A maize cytokinin gene encoding an *O*-glucosyltransferase specific to *cis*-zeatin

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Zeatin is a naturally occurring cytokinin. Biosynthesis and metabolism studies of zeatin have been directed mostly at the *trans* isomer, although *cis*-zeatin and its riboside occur as major components in some plant species. It is not known whether parallel regulatory pathways exist for the two isomers. Based on the sequence of the gene *ZOG1* encoding a *trans*-zeatin *O*-glucosyltransferase from *Phaseolus* (EC 2.4.1.203), a *cis*-zeatin-specific *O*-glucosyltransferase was isolated from maize. This gene, *cisZOG1*, contains an ORF of 1,401 nucleotides encoding a protein of 51.1 kDa with 41% identity to the *Phaseolus* ZOG1 protein. Unexpectedly, the maize enzyme recognizes as substrates *cis*-zeatin and UDP-glucose but not *cis*-ribosylzeatin, *trans*-zeatin, or *trans*-ribosylzeatin. This finding indicates the existence of cis-specific regulatory elements in plants and suggests that *cis*-zeatin and derivatives may be more important in cytokinin homeostasis than currently recognized.

Cytokinins are essential hormones for plant growth. In addition to promoting cell division and differentiation in tissue culture, cytokinins regulate a host of developmental events in whole plants such as bud formation, leaf expansion, delay of senescence, promotion of seed germination, and chloroplast formation (reviewed in ref. 1). Naturally occurring cytokinins are adenine derivatives with a side chain at the N^6-position; cytokinins with a hydroxylated isoprenoid side chain such as *trans*-zeatin, first identified in maize (2), are major constituents in plants. Studies on the biosynthesis and metabolism of cytokinins have centered on *trans*-zeatin, although *cis*-zeatin and its derivatives have been isolated from a number of species including potato (3, 4), *Mercurialis* (5), hops (6), rice (7–9), wheat (10), oats (10), and chickpeas (11). This emphasis on the *trans* isomers may be attributed to their higher biological activity.

Two pathways for cytokinin biosynthesis have been proposed. The first is the direct pathway, involving formation of N^6-isopentenyladenosine monophosphate from AMP and dimethylallyl pyrophosphate, followed by hydroxylation of the side chain to form *trans*-zeatin-type cytokinins. In support of this pathway, isopentenyltransferase activity in tobacco (12) and hydroxylation activity in cauliflower (13) have been reported. The discovery of the *Agrobacterium ipt* gene (14, 15) lends support to the existence of a similar biosynthetic pathway in plants. However, plant genes encoding such enzymes have not been identified, and indirect evidence suggests the existence of a biosynthetic pathway for zeatin without isopentenyl intermediates (16, 17). The second, indirect pathway involves release of cytokinins by turnover of tRNAs containing *cis*-zeatin. Because *cis*-zeatin is much less active in bioassays, conversion to the trans isomer by enzymes such as the *cis-trans* isomerase of zeatin (18) may be required. The relative contribution by each pathway and the biosynthetic steps involved remain uncertain (19).

Changes affecting the N^6-side chain of zeatin are important, because even small substitutions have pronounced effects on cytokinin activity (20). The most frequent modifications of the *trans*-zeatin side chain include reduction to dihydrozeatin (21), conjugation to *O*-glycosides (22), and oxidative degradation (reviewed in ref. 23). Comparable metabolic conversions for the side chain of *cis*-zeatin may occur. However, enzymes such as zeatin reductase and *O*-glycosyltransferases are specific to *trans*-zeatin (21, 22). Cytokinin oxidases degrade cytokinins with an unsaturated N^6-side chain, and therefore, both *trans*- and *cis*-zeatin are susceptible (23). Enzymes specific to *cis*-zeatin have not been reported.

Plant cytokinin genes have been elusive, and only recently have genes encoding the zeatin *O*-glucosyltransferase *(ZOG1)* and *O*-xylosyltransferase (*ZOX1*) from *Phaseolus* (24, 25) as well as a cytokinin oxidase from maize (26, 27) been cloned. The *ZOG1* and *ZOX1* genes code for enzymes specific to *trans*-zeatin. Here we report the isolation and characterization of a gene encoding a *cis*-zeatin specific *O*-glucosyltransferase from maize. The finding of a gene and enzyme specific for *cis*-zeatin indicates the need to reassess the function of *cis*-zeatin and its derivatives in cytokinin homeostasis.

Materials and Methods

Plant Materials. Unless otherwise specified, DNA, cDNA libraries, and expressed sequence tags (ESTs) were derived from inbred B73 of *Zea mays*.

Synthesis of Specific Probes to Screen a cDNA Library. The sequence of *ZOG1*, encoding a zeatin *O*-glucosyltransferase of *Phaseolus lunatus* (24) was screened against the maize EST database at Pioneer Hi-Bred International. Candidate ESTs were analyzed further. Specific PCR primers were designed based on the consensus sequence of the UDP-glycosyltransferase signature region of zeatin *O*-glycosyltransferases and a specific region of a selected EST representing an incomplete cDNA. The primers were then used to amplify maize genomic DNA by PCR (28) to obtain a probe for the screening of a cDNA library (see *Results* for primer sequences).

Construction and Screening of a cDNA Library from Maize Kernels. Total RNA was isolated from maize kernels at 35 days after pollination of inbred B73 by using TriPure reagent following the manufacturer's protocols (Roche Molecular Biochemicals). mRNA was then isolated by using the PolyATtract System III (Promega), according to the manufacturer's instructions. The expression/cloning library was constructed by using the SuperScript Plasmid System for cDNA synthesis and plasmid cloning

Abbreviations: EST, expressed sequence tag; RT-PCR, reverse transcription–PCR.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF318075).

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(GIBCO/BRL). First-strand synthesis was primed with a *Not*I–oligo d(T) primer–adapter. After second-strand synthesis, *Sal*I adapters were ligated to the cDNA, which was subsequently digested with *Not*I and size-fractionated. cDNA was directionally inserted into pSPORT1 plasmid (GIBCO/BRL) following the manufacturer's procedure. The cDNA library was screened with ^{32}P-labeled probes by using standard hybridization protocols. Clones were sequenced by the Central Services Laboratory (Center for Gene Research and Biotechnology, Oregon State University, Corvallis) with Applied Biosystems sequence analyzers. Primers were ordered from GIBCO/BRL.

Isolation of Recombinant Proteins. To obtain recombinant proteins, the ORF of the selected cDNA was amplified by PCR by using primers that generated products with a *Nco*I site at the 5′ and a *Xba*I site at the 3′ terminus. The PCR product was digested with *Nco*I and *Xba*I and ligated into Ptrc99A vector (Amersham Pharmacia), which was modified to generate a translational fusion with seven histidine residues at the N terminus of the protein. After transformation into the XL1 Blue cell line, colonies were selected on LB-AMP plates. Individual colonies were grown overnight in LB-AMP medium, and 3 ml of the culture was used to inoculate 300 ml of LB-AMP medium. Induction was achieved with 2 mM isopropyl β-D-thiogalactoside after cells were allowed to grow for 3–4 h (OD at 595 nm of 0.8–1.0). After 4 h, cells were collected and frozen at −80°C overnight, and resuspended in 25 ml of 0.2 M Tris (pH 8.0) containing 0.2 mg/ml lysozyme, 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 μl/ml benzonase (Novagen), and 1.5 μl/ml protease inhibitor mixture Set III (Calbiochem). Samples were incubated on ice for 30 min and sonicated with two 15-sec bursts to release proteins. Soluble proteins were collected after centrifugation and purified on a nickel column (Novagen) by sequential elution with 15, 60, 100, and 500 mM imidazole. Subsequently, imidazole was removed by Centriprep YM-10 (Millipore) ultrafiltration. Both crude extracts and column-purified protein were used for enzyme assays as specified in *Results*.

Western Blot Analyses. For Western blotting, the procedures of Martin *et al.* (29) were followed. The antibody against the histidine tag was obtained from Sigma.

Enzyme Assays and Analysis of Reaction Products. Enzyme activity was determined as reported (22). Briefly, ^{14}C-labeled cytokinins (specific activity of 24 mCi/mmol; 1 Ci = 37 GBq) and glycosyl donor (4 mM UDP-glucose or other glycosyl donors, as specified in *Results*) in 0.17 M Tris (pH 8.0) were incubated with recombinant protein. Reaction products were analyzed by HPLC (22). To characterize the reaction product further, it was treated with β-glucosidase for 2 h as described (30), after which the products were analyzed by HPLC (30).

LC-MS. After enzyme assays with *cis*-zeatin as the substrate and separation of products by HPLC, fractions containing the product (and corresponding to the expected elution of *O*-glucosyl-*cis*-zeatin) were reduced in volume by rotary evaporation and subjected to LC-MS analyses. The chromatography system consisted of a Shimadzu HPLC, a Rheodyne 8125 injector with 5 μl of sample loop, and a 5-μm particle Luna C18(2) 100-mm × 0.32-mm column. Compounds were separated by a linear gradient of 10% to 60% (vol/vol) methanol in 25 min at a flow rate of 5 μl/min. Both the water and methanol HPLC solvents contained 0.1% acetic acid and 0.01% trifluoroacetic acid. A Sciex API III+ triple quadrupole Ionspray Mass Spectrometer (Perkin-Elmer) was used, with an ionspray source. MS/MS spectra were acquired at an orifice voltage of 80 V, a collision energy of 15 eV, and an argon collision gas thickness of 230.

Isolation of the Genomic Sequence. The genomic sequence was isolated by PCR. DNA was isolated from *Z. mays* (B73) by using a modified CTAB (hexadecyltrimethylammonium bromide) method (31). PCR was performed with primers homologous to the 5′ and 3′ regions of the cDNA clone. The products obtained from PCRs were analyzed on a 1% Sea Plaque gel. Bands of interest were excised and DNA was purified with a Qiaex II Gel Extraction Kit (Qiagen, Chatsworth, CA). The products were ligated into a pGem-T Easy Vector (Promega) for sequencing.

Analyses of mRNA Levels. Reverse transcription–PCR (RT-PCR) and molecular beacons (32, 33) were used to determine the relative levels of *cisZOG1* message. Total RNA was isolated from roots, stems, and leaves of 4-week-old corn seedlings, prefertilization cobs, and kernels at 13 days after pollination, according to Chang *et al.* (34). After overnight precipitation with lithium chloride, the pellet was dissolved in SSTE (1 M NaCl/0.5% SDS/10 mM Tris, pH 8.0/1 mM EDTA) and applied to the QIAshredder spin column (RNeasy Plant Mini Kit; Qiagen). The RNeasy Plant Mini Kit protocol was followed subsequently. RNA was eluted with 100 μl of RNase-free water and treated with RQ1 RNase-free DNase (Promega). The DNase was heat inactivated before RT-PCR. The RNA was quantified with a spectrophotometer and stored at −80°C.

Approximately 1 μg of total RNA was used for RT-PCR. Ribosomal RNA (18S) was used as a control. Gene-specific primers were synthesized by GIBCO/BRL, and molecular beacons were synthesized by Operon Technologies(Alameda, CA). Primers and beacons were designed by using the PRIMER 3 program (by Steve Rozen and Helen J. Skaletsky (1998); available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). The melting temperature of the stem was predicted with the DNA MFOLD program of Zuker (35). A Gene AMP Thermostable rTh Reverse Transcriptase RNA PCR Kit (Perkin–Elmer) was used for RT-PCR according to the manufacturer's instructions. The RT reaction was incubated for 5 min at 53°C and 20 min at 70°C in a Robocycler 40 (Stratagene). The RT products were added to the recommended PCR master mix, 1 μM primers, and beacon. PCR was performed on the ABI Prism 7700 Sequence Detection System (PE Biosystems, Branchburg, NJ). The amplification conditions were 1 cycle for 2 min at 94°C, followed by 50 cycles for 35 sec at 94°C, for 1 min at 55°C, and for 30 sec at 72°C. The increase in fluorescence was measured at the 55°C annealing step. A dilution series of root RNA was used to generate a standard curve. *cisZOG1* product was normalized by using 18S as an endogenous control and calculated relative to root message levels.

Primers for *cisZOG1* were CGCAGTGGCTCATGAAGG (right) and CCGACATATACGCCGAGT (left), and the beacon was 5′-fluorescein-CGCTCGCGCCATGTTCCTGTCCG-AGTCGAGCG-3′-dabcyl. Primers for 18S were AGTTTGAG-GCAATAACAGGTCT (right) and GATGAAATTTCCCAA-GATTACC (left), and the beacon was 5′-fluorescein-CGCACGTCGCTACACTGATGTATCCAACGAGTACG-TGCG-3′-dabcyl.

Sequence Analyses. Similarity search of the *cisZOG1* amino acid sequence was performed with the BLAST algorithm (36). Sequence alignments were determined with Genetics Computer Group software (Madison, WI).

Results

Identification of a Candidate EST and Generation of a Specific Probe to Screen cDNA Libraries. The sequence of *ZOG1* (zeatin *O*-glucosyltransferase) from *P. lunatus* was compared with sequences in the maize EST database at Pioneer Hi-Bred International. A number of ESTs with various degrees of homology were identified. The partial sequence of an EST, cdmah36, was

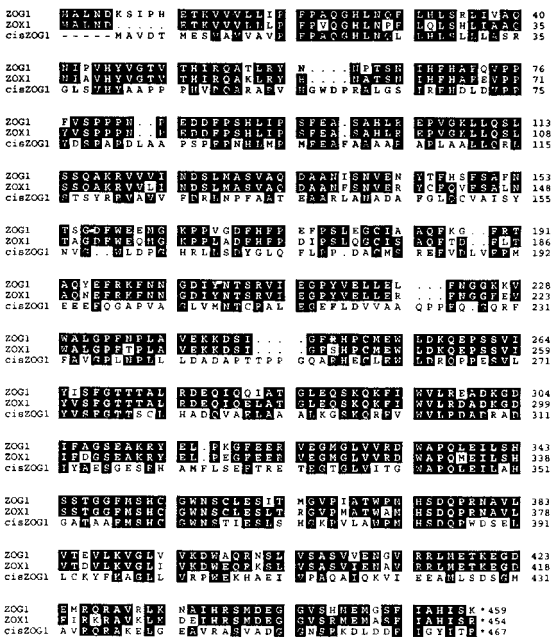

Fig. 1. Comparison of the amino acid sequence of maize *cisZOG1* (AF318075) with *Phaseolus ZOX1 and ZOG1* with PILEUP (Genetics Computer Group). Homology between sequences is indicated by black boxes.

judged to have the highest homology (49% identity) with *ZOG1*. Based on the consensus sequence of UDP-glycosyltransferases, *ZOG1*, and cdmah36, a pair of PCR primers (CTACGTCTCGTTCGGCACGACTTC and CAGCGTGGAGTTCCAACCGCAGTG) was used to amplify maize genomic DNA. A 292-bp fragment was generated and designated as *CORN2*.

cDNA Cloning and Characterization of the Gene Product. Probing the cDNA library constructed from kernels at 35 days after pollination of the inbred B73 with ^{32}P-labeled *CORN2* resulted in the isolation of a cDNA of 1,551 nucleotides. The cDNA contained an ORF of 1,401 bp encoding a protein of 51.1 kDa. The ORF is 50% identical to *ZOG1* at the DNA level and 41% identical at the amino acid level (Fig. 1). Western analyses showed that the recombinant protein was not antigenic to monoclonal antibodies specific to the ZOG1 enzyme of *Phaseolus* (ref. 29 and data not shown).

Enzyme Activity of the Recombinant Protein. The ORF of the cDNA was cloned into the modified pTrc99A vector containing a histidine tag at the N terminus. Induction by isopropyl β-D-thiogalactoside resulted in the synthesis of the insert-directed protein, which was purified by a nickel column (Fig. 2). The purified protein (Fig. 2*A*, lanes 5 and 6) was antigenic to antibodies specific to the histidine tag (Fig. 2*B*, lanes 5 and 6). Substrate specificity of the recombinant protein was initially tested with unpurified proteins by using a number of cytokinins and glycosyl donor substrates in all possible combinations. The cytokinins tested included *trans*- and *cis*-zeatin, dihydrozeatin, and the respective nucleosides. Potential sugar donors tested were UDP-glucose, UDP-xylose, UDP-glucuronic acid, UDP-galactose, ADP-glucose, and UDP-mannose. Enzymatic product was formed only in the presence of *cis*-zeatin and UDP-glucose. Both crude and purified proteins were then used in assays with *cis*-[^{14}C]zeatin and UDP-glucose. The reaction product eluted from the HPLC column at the expected position of the *O*-glucoside of *cis*-zeatin (Fig. 3 *A* and *B*) 4 min earlier than *cis*-zeatin. This product was formed in reactions mediated by

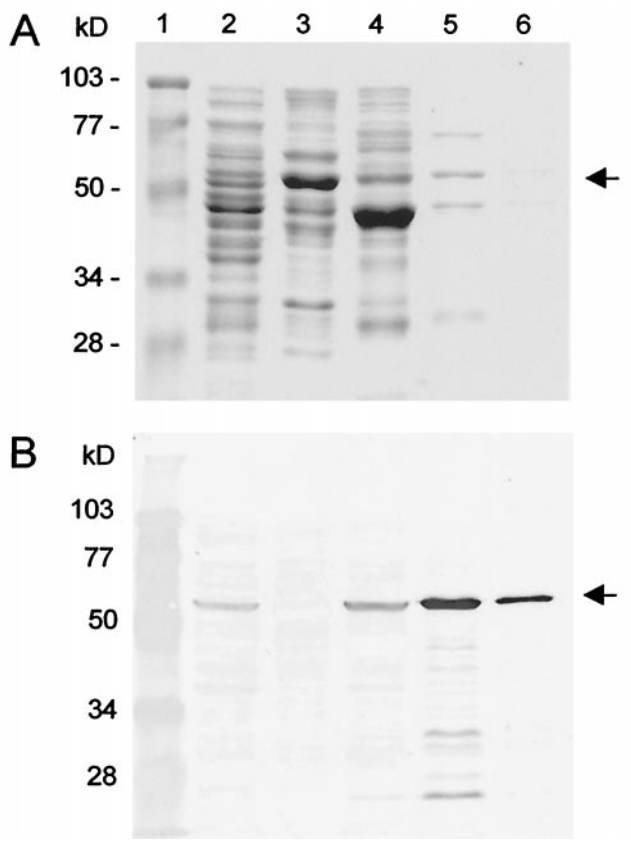

Fig. 2. SDS gel (*A*) and corresponding Western blot (*B*) of protein samples isolated from *Escherichia coli* cells harboring the pTrc plasmid with the *cisZOG1* gene. Lane 1, molecular mass markers; lane 2, protein from 60 μl of cells; lanes 3, 4, 5, and 6, eluate from a nickel column with 15, 60, 100, and 500 mM imidazole after application of protein from 1.5 ml of cells to the column. The arrows indicate the position of cisZOG1 protein. The Western blot was developed with His-tag specific antibody. kD, kilodalton.

both the crude cell extract and purified proteins (from fractions eluted with 100 and 500 mM imidazole, after removal of imidazole by Centriprep ultrafiltration). No such enzymatic product was produced in incubations with comparably obtained proteins from *E. coli* without the insert or with unrelated inserts. The authenticity of the labeled product as *O*-glucosyl-*cis*-zeatin was confirmed by treatment with β-glucosidase, resulting in conversion back to *cis*-zeatin (Fig. 3*C*). In addition, the MS profile of the product (Fig. 4) exhibited the characteristic spectrum of *cis*-zeatin but had the expected molecular mass of its *O*-glucoside and was very similar to that of *O*-glucosyl-*trans*-zeatin (it should be noted that no *O*-glucosyl-*cis*-zeatin standard is available). Therefore, the protein encoded by the ORF is a cytokinin metabolic enzyme that can catalyze the formation of *O*-glucosyl-*cis*-zeatin from *cis*-zeatin and UDP-glucose (Fig. 5).

Genomic Sequence of the *cis*-Zeatin *O*-Glucosyltransferase. Genomic DNA was amplified by using primers flanking the cDNA. The sequence of the PCR product was identical to the cDNA, indicating that the gene has no intron. The gene is designated as *cisZOG1* (for *cis*-zeatin *O*-glucosyltransferase). Based on BLAST search of public databases, the gene is unique but has some homology to other glucosyltransferases, particularly at the 3′ end containing the putative UDP-glucose-binding site. The most closely related genes are *ZOG1* and *ZOX1* of *Phaseolus* (24, 25).

Expression of *cisZOG1*. To determine the level of *cisZOG1* transcription in maize, corresponding mRNA levels were measured

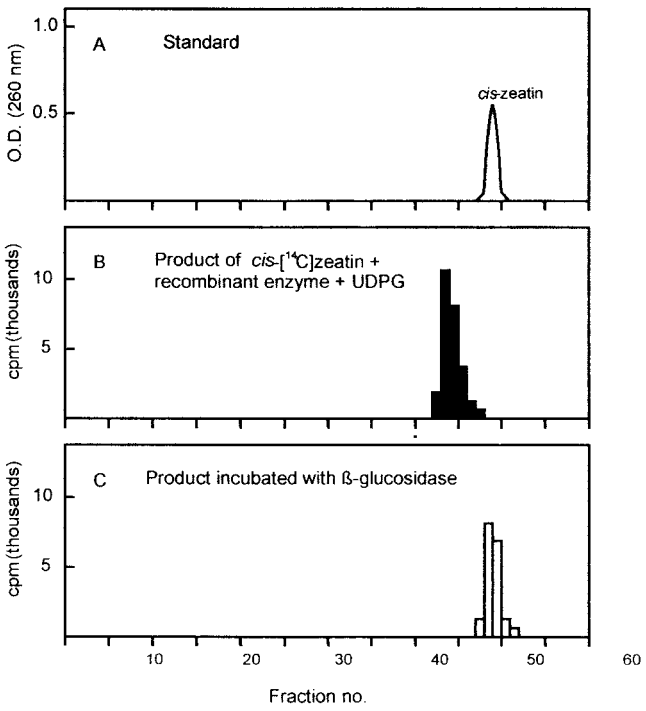

Fig. 3. Elution positions of *cis*-zeatin standard and products of the enzymatic reaction after HPLC as reported (22). (*A*) *cis*-zeatin standard. (*B*) Product of enzymatic reaction after incubating recombinant protein with *cis*-[^{14}C]zeatin (45,000 cpm) and UDP-glucose (4 mM) in 0.17 M Tris, pH 8.0. (*C*) Reaction product obtained from *B* treated with β-glucosidase for 2 h as reported (30), resulting in formation of *cis*-zeatin.

by RT-PCR combined with molecular beacons (32, 33) that are capable of distinguishing sequences that differ in a single nucleotide. By using this method and the measurement of 18S

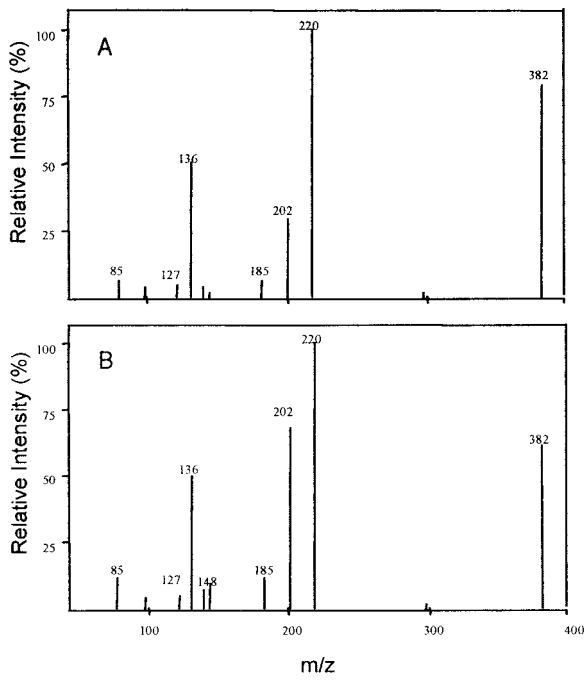

Fig. 4. Mass spectra of (*A*) reaction product obtained from incubating *cis*-zeatin with recombinant protein and (*B*) *O*-glucosyl-*trans*-zeatin standard.

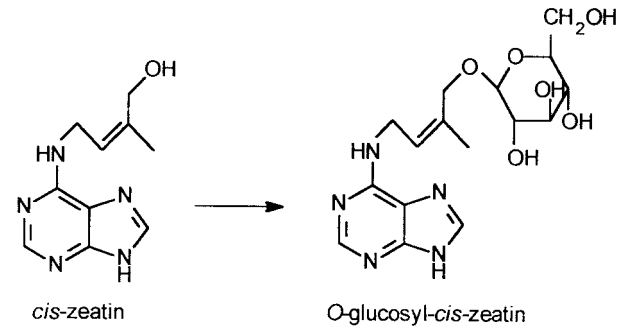

Fig. 5. Reaction mediated by the *cis*-zeatin *O*-glucosyltransferase.

rRNA as standards, the highest level of *cisZOG1* mRNA was found in the roots, with significantly lower levels in the cob and kernels, and very low levels in the leaves (Fig. 6). No appreciable *cisZOG1* mRNA could be detected in stems.

Discussion

The discovery of a gene and enzyme specific to the metabolism of *cis*-zeatin was unexpected, because no regulatory mechanisms specific to *cis*-zeatin have been detected before. Moreover, *cis*-zeatin or its derivatives have not been reported for maize, although recent analyses indicate that cis isomers are present in this species (R. Vankova, R.C.M., M.C.M., and D.W.S.M., unpublished results). The only relevant reports concern the identification of *cis*-zeatin-*O*-glucoside and its riboside in developing rice kernels (9) and the tentative identification of the riboside in root exudates of *Urtica* (37). The implication of the current finding is that pathways parallel to those for *trans*-zeatin exist for the metabolism of *cis*-zeatin, served by *cis*-specific enzymes. It also suggests that *cis*-zeatin derivatives are more prevalent and perhaps more relevant to cytokinin biology than previously thought. Because of the low activity of *cis*-zeatin and its derivatives in bioassays (38), they are presumed to have limited growth-promoting properties in whole plants. However, these compounds may serve other unique functions, particularly in species such as chickpeas (11), rice (7–9) or *Mercurialis* (5), in which the *cis*-isomers are the predominant cytokinins in some organs. For example, *cis*-zeatin and derivatives found in rice

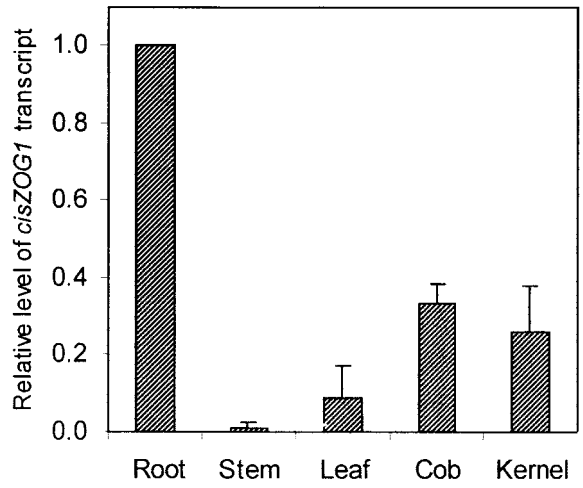

Fig. 6. Relative levels of *cisZOG1* transcript in maize tissues (with root transcript = 1) determined by RT-PCR and molecular beacons. Fluorescence for *cisZOG1* in each sample was normalized to the 18S control. Data are averages of three experiments (standard deviation indicated).

roots (7–9) and potato stolons (3, 4) may be transport forms, converted to *trans* isomers in above-ground tissues where light and *cis-trans* isomerases can mediate the isomerization. The relatively high level of *cisZOG1* message in maize roots is compatible with this hypothesis. It is also possible that *cis*-isomers have regulatory functions or are required for specific developmental events yet to be identified. Notwithstanding these unanswered questions, it seems that future measurement of cytokinins in maize and other species will have to take into account *cis*-zeatin and its derivatives. A corollary of a *cis*-specific metabolic pathway is the possibility of a direct biosynthetic route for *cis*-zeatin, independent of tRNA breakdown.

The *cisZOG1* expression studies indicated relatively high levels of transcript in maize roots and much lower levels in cobs and kernels. This expression pattern contrasts to that of the *ZOG1*/*ZOX1* genes of *Phaseolus*, where Northern and Western analyses detected high levels in the immature seeds and comparatively low levels in the roots (24, 25). Because *O*-glucosyl conjugates are considered storage products, formed when cytokinins occur in excess, the distribution differences may reflect differential accumulation of the two isomers in plant parts or differences in accumulation between these two species.

Isolation of maize genes based on sequences of *Phaseolus ZOG*/*ZOX* genes was greatly enhanced by the availability of an EST database. However, the function of candidate genes is less predictable. Although several other cDNAs with weak homology to *ZOG1* were isolated, none seem to have *trans*-zeatin *O*-glucosyltransferase activity (R.C.M., M.C.M., and D.W.S.M., unpublished results). As the *O*-glucoside of *trans*-zeatin has been identified in maize (39, 40), it is expected that genes coding for *O*-glucosyltransferase(s) specific to *trans*-zeatin should occur. The search for such genes and enzymes is continuing based on probe hybridization and immunoscreening.

Probing of genomic libraries of inbreds B73 and W64 with *cisZOG1* resulted in the isolation of additional genomic clones (R.C.M., M.C.M., and D.W.S.M., unpublished results). Preliminary results indicate that B73 contains another gene highly homologous to *cisZOG1*. The partial sequence (1,045 bp) of the ORF is 98% identical to that of *cisZOG1*. However, the 5′ upstream sequences differ considerably, which may indicate differential regulation of the expression of this homolog. (It should be noted that the molecular beacon used to measure *cisZOG1* expression is specific to *cisZOG1*.) All positive genomic clones from W64 contain the same gene. The ORF sequence differs from that of *cisZOG1* in 11 nucleotides (five amino acids) and may represent the single copy of a functional duplicate in this genotype. Thus far, none of the zeatin *O*-glucosyltransferase homologues we have isolated from *Phaseolus*, maize, or *Glycine* contain introns, a property often observed with glucosyltransferase genes.

We have now identified a collection of genes encoding enzymes with similar functions but having distinct substrate specificity. These genes are members of a large family of glycosyltransferases, with limited homology in the 5′ half of the genes but with higher homology at the 3′ end containing the UDP-glycosyltransferase signature. By establishing the three-dimensional structure of the proteins, it may be possible to determine the substrate-binding domains. Such information will be valuable in defining the substrates of other glucosyltransferases as well as identifying additional zeatin-related enzymes and receptors.

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