose (Pharmacia) at room temperature for 30 min. To remove the GroEL proteins which co-purified with UGTs 76C1 and 76C2, the beads were washed with PBS followed by a wash with 20 mM ATP and 20 mM MgCl$_2$ at room temperature for 30 min prior to the elution of the recombinant proteins with 20 mM reduced-form glutathione, 100 mM Tris-HCl, pH 8.0, 120 mM NaCl.

Steady-State Enzyme Kinetic Measurements—Michaelis-Menten kinetics of the enzymes were measured over a range of 0–0.5 mM of cytokinin substrates in the presence of 1 µg of recombinant protein, 100 mM MES, pH 7.0, 2.5 mM UDP-glucose, 0.5 mM ATP, 50 mM MgCl$_2$. The reactions (200 µl) were carried out at 30 °C for 1 h. The kinetic parameters were derived using Hyperbolic Regression Analysis of Hyper32 programme available from http://homepage.ntworld.com/john.easterby (Copyright J. S. Easterby).
HPLC Analysis of the in Vitro Reaction Mixtures—Reverse-phase HPLC (SpectraSYSTEM HPLC systems and UV6000LP Photodiode Array Detector, ThermoQuest) analysis was carried out using a Columbus 5 µ C18 column (250 × 4.60 mm, Phenomenex) maintained at 30 °C. The data were acquired and analysed using the software ChromQuest version 2.51. Two different HPLC methods were developed for the analysis of cytokinins and their analogues. Each recombinant UGT activity assay containing a single substrate was analysed using one of the methods described in the following.

The first HPLC method employed a linear gradient of 10–100% methanol against H2O (all solutions contained 2.5 ml/L of glacial acetic acid and 0.4 ml/L of triethylamine) with a flow rate at 1 ml/min over 25 min. Each peak on the chromatogram was scanned between 200–400 nm (photodiode array profile) and was integrated at 270 nm. This method was used to analyse kinetin (retention time, $R_t = 14.0$ min), kinetin-3-$N$-glucoside ($R_t = 7.0$ min), kinetin-7-$N$-glucoside ($R_t = 10.4$ min), kinetin-9-$N$-glucoside ($R_t = 11.7$ min), $N^\delta$-isopentenyladenine ($R_t = 16.6$ min), $N^\delta$-isopentenyladenine-7-$N$-glucoside ($R_t = 12.2$ min), $N^\delta$-isopentenyladenine-9-$N$-glucoside ($R_t = 14.5$ min), $N^\delta$-benzyladenine ($R_t = 16.2$ min), $N^\delta$-benzyladenine-3-$N$-glucoside ($R_t = 9.1$ min), $N^\delta$-benzyladenine-7-$N$-glucoside ($R_t = 12.5$ min), $N^\delta$-benzyladenine-9-$N$-glucoside ($R_t = 14.0$ min), olomoucine ($R_t = 16.4$ min), olomoucine glucoside ($R_t = 15.1$ min), thidiazuron ($R_t = 17.1$ min), ortho-topolin ($R_t = 15.4$ min), meta-topolin ($R_t = 13.4$ min), meta-topolin-9-$N$-glucoside ($R_t = 11.5$ min), 3-isobutyl-1-methylxanthine ($R_t = 15.9$ min), N-(2-chloro-4-pydiryl)-$N^\prime$-phenylurea ($R_t = 19.1$ min) and 3-methyl-7-pentylamino-pyrazolo[4,3-D]-pyrimidine ($R_t = 17.0$ min).

