A Maize Homologue of the Bacterial CMP-3-Deoxy-d-manno-2-octulosonate (KDO) Synthetases

SIMILAR PATHWAYS OPERATE IN PLANTS AND BACTERIA FOR THE ACTIVATION OF KDO PRIOR TO ITS INCORPORATION INTO OUTER CELLULAR ENVELOPES

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The eight-carbon acid sugar 3-deoxy-d-manno-2-octulosonate (KDO) is an essential component of Gram-negative bacterial cell walls and capsular polysaccharides. KDO is incorporated into these polymers as CMP-KDO, which is produced in an unusual activation step catalyzed by the enzyme CMP-KDO synthetase. CMP-KDO synthetase activity has traditionally been considered exclusive to Gram-negative bacteria. CMP-KDO synthetase inhibitors attract great interest owing to their potential as selective bactericides. The sugar KDO is also a component of the rhamnogalacturonan II pectin fraction of the primary cell walls of higher plants and of the cell wall polysaccharides of some green algae. However, the metabolic pathway leading to its incorporation into the plant cell wall is unknown. This paper describes the isolation and characterization of a maize gene, which codes for a protein very similar in sequence and activity to prokaryotic CMP-KDO synthetases. Remarkably, the maize gene can complement a CMP-KDO synthetase (kdsB) Salmonella typhimurium mutant defective in cell wall synthesis. ZmCKS activity is novel in eukaryotes. The evolutionary origin of ZmCKS is discussed in relation to the high degree of conservation between the plant and bacterial genes and its atypical codon usage in maize.

The acid sugar 3-deoxy-d-manno-octulosonate (KDO) is an important component of the lipopolysaccharides found on the outer surface of Gram-negative bacteria (1, 2). The acid sugar 3-deoxy-d-manno-octulosonate (KDO) is also found in some capsular polysaccharides of the Gram-negative bacteria (3–5).

The incorporation of KDO into lipopolysaccharides is catalyzed by membrane-bound KDO-transferase that requires a nucleotide derivative of the molecule, CMP-KDO (1). The synthesis of this activated form of KDO is catalyzed by the enzyme CMP-KDO synthetase (E.C. 2.7.7.38) in a reaction that takes place in the bacterial cytoplasm.

KDO + CTP → CMP-KDO + pyrophosphate

Activation of a sugar by its coupling to monophosphonucleosides rather than to diphosphonucleosides is very unusual. The only other known sugar activation reaction with a similar mechanism is the formation of CMP-N-acetylmuramidine (6).

CMP-KDO synthetase is an essential gene product for the growth of most Gram-negative bacterial cells. Temperature-sensitive mutations in CMP-KDO synthetase (kdsB gene) are lethal at the nonpermissive temperature because the incorporation of KDO into lipid A precursors is blocked. These lipid A precursors then accumulate, inhibit cell growth, and ultimately cause death (7, 8). A second, nonessential CMP-KDO synthetase gene involved in the synthesis of capsular polysaccharides has also been described in Escherichia coli (kpsU gene; Ref. 9).

CMP-KDO synthetases are traditionally considered unique to Gram-negative bacteria. This and their essential role in bacterial viability make these enzymes an attractive target for the design of very selective antimicrobial agents (10, 11). However, the sugar KDO is not restricted to Gram-negative bacteria. It is also a component of the rhamnogalacturonan II pectin fraction of the primary cell walls of most plants (12–15) and also of the cell wall polysaccharides of some green algae (16, 17). Pectins are a heterogeneous group of polymers that characteristically contain acid sugars such as glucuronic and galacturonic acids and that are present in the middle lamella of the primary cell walls of higher plants. Pectins are composed of three polysaccharide components: homogalacturonans, rhamnogalacturonan I, and rhamnogalacturonan II (18). Rhamnogalacturonan II is a small, nonabundant, and highly complex carbohydrate with a very diverse array of sugars showing different linkages. It includes sugars normally considered unusual in plants, such as KDO. However, rhamnogalacturonan II is present in the primary cell walls of most plants, including dicots, monocots, and gymnosperms (13, 19). Pectins form a gel phase in the primary cell wall in which the cellulose-hemicellulose network is embedded. They are also the source of bioactive oligosaccharides (20). However, the precise function of the rhamnogalacturonan II fraction, the specific contribution of KDO to its function, and the metabolic pathway leading to its synthesis, activation, and incorporation of KDO into this plant polysaccharide remain unknown.