The second HPLC method employed a linear gradient of 10–60% methanol against H2O (all solutions contained 2.5 ml/L of glacial acetic acid and 0.4 ml/L of triethylamine) with a flow rate at 1 ml/min over 25 min. Each peak on the chromatogram was scanned between 200–400 nm (photodiode array profile) and was integrated at 270 nm. This method was used
to analyse trans-zeatin ($R_t = 14.5$ min), trans-zeatin-7-N-glucoside ($R_t = 11.3$ min), trans-zeatin-9-N-glucoside ($R_t = 12.6$ min), trans-zeatin-O-glucoside ($R_t = 12.9$ min), trans-zeatin-9-N-riboside ($R_t = 16.3$ min), trans-zeatin-9-N-ribosyl-O-glucoside ($R_t = 14.5$ min), trans-zeatin-7-N-glucosyl-O-glucoside ($R_t = 10.5$ min), trans-zeatin-9-N-glucosyl-O-glucoside ($R_t = 11.1$ min), cis-zeatin ($R_t = 13.5$ min), cis-zeatin-7-N-glucoside ($R_t = 11.2$ min), cis-zeatin-9-N-glucoside ($R_t = 11.8$ min), cis-zeatin-O-glucoside ($R_t = 12.2$ min), dihydrozeatin ($R_t = 15.0$ min), dihydrozeatin-7-N-glucoside ($R_t = 11.7$ min), dihydrozeatin-9-N-glucoside ($R_t = 13.0$ min), dihydrozeatin-O-glucoside ($R_t = 14.0$ min), dihydrozeatin-9-N-glucosyl-O-glucoside ($R_t = 12.5$ min).

Adenine ($R_t = 12.9$ min) and guanine ($R_t = 8.8$ min) were analysed by a linear gradient of 10–20% methanol against 50 mM ammonium formate buffer pH 4.6 over 20 min with a flow rate at 0.5 ml/min. Each peak on the chromatogram was scanned between 200–400 nm (photodiode array profile) and was integrated at 254 nm.

Identification and Quantification of the Glucosides Synthesised During the in Vitro Reactions—The nature of glucosides synthesised by recombinant UGTs in this study was confirmed by comparing the photodiode array profile and the retention time of the glucosides on the chromatogram with the authentic compounds when these were available (see Materials section). For those glucosides that were not available commercially, their chromatograms were compared with the glucosides of similar chemical structures. Kinetin-7-N-glucoside and kinetin-9-N-glucoside were assigned by comparing with the chromatogram that separating $N^6$-benzyladenine and its glucosides. cis-Zeatin-7-N-glucoside, cis-zeatin-9-N-glucoside and cis-zeatin-O-glucoside were assigned similarly using the chromatogram separating trans-zeatin and trans-zeatin glucosides. trans-Zeatin-7-N-glucosyl-O-glucoside, dihydrozeatin-9-N-glucosyl-O-glucoside and olomoucine glucoside were confirmed by hydrolysis of the O-glucosidic bond using β-glucosidase leading to the formation trans-zeatin-7-N-glucoside,
dihydrozeatin-9-N-glucoside and olomoucine respectively. The glucosides were quantified using either the extinction coefficient of the authentic glucosides or the extinction coefficient of the aglycone after hydrolysis of the corresponding glucosides.

**Over-Expression of UGT76C1 in Arabidopsis**—The binary plasmid pJR1Ri carrying UGT76C1 cDNA was introduced into *A. thaliana* via *Agrobacterium tumefaciens*-mediated vacuum infiltration (42). To select the transgenic plants, seeds collected from vacuum-infiltrated plants were surfaced sterilized with 10% (v/v) Chloros solution (Scientific Laboratory Supplies, Nottingham, U.K.) with drop of Tween 80 followed by 70% (v/v) ethanol, and were germinated on ATS agar (10 g/L sucrose, 5 ml/L of 1 M KNO₃, 2.5 ml/L of 1 M KPO₄, pH 5.5, 2 ml/L of 1 M MgSO₄, 2 ml/L of 1 M Ca(NO₃)₂, 2.5 ml/L of 20 mM EDTA, 1 ml/L micronutrients and 0.8% [w/v] Oxoid agar [Oxoid Ltd, Hampshire, U.K.]) containing kanamycin (50 mg/L) and cefotaxime (50 mg/L). All plants were homozygous for the transgene. The control plant, designated Ri, was transformed with an empty pJR1Ri vector.

**Northern Blot Analysis**—Total RNA was extracted from the transgenic plants following the method of Verwoerd *et al.* (43). An aliquot of total RNA (10 µg) was separated on 0.6 M formaldehyde/1.4% (w/v) agarose containing 0.5 µg/ml ethidium bromide, blotted onto Hybond-N membrane, UV-crosslinked (120 mJ), and probed with radio-labelled DNA fragments prepared with random primers.

**Crude Protein Extraction from Transgenic Plants and the Glucosyltransferase Assay**—To obtain the crude protein extract from the transgenic plants, 1 g of frozen tissue was ground to fine powder in liquid nitrogen using pestle and mortar. 1 ml of extraction buffer (25 mM Tris-MES, pH 6.5, 10% [v/v] glycerol, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1% polyvinylpolypyrrolidone [w/w of aerial tissue]) was added to the powder and the slurry was thawed on ice. Subsequently the slurry was mixed vigorously and centrifuged at 10500 × g, 4 °C for 20 min. 850 µl of the supernatant were collected and
transferred to a 1.5 ml microfuge tube and were further centrifuged at 15000 \times g, 4 \, ^\circ \text{C} \text{ for 5 min. The resulting supernatant was collected for glucosyltransferase activity assay. The protein concentration assay was carried out with Bio-Rad Protein Assay Dye using bovine serum albumin as reference.}

To investigate the glucosyltransferase activity of the crude protein extracts prepared from plant tissues, 50 \, \mu l crude protein extracts (containing 0.1–0.3 mg of total protein) were mixed with 1 mM \textit{trans}-zeatin, 5 mM UDP-glucose and 100 mM MES, pH 7.0 in a 100-\mu l reaction. The reactions were incubated at 30 \, ^\circ \text{C} \text{ for 1 h and were stopped by the addition of 10 \, \mu l of trichloroacetic acid (240 mg/ml). The reaction mixtures were analysed subsequently using reverse-phase HPLC following the method described above.}

\textit{Exogenous Application of trans-Zeatin to Transgenic Plants Over-Expressing UGT76C1 cDNA}—Transgenic line 4/1 and a control line Ri were selected and sprayed with \textit{trans}-zeatin exogenously. \textit{trans}-Zeatin was dissolved in distilled water containing 1\% DMSO and 0.05\% Tween 20 to a final concentration of 0.5 mM, and was sprayed either on its own or together with the inhibitors (1 mM 3-isobutyl-1-methylxanthine or 0.05 mM olomoucine) onto the plants using a spray bottle. At appropriate time points, approximately 1 g of aerial tissues from each line was collected, frozen in liquid nitrogen, and stored at \textendash 80 \, ^\circ \text{C} \text{ prior to the extraction.}

\textit{HPLC Analysis of the trans-Zeatin Glucosides in Transgenic Plants}—To analyse the amount of the glucosides of interest in the transgenic plants, 1 g of plant tissue was ground to fine powder and was extracted with 10 ml of 80\% methanol. The slurry was left at room temperature for 1 h followed by centrifugation at 5000 \times g \text{ for 5 min. After filtration, the supernatant was collected, concentrated in vacuo and analysed with HPLC. The HPLC analysis was carried out as described above with some modifications. The methanol extract was analysed with a linear gradient of methanol in H_{2}O (all solutions contained 2.5 ml/L of
glacial acetic acid and 0.4 ml/L of triethylamine) at 1 ml/min from 10–100% over 40 min, 30 °C and monitored between 200–400 nm. The peaks corresponding to the trans-zeatin glucosides were integrated at 270 nm and were quantified using the extinction coefficient of authentic glucosides. 0.1 mM $N^6$-isopentenyladenine was added as internal control at the beginning of the extraction to monitor the recovery rate.
RESULTS

Recombinant UGTs of Arabidopsis Recognise Cytokinins—Members of the multigene family of UGT sequences of Arabidopsis were expressed as recombinant fusion proteins in E. coli, purified by affinity chromatography and screened in vitro for activity against each of the five classical cytokinins: trans-zeatin, dihydrozeatin, N\(^6\)-benzyladenine, N\(^6\)-isopentenyladenine and kinetin. Only five from the 105 sequences analysed (Table S1) were found to have activity towards the cytokinins. Two UGTs, 76C1 and 76C2, can glucosylate all of the individual cytokinins at either the N\(^7\) or N\(^9\) position, and three UGTs, 85A1, 73C5 and 73C1, can glucosylate the OH group on the N\(^6\) side chain of trans-zeatin and dihydrozeatin. Representative profiles from standard HPLC methods are shown in Figure 1 to illustrate the separation of the different glucosides of trans-zeatin and N\(^6\)-benzyladenine. The specific activity of the UGTs against the five substrates is summarised in Table I.

To gain insight into substrate recognition by these five enzymes, their activity towards a further thirteen compounds was assayed and results shown in Table II. Interestingly, O-glucosylation of trans-zeatin greatly reduced the activity of the N-glucosyltransferases towards the substrate. In a similar manner, N-glucosylation of dihydrozeatin abolished activity of the O-glucosyltransferase, UGT85A1. These data imply that glucosylation at one site on the cytokinin strongly influences glucosylation at the second site. In contrast to earlier studies on cytokinin O-glycosyltransferases from other plant species (27, 28), recombinant UGTs from Arabidopsis recognised both trans- and cis-zeatin in vitro.

Figure 2A shows an SDS-PAGE analysis of the five UGTs capable of glucosylation of cytokinins in vitro. The molecular weights of the fusion proteins range between 66 kDa and 84 kDa. Additional smaller-sized polypeptides were found in the affinity-purified samples of the UGTs 76C1 and 76C2 fusion proteins. Following an in-gel tryptic digestion and by MALDI-MS analysis, these polypeptides were unambiguously identified as the bacterial
chaperonin GroEL with a combined Mowse score of 1200 from a Mascot search against all entries of the NCBI nr database. A total of 27 peptides from the protein were observed in the MALDI-MS spectrum of the tryptic digest, which together accounted for 66% of the amino acid sequence of the protein. CID-MS/MS spectra of six of these peptides gave Mascot MS/MS-ion-search scores with confidences greater than 99.99%, and an additional three gave scores with confidences greater than 99%. As described in the Experimental Procedures, the GST affinity matrix binding the UGT fusion protein/GroEL complexes was pre-treated with 20 mM ATP and 20 mM MgCl₂ to remove the bacterial chaperonin prior to elution of the UGTs with the reduced form of glutathione. As shown in Figure 2B, an incubation of 30 min was sufficient to remove the chaperonin, and the UGTs were used in the following kinetic analysis. No effect of the treatment on UGT activities was observed (data not shown).

**Steady-State Kinetics of the N-Glucosyltransferases UGTs 76C1 and 76C2**—Results in Figure 3 summarise the optimisation of reaction conditions for the N-glucosyltransferases. Both UGTs, 76C1 and 76C2, have a pH optimum of ~7.0 and their activities can be enhanced ~1.5-fold by the addition of 1 mM DTT. The reactions are linear up to 60 min. Under these conditions, both UGTs displayed high $k_{cat}/K_m$ values towards $N^6$-benzyladenine and $N^6$-isopentenyladenine (>10 mM⁻¹s⁻¹) as shown in Table III. Taken together with their low $K_m$ values towards these two substrates, it is clear that $N^6$-benzyladenine and $N^6$-isopentenyladenine are the preferred substrates for UGTs 76C1 and 76C2 from those tested. The $K_m$ values of UGTs 76C1 and 76C2 towards $N^6$-benzyladenine, 0.09 mM and 0.04 mM respectively, are similar to those reported in the study (28) of cis-ZOG1 and cis-ZOG2 towards cis-zeatin (0.046 mM and 0.096 mM respectively). There are no kinetic data from other cytokinin-O-glycosyltransferases available for comparison.

**Inhibition of the Cytokinin N-Glucosyltransferases**—In early studies of the metabolism of leaf discs, co-incubation of substituted xanthines or olomoucine (6-benzylamino-2-[2-
hydroxyethylamino]-9-methylpurine) with cytokinins was found to decrease the level of N-glucosides in tissue extracts (45–47). It was suggested that these compounds might directly inhibit the activity of the N-glucosyltransferase(s). Figure 4 shows the effects of a substituted xanthine (3-isobutyl-1-methylxanthine) and olomoucine on the activity of UGTs 76C1 and 76C2. It can be seen that both compounds inhibit the activities of UGTs (Fig. 4A). Lineweaver-Burk plots of the inhibition experiments showed that the trendlines intercepted on the Y-axis suggesting that these compounds are competitive inhibitors (Fig. 4B and 4C). Comparing the $K_i$ values of the two inhibitors, olomoucine is clearly more effective. The $K_i$ value of olomoucine derived in this study is near-identical to that reported previously when crude enzyme extracts were used to analyse N-glucosyltransferase activity (47).

**Analysis of Transgenic Arabidopsis Plants in which UGT76C1 is Constitutively Over-Expressed**—The cauliflower mosaic virus 35S (CaMV35S) promoter was used to drive the expression of UGT76C1 cDNA in transgenic Arabidopsis. Eight independent homozygous lines were obtained and 4-week-old aerial tissue was collected and subjected to Northern analysis. Figure 5A shows that six transgenic lines (1/1, 4/1, 14/3, 15/1, 18/1 and 19/3) expressed high steady-state levels of UGT76C1 mRNA in comparison to those observed in the wild-type (Wt) and empty-vector control (Ri) plants. Two lines (3/1 and 6/4) showed negligible expression of UGT76C1. Leaves from the transgenic plants were harvested, extracted and analysed for enzyme activity towards trans-zeatin. The results are shown in Figure 5B and demonstrate that steady-state levels of mRNA correlate with enzyme activity to form N-glucosides. No significant enzyme activity to form O-glucosides was detected in the crude extracts (data not shown). The consequences of applying exogenous cytokinin to the transgenic line over-expressing UGT76C1 was analysed and the results compared to those obtained with the vector-only control. Figure 6A shows the HPLC traces of extracts from leaves harvested at 6 h following spray treatment with cytokinin or buffer-only. The results
indicate that there are negligible levels of the 7-N-glucoside in either the Ri control or the over-expressor after spraying with buffer. This indicates that over-expression of the UGT alone does not lead to increased level of product. On application of cytokinin, 7-N-glucoside can be detected in the Ri control, but the level was much greater in the transgenic line. Only trace levels of other glucosides were ever detected. Figure 6B illustrates a time-course of accumulation of the 7-N-glucoside and the effects of co-spraying the trans-zeatin with inhibitors of the N-glucosyltransferase. In the presence of either of the inhibitors, the level of 7-N-glucoside in the leaves of the control or those of the over-expressor line was substantially reduced.
DISCUSSION

Glucosylation of cytokinins is a well-recognised modification that is thought to play an important role in hormonal homeostasis. Whilst O-glucosylation and N-glucosylation of cytokinins have been described and reviewed, only O-glucosylation has been investigated in depth due to the availability of genes encoding the enzymes and biochemical analyses of their recombinant products (3). Genes encoding these enzymes from several plant species have been identified by Mok and co-workers, including ZOG1 from Phaseolus lunatus, ZOX1 from Phaseolus vulgaris, and cis-ZOG1 and cis-ZOG2 from maize (24, 25, 27, 28). In contrast, nothing has been known of the N-glucosyltransferases of cytokinins, except early biochemical studies on partially purified enzyme preparations from a range of different plant species (29, 30, 35). We now report the identification of two UGTs from Arabidopsis that recognise the classical cytokinins and form the 7-N- and 9-N-glucosides within an in vitro assay. In addition, we also found three UGTs that form the O-glucoside with trans-zeatin, cis-zeatin and dihydrozeatin. Whilst we do not know as yet whether cytokinins are the natural substrates of these enzymes in vivo, we have confirmed that the recombinant UGT76C1 can function in planta, continue to recognise cytokinin as a substrate and synthesise the 7-N-glucoside. The genes we have identified can now be used in a number of genetic studies to define their endogenous substrates and in particular, the consequences of N-glucosylation on cytokinin homeostasis.

Three N-glucosides of cytokinin have been described in the literature (29, 30, 34, 35, 48). These include the products of glucosylation at the N7 and N6 positions, and at the N3 position. In this study, none of the 105 recombinant UGTs that were assayed produced a 3-N-glucoside with any of the cytokinins. Whilst 3-N-glucosides have been reported in some plant species such as radish (34), to our knowledge their existence in Arabidopsis has not been investigated previously.
The UGTs, 76C1 and 76C2, catalysed the formation of both the 7-N- and the 9-N-glucoside of each of the cytokinins analysed. This observation may be due to the known tautomeration of adenine, in which a hydrogen atom attaches either to the \( \tilde{N}^7 \) (7-H tautomer) or the \( N^9 \) position (9-H tautomer) of the molecule. In these circumstances in aqueous solutions, the 7-H tautomer is the favoured state (49) leading to an increased availability of the 7-N for glucosylation by the UGTs. This may be the explanation for the higher levels of the 7-N-glucoside produced by the two UGTs in vitro.

\( N \)-glucosylation has attracted considerable interest over many years, since it is considered to be a major barrier to the successful use of cytokinins in field applications (45, 46). As a consequence there have been a number of studies to investigate the ways in which the process could be inhibited (45–47). For example, the efficacy of several structurally-related compounds were assayed in tissue extracts and led to the identification of substituted xanthines as useful inhibitors. It was speculated that compounds such as those would act as inhibitors of the \( N \)-glucosyltransferases, but this could not be proven due to the lack of purified enzyme(s). We now confirm, using the recombinant UGTs, that both 3-isobutyl-1-methylxanthine and olomoucine are highly effective inhibitors of \( N \)-glucosyltransferases.

Among the five UGTs reported in this study, only UGTs 76C1 and 76C2 are closely-related with 60% identity at the amino acid level. When these sequences are compared to the enzymes identified in Zea and Phaseolus by Mok and co-workers, only low sequence similarity was observed (<30%). The Zea and Phaseolus UGTs form a unique branch on the phylogenetic tree containing 107 Arabidopsis UGTs (Fig. 7). It is possible that the Zea and Phaseolus UGTs evolved from a common ancestor distinct from those identified in Arabidopsis (39, 40). Within the Arabidopsis UGT family, the UGTs capable of glucosylating cytokinins have evolved from three separate branches. To-date, the two branches containing UGTs 76C1, 76C2 and 85A1 have not been associated with any catalytic activity. In contrast,
UGTs 73C1 and 73C5 evolved from the branch which consists of UGTs that recognise a number of metabolites. For example, UGT73C6 has been reported as a flavonoid-7-O-glycosyltransferase (58). The UGTs in the same branch are also capable of conjugating the model substrates hydroxycoumarins in vitro (53). In addition, UGT73C5 has been described in another recent study as a glycosyltransferase responsible for fungal toxin detoxification in planta (51). It is possible UGT73C5 recognises multiple endogenous compounds and xenobiotics including cytokinins, flavonoids, hydroxycoumarins and fungal toxins. Alternatively, the in vitro analysis may only reflect catalytic activity towards a structural feature(s) common to a range of secondary metabolites.

Recent studies have analysed the effects of cytokinin application on global gene expression using a DNA microarray strategy (50). Expression profiling using an 8300-element Arabidopsis Affychip examined cytokinin-responsive genes over a 24 h time period. The expression of the cytokinin oxidase genes was significantly up-regulated in these experiments. Only two of the UGTs described in this report were represented in the Affychip – UGTs 73C5 and 73C1, and the expression of neither of these genes was up-regulated.

Our studies have revealed a number of UGTs with catalytic activities towards important plant metabolites such as hydroxycinnamates, hydroxybenzoates and hormones, such as auxin (41, 44, 52–55). The constitutive over-expression of these UGTs has been found to lead to a diversity of effects, dependent on the aglycone that is glucosylated (54, 56). For example, when UGT84B1 recognising IAA was constitutively over-expressed, the transgenic lines showed many features typical of an auxin-deficient phenotype (56). The lack of gravitropism displayed by the root system of these plants was recovered by application of an auxin analogue 2,4-D, that was not glucosylated by the UGT (55, 56). Metabolite profiling of the transgenic lines confirmed very high levels of the IAA-glucose ester, but levels of IAA were also elevated, suggesting that changing the IAA conjugate levels in the plant caused a
complex set of events to occur (56). As yet, we have only a preliminary analysis of the transgenic lines described in this study over-expressing the UGT76C1. However, under normal growth conditions, over-expression of the cytokinin N-glucosyltransferase seemingly has little effect on phenotype, even though the enzyme was confirmed to be active in planta and able to conjugate exogenous cytokinin. In contrast, the studies of Mok and co-workers clearly showed a distinct phenotype when an O-glucosyltransferase from Phaseolus lunatus (24) was over-expressed constitutively in tobacco callus (57). The O-glucoside was found to accumulate and the cell cultures required 10-fold higher concentrations of trans-zeatin for the induction of differentiation.

Detailed analyses of transgenic Arabidopsis lines in which the five glycosyltransferases of cytokinins are silenced or over-expressed are now possible and will provide greater insight into the role of glucosylation in cytokinin homeostasis.

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FOOTNOTES

The nucleotide sequences corresponding to the UGTs described in this study have been submitted to the GenBank™/EBI Data Bank and have been published previously (39, 40). The accession numbers of these sequences are: AB017060 (UGT76C1), AB005237 (UGT76C2), AC006551 (UGT85A1), AC006282 (UGT73C5) and AC006282 (UGT73C1).
FIGURE LEGENDS

FIG. 1. **HPLC analysis of cytokinins and their glucoconjugates.** The reaction mixes contained a single cytokinin (A, trans-zeatin; B, \(N^6\)-benzyladenine) incubated with UGT 76C1, 76C2 or 85A1. For trans-zeatin conjugates, a linear gradient of methanol from 10–60% at 1 ml/min flow rate over 25 min was used, for \(N^6\)-benzyladenine conjugates, a linear gradient of methanol from 10–100% was used (all solutions contained 2.5 ml/L of glacial acetic acid and 0.4 ml/L of triethylamine).

FIG. 2. **SDS-PAGE analysis of the five recombinant UGTs capable of cytokinin glucosylation in vitro.** (A) The UGTs were analysed on a 10% (w/v) polyacrylamide gel and visualised with Coomassie Brilliant Blue staining. The smaller polypeptide co-purifying with UGTs 76C1 and 76C2 fusion proteins was confirmed to be the bacterial chaperonin GroEL. (B) SDS-PAGE analysis of UGTs 76C1 and 76C2 eluted from the affinity matrix post-treated with MgCl₂/ATP.

FIG. 3. **Biochemical characterisation of UGTs 76C1 and 76C2.** (A) The pH optimum of the UGTs was measured over the range pH 5.0–9.0 in the reactions containing 0.5 mM trans-zeatin and 100 mM buffer. The buffers analysed include MES buffer (pH 5.0–7.0), phosphate buffer (pH 6.0–8.0) and Tris-HCl buffer (pH 7.0–9.0). (B) The effect of DTT on the UGT activity was studied by the addition of various concentrations of DTT into the reactions containing 0.5 mM trans-zeatin, 100 mM MES, pH 7.0. The reactions described in (A) and (B) were carried out at 30 °C for 1 h. (C) The time course of the UGT activity was studied by measuring the amount of glucoside formed by 1 µg of recombinant enzyme in 100 mM MES, pH 7.0 at 30 °C in the presence of 0.5 mM trans-zeatin. The results represent means ± S.D. from three replicates.
Fig. 4. Inhibition of the activity of UGTs 76C1 and 76C2. (A) The inhibition of the activity of UGTs 76C1 and 76C2 was studied by addition of two potential inhibitors into the reactions containing 0.5 mM trans-zeatin and 100 mM MES, pH 7.0 incubated at 30 °C for 1 h. The results represent the means ± range from two replicates. (B) Characterisation of the inhibition of 3-isobutyl-1-methylxanthine to the UGT activity. The UGT activity was measured in the reactions containing 0–0.1 mM trans-zeatin, 1 mM DTT, 100 mM MES, pH 7.0 and 0.25 mM/0.50 mM 3-isobutyl-1-methylxanthine. (C) Characterisation of the inhibition of olomoucine to the UGT activity. The reactions were set up as described in (B) except the inhibitor 3-isobutyl-1-methylxanthine was replaced by 5 µM/10 µM olomoucine and the activity was measured with 0–0.3 mM trans-zeatin.

Fig. 5. Analysis of the transgenic plants overexpressing UGT76C1 cDNA using the CaMV35S promoter. (A) Aerial tissues from 4-week-old transgenic plants, the control plant Ri and the wild-type plant (Wt) were used to analyse the steady-state levels of mRNA. Total RNA (10 µg) was analysed by 1.4% (w/v) agarose gel and visualised with ethidium bromide staining (bottom). The RNA was then transferred onto nylon membrane and subsequently probed with radiolabelled DNA fragments of UGT76C1 (top panel) and β-ATPase (middle panel). (B) Crude protein extracts were prepared from 4-week-old aerial tissues of the plants described above. The UGT activity in the crude protein extracts was measured following the method described in “Experimental Procedures”. The specific enzyme activity was expressed as pmol of trans-zeatin glucosylated to form 7-N- and 9-N-glucosides per second (pkat) by 1 mg of protein in 1 h of reaction time at 30 °C.
**FIG. 6.** Exogenous application of trans-zeatin to transgenic plants over-expressing UGT76C1 cDNA. (A) The HPLC trace (monitored at 270 nm) of the extracts from the transgenic line 4/1 and the control line Ri sprayed with 0.5 mM trans-zeatin or buffer (1% DMSO and 0.05% Tween 20). Peak 1, trans-zeatin-7-N-glucoside; Peak 2, trans-zeatin-9-N-glucoside and trans-zeatin-O-glucoside (overlapped); Peak 3, trans-zeatin applied exogenously; Peak 4, N^6^-isopentenyladenine added as an internal control at the beginning of the extraction process. The extracts were analysed with a linear gradient of methanol in H_2O from 10–100% (all solutions contained 2.5 ml/L of glacial acetic acid and 0.4 ml/L of triethylamine) over 40 min at 1 ml/min and monitored at 270 nm. (B) The trans-zeatin-7-N-glucoside levels in the plants sprayed with 0.5 mM trans-zeatin in the presence or absence of inhibitors (1 mM 3-isobutyl-1-methylxanthine [IMX] or 0.05 mM olomoucine).

**FIG. 7.** Phylogenetic tree of the amino acid sequences of Arabidopsis UGTs and other cytokinin-O-glycosyltransferases. The phylogeny of 107 Arabidopsis UGTs and 4 cytokinin-O-glycosyltransferases reported previously (ZOG1, accession no. AF101972; ZOX1, accession no. AF116858; cis-ZOG1, accession no. AF318075; cis-ZOG2, accession no. AY082660) (24, 25, 27, 28) was analysed by the neighbour-joining method using the distance matrix derived from the multiple alignment of CLUSTAL W (EMBL, Heidelberg, Germany). The phylogenetic tree was drawn using the software PhyloDraw version 0.8 (Graphics Application Lab., Pusan National University, South Korea). The UGT activities reported in the literature (41, 52, 54, 55, 59), except those conjugating the model substrates hydroxycoumarins (53), are shown in bold. Asterisks indicate the UGTs reported in this study.
**Table I**

*Specific activity of UGTs 76C1, 76C2, 85A1, 73C5 and 73C1 towards cytokinins*

The specific enzyme activity was defined as nmol of substrates converted into glucose conjugates per second (nanokatal, nkat) by 1 mg of protein. –, no significant activity detected (< 0.01 nkat/mg protein).

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TABLE II

Specific activity of UGTs 76C1, 76C2, 85A1, 73C5 and 73C1 towards other related compounds

Thirteen additional compounds related to cytokinins were analysed against the five UGTs that glucosylate cytokinins. Four compounds that can be glucosylated by these UGTs are listed in the Table. The specific enzyme activity was defined as nmol of substrates converted into glucose conjugates per second (nanokatal, nkat) by 1 mg of protein. The remaining nine compounds that cannot be glucosylated by these UGTs are shown in the footnote. –, no significant activity detected (< 0.01 nkat/mg protein).

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</table>

* the compounds that cannot be glucosylated in this study:
TABLE III

The steady-state enzyme kinetic study of UGTs 76C1 and 76C2 towards five classical cytokinins

The steady-state kinetics of UGTs 76C1 and 76C2 were analysed over a range of 0–0.5 mM cytokinins. The reactions were performed in 100 mM MES, pH 7.0 at 30 °C for 1 h. The kinetic parameters $K_m$ and $V_{max}$ were derived using the Hyperbolic Regression Analysis of the Hyper32 programme available from http://homepage.ntworld.com/john.easterby (Copyright J. S. Easterby), and the $k_{cat}$ values were calculated from the $V_{max}$ values. The results represent the means from two replicates ± range.

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Figure 1

A  trans-zeatin

B  N6-benzyladenine

76C1

76C2

85A1

aglycone

9-N-Glc

7-N-Glc

O-Glc

3-N-Glc
Figure 2

A

B

Figure 2
Figure 3
Figure 4
A  

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B  

![Graph](image4.png)

Figure 5
Figure 6
Figure 7
**Supplementary Table S1.** The list of 105 glycosyltransferases that were screened *in vitro* towards cytokinins.

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Bingkai Hou, Eng-Kiat Lim, Gillian S. Higgins and Dianna J. Bowles

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