This paper describes the isolation of a maize gene, ZmCKS, that codes for a protein homologous in sequence and activity to the prokaryotic CMP-KDO synthetases. ZmCKS is probably
involved in the activation of KDO prior to its incorporation into the cell wall pectins. The maize gene can complement a Salmonella typhimurium mutant for the kdsB gene, defective in bacterial cell wall synthesis. This indicates a remarkable conservation of the incorporation pathways of KDO into the cell walls of bacteria and plants. The unusually high sequence homology between the maize gene and its prokaryotic counterparts and the deviation of the normal codon usage of maize shown by ZmCKS suggest a horizontal transfer event originally introduced ZmCKS related genes into plant genomes.

**EXPERIMENTAL PROCEDURES**

**DNA Manipulations**—Standard DNA manipulation techniques were used as described by Sambrook et al. (21). The ZmCKS cDNA clone was isolated from a previously described cDNA library synthesized from maize (Zea mays L. line A69Y) endosperm RNA (22). A genomic fragment corresponding to ZmCKS was obtained by PCR amplification from genomic DNA of the maize line A69Y using primers 9–2E1 5'-CACAG-GATCCACGGCCTGGGATGAC3' (nucleotide positions 211–228 in Fig. 1) plus a BamHI target 5'-end extension shown here underlined) and 9–2EB 5'-ACACAGAGTCTCGAATTCCTGCTGGTCG3' (nucleotide positions 954–937 in Fig. 1) plus a BglII target 5'-end extension shown here underlined), which encodes the coding region of ZmCKS between amino acid position 51 and the translation stop codon (see Fig. 1). A unique 2.2-kilobase pair amplified fragment was obtained after 35 PCR cycles (15 s at 95 °C, 30 s at 50 °C, and 2 min and 30 s at 72 °C), cloned in plBlueScript, and sequenced.

**RT-PCR Analysis**—mRNA was prepared from total RNA using oligotex particles (Qiagen GmbH). 20-μg mRNA samples were reverse-transcribed with superscriptII Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) using oligo(dt) primer in a reaction of 20 μl. 0.5 μl of the first strand of cDNA was used in a PCR reaction using primers 9–2E1 and 9–2EB and a PCR program (30 cycles of 15 s at 95 °C, 30 s at 50 °C, and 1 min 30 s at 72 °C). Southern and Northern Blot Analysis—Southern and Northern blots were prepared using standard procedures (21). For Southern blots, 15 μg of genomic DNA from the maize line A69Y, E. coli, Arabidopsis thaliana, Lycopersicon esculentum, Hordeum vulgare, or Triticum aestivum were completely digested with the appropriate restriction endonuclease and separated on a 0.8% agarose gel before transfer. Northern blots were prepared with 2-μg mRNA samples and run in formaldehyde gels. Transfer to nylon membranes was in 10× SSC. Filters were prehybridized and hybridized as in the standard hybridization conditions. 0.5 μg of a digoxigenin-labeled probe comprising the coding region of ZmCKS starting at amino acid position 51 (see Fig. 1). The probe was prepared by PCR amplification of the ZmCKS cDNA clone using primers 9–2E1 and 9–2EB (see Fig. 1) and a 1:4 DUTP-DIG dTTP ratio. Filters were washed and further processed as recommended by the supplier (Roche Molecular Biochemicals).

**Expression of ZmCKS in E. coli**—ΔZmCKS, a truncated derivative of the ZmCKS DNA clone, was generated by PCR using primers 9–2E1 and 9–2EB (see Fig. 1). The PCR product was digested with the restriction enzymes BamHI and BglII and cloned into the pQE42 expression vector (22) (Qiagen GmbH) to yield pQE42-ΔZmCKS. The pQE42 vector provides a start codon and a His tag, resulting in a vector-derived 12-amino acid N-terminal extension of sequence MRSGHHHHHHGS that is fused to the first residue of the ZmCKS protein, the serine at position 51 in Fig. 1. The resulting protein expressed from pQE42-ΔZmCKS has a predicted molecular mass of 29 kDa. The cloning junctions of the construct were checked by sequencing prior to the expression studies. E. coli cells of the strain SGI3009 containing the plasmid pREP4 expressing the lac repressor protein (23) were electroporated with plasmid pQE42-ZmCKS. In a parallel negative control experiment the same strain was electroporated with an “empty” pQE42 vector. Transformed bacteria of both classes were separately grown at 37 °C in 800 ml of LB medium containing both ampicillin (100 μg/ml) and kanamycin (25 μg/ml). Once the cultures reached an optical density (600 nm) of 0.6, they were induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The cultures were subsequently incubated at 37 °C for 5 h. Bacteria were harvested by centrifugation and resuspended in 20 ml of lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme, 0.5 mM PMSF). All subsequent purification steps were done at 4 °C. Bacterial suspensions were incubated on ice for 30 min and sonicated, and the cellular debris was removed by centrifugation. The supernatant was passed through a Ni-NTA agarose resin column which binds to the His tag contained in ΔZmCKS. The resin was subsequently washed with approximately 80 ml of washing buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.5 mM PMSF) until the absorbance at 280 nm of the eluting fractions was lower than 0.01. Finally, proteins retained at the column were eluted from the Ni-NTA agarose in 3 ml of elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 250 mM imidazole, 0.5 mM PMSF), concentrated by ultrafiltration using Centricron YM-10 membranes (Millipore Corp.), and protein concentration was estimated by the Bradford method. Typically, 1.2 mg of protein was obtained in this final fraction from 800 ml of bacterial culture; quantification after SDS-PAGE and Coomassie Blue staining indicated that ΔZmCKS represented approximately 80% of the purified proteins (see Fig. 5a, lane S). A second major band present in these preparations, with a slightly higher electrophoretic mobility than ΔZmCKS (see Fig. 5a, lane S), appears to be a degradation product of the protein because the band almost disappeared when proteinase inhibitors were used throughout the protein preparation procedure.

A much lower yield in protein was obtained from bacteria carrying the empty pQE42 plasmid. The proteins retained in the column were nevertheless concentrated so that an amount of protein equivalent to that of ΔZmCKS could be used in SDS-PAGE and enzymatic activity analysis. No trace of any protein resembling ΔZmCKS in SDS-PAGE mobility was observed in this fraction (see Fig. 5a, lane C). Enzymatic Activity Assays—The enzymatic CMP-KDO synthetase activity of ΔZmCKS was assayed as described previously (24). Briefly, different amounts of the recombinant ΔZmCKS protein fraction and of the negative control sample (5–20 μl, corresponding to 19–114 μg) (see Fig. 5) were incubated at 30 °C in a final volume of 1 ml. Reactions contained 200 mM Tris acetate buffer (final pH, 9.5), 10 mM MgCl2, 2 mM KDO, and 10 mM CTP, UTP, or ATP. Reactions were quenched by the addition of two volumes of ice-cold ethanol. The CMP-KDO production was determined by a modification of the thioarbituric acid assay followed by comparison of the resulting optical density at 549 nm with that of known amounts of KDO standards processed in the same way (24). One unit of enzyme activity equals 1 μmol of CMP-KDO formed per minute (24). For the determination of the incorporation of the sugar monophosphates of ΔZmCKS, reactions contained a fixed amount of the recombinant protein (0.03 units) and variable amounts of either CTP or UTP. To measure the production of pyrophosphate and the KDO derivative, the reaction was allowed to proceed for 15 min with 0.1 unit of ΔZmCKS. After quenching the reaction, the levels of the nucleotide derivative of KDO produced were measured in one aliquot of the reaction mixture as in the standard enzymatic assay (24), while the constitutive expression of the lac repressor protein (22) and either pQE42-ΔZmCKS or an empty pQE42. In both cases, transformed bacteria were selected by growing at 30 °C on LB agar plates containing ampicillin (resistance provided by the pREP4 plasmid) and kanamycin (resistance provided by pREP4). Twelve independent colonies from the first transformation experiment and four from the control were selected for further study. The sixteen isolates were plated on two sets of LB plates containing ampicillin and kanamycin but differing in the presence or absence of 1 mM IPTG inducer. After 2 h of incubation at 30 °C (26), one plate of each set was incubated overnight at the restrictive temperature (42 °C), and the other was incubated at a permissive temperature (30 °C).

**RESULTS**

**Isolation of a cDNA Coding for Maize CMP-KDO Synthetase**—In the course of screening for maize endosperm genes, a 1.3-kilobase cDNA clone (ZmCKS) was isolated that contained a potential open reading frame for a protein of 298 amino acids and a predicted molecular mass of 32 kDa (Fig. 1) and that was highly homologous in sequence (35–41% amino acid similarity) to the CMP-KDO synthetases of Gram-negative bacteria. The maize clone also showed clear homology to an anonymous partial EST from the gymnosperm Pinus taeda (Fig. 2). The ZmCKS cDNA clone was probably nearly full length because it was very similar in size to the corresponding mRNA detected by
Northern analysis (see Fig. 4). The first methionine codon of the ZmCKS open reading frame is in a good sequence context for a monocot translation start site (27) and appears to be followed by a typical N-terminal signal peptide (underlined in Fig. 1). The most likely signal peptide cleavage site is located between residues 40 and 41, and the resulting N terminus of the mature protein closely corresponds with those of the prokaryotic CMP-KDO synthetases (Fig. 2). The mature ZmCKS protein would have a molecular mass of 29 kDa, slightly higher than that of most of the bacterial enzymes (27 kDa). None of the reported bacterial CMP-KDO synthetases possess an N-terminal signal peptide (Fig. 2). The leader peptide of ZmCKS could have joined the prokaryotic mature protein region during the evolution of the eukaryotic gene and targeted the enzyme toward a specific subcellular compartment. Signs of this composite evolutionary origin of ZmCKS were sought by comparing the codon usage bias of its signal peptide and mature protein coding regions. The codon adaptation index (CAI) developed by Sharp and Li (28) was used for this. The CAI measures the extent to which codon usage of a particular gene agrees with a reference set from other genes of the same species. Here, use was made of the data set of 676 coding sequences from Z. mays compiled by Nakamura et al. (29). Typically, CAI values range between 0.7 and 0.8 depending on factors such as the expression level or the GC content of the gene analyzed (not shown). In the case of ZmCKS, the complete coding region had a CAI of 0.64, whereas the region corresponding to the mature protein showed a CAI of 0.63, a figure significantly lower than that of the leader peptide (0.78). A comparison was also made of the codon usage of ZmCKS with respect to a reference set of highly expressed maize genes (as compiled in CGC, Wisconsin Package Programs, January 1991): those under the highest evolutionary pressure to adapt their codon usage to the optimum value of the species. In this case, a typical nonhighly expressed maize gene showed a CAI value between 0.36 and 0.52 (data not shown). ZmCKS, however, showed CAI values of 0.29 for the whole coding region, 0.25 for the “mature” protein, and 0.71 for the “signal peptide” coding sequence, further confirming that the part of the gene that could be aligned with the prokaryotic genes shows very poor adaptation to maize codon usage. This is in contrast to the signal peptide coding sequence, which showed perfect adaptation. These differences could result from different evolutionary origins for the two regions of the ZmCKS gene.

To assess the identification of ZmCKS as a maize gene, a genomic fragment amplified by PCR using primers derived from the cDNA sequence (at positions indicated in Fig. 1) was cloned. Complete sequencing of this clone (EMBL accession number AJ250331) showed, after comparison with the corresponding cDNA sequence, that the coding region of the gene is interrupted by the presence of seven introns located at nucleotide positions 303, 349, 436, 561, 701, 753, and 864. These introns are 506, 84, 94, 264, 76, 104, and 229 nucleotides long, respectively (Fig. 1). In all cases the left and right splice sites are the canonical GT and AG dinucleotides.

Southern blot analysis of maize genomic DNA probed with ZmCKS cDNA indicated the presence of a unique copy of this gene per haploid genome (Fig. 3, left panel). Related genes were detectable by low stringency hybridizations with the genomes of two other cereal species, wheat and barley, and with the Gram-negative bacterium E. coli, but no signal was detected in

**Fig. 1. Sequence of maize ZmCKS and its deduced amino acid sequence.** The putative N-terminal signal peptide is shown underlined. Horizontal arrows indicate the positions and lengths of the oligonucleotides 9–2E1 and 9–2Eb. Vertical arrows mark the locations of seven introns in the corresponding genomic sequence.

**Fig. 2. Amino acid alignment of ZmCKS and CKS proteins from the Gram-negative bacteria E. coli (EclCKS, LCKS product of the kdsB gene, EMBL accession number J02614; EchCKS, K-CKS product of the kpsU gene, EMBL accession number XT4567), Hemeophilus influenzae (HfCKS, EMBL accession number U32691), Chlamydia trachomatis (CtCKS, EMBL accession number U15192), and Helicobacter pylori (HpCKS, EMBL accession number AE000543). PtCKS is an anonymous EST from P. taeda (EMBL accession number AA739505). Single-letter amino acid code is used. X in the PtCKS sequence designates an undetermined amino acid.
Arabidopsis or tomato genomic DNA (Fig. 3, right panel). Further, no sequences were found homologous to ZmCKS in the data bases containing the complete genomic sequence of the eukaryotic species Saccharomyces cerevisiae and Caenorhabditis elegans.

As in prokaryotes, the incorporation of KDO into plant pec-
tins probably requires its previous activation as CMP-KDO. ZmCKS would catalyze this activation step. Consequently, given the ubiquitous presence of this polysaccharide in plant cell walls, it is not surprising that ZmCKS messenger RNA could be detected by RT-PCR in every vegetative and reproductive maize tissue tested (Fig. 4A). The gene also seems to be constitutively expressed throughout development because only a slight variation was observed in its mRNA levels during seed maturation (Fig. 4B). The maximum levels of accumulation of mRNA are reached between 8 and 14 days after pollination, when the cellularization process, and therefore a very active synthesis of primary cell walls, takes place in the endosperm.

ZmCKS Has CMP-KDO Synthetase Activity—The close se-
quence similarity between ZmCKS and Gram-negative CMP-
KDO synthetases prompted the investigation of the enzymatic activity of the maize protein. To this end, a truncated deriv-
ae of the ZmCKS cDNA, ΔZmCKS, was prepared. ΔZmCKS lacks 50 amino acids placed at the N terminus of the ZmCKS protein, including the putative signal peptide (shown underlined in Fig. 1) and a short N-terminal extension present in the maize clone but not in its prokaryotic counterparts (Fig. 2).

ΔZmCKS was cloned in the bacterial expression vector pQE42 (Qiagen GmbH) by replacement of its DHFR gene. The resulting pQE42-ΔZmCKS plasmid, in which the expression of ΔZmCKS is under the control of the lac promoter, was transformed into the E. coli strain pREP4-SG13009, which constitutively expresses the lac repressor protein. The recombinant ΔZmCKS protein contained at its N terminus a His$_6$ tag and was partially purified by binding to Ni-NTA agarose resin under native conditions (Fig. 5a, lane S). ΔZmCKS was shown to catalyze the production of a nucleotide derivative of KDO when assayed as described by Ray and Benedict (24). The specific activity of our ΔZmCKS preparation at 30 °C was 0.7 unit/mg total protein. As a negative control experiment, a protein extract from bacteria carrying an empty expression plasmid was incubated in the same way with the Ni-NTA agarose resin. The proteins retained in the resin (Fig. 5a, lane C) did not show any enzymatic activity, excluding the possibility that any of the two E. coli CMP-KDO synthetases is contaminating the ΔZmCKS preparation.

To confirm that ΔZmCKS catalyzes the synthesis of CMP-
KDO and not that of CDP-KDO, the levels of pyrophosphate and KDO derivative produced in the reaction were measured. The production of pyrophosphate is only possible if ΔZmCKS catalyzes the synthesis of CMP-KDO. Our results indicate that, when assayed as described under “Experimental Procedures,” 0.1 unit of ΔZmCKS catalyze the synthesis of 520 nmol of the KDO derivative and 470 nmol of pyrophosphate (a 1.1:1 molar relation). Consequently, ΔZmCKS has CMP-KDO synthetase activity (Fig. 5).

As with its bacterial homologues, the CMP-KDO synthetase activity of ΔZmCKS showed an optimum pH of 9.5 and was inactive when ATP was used as the nucleotide donor. The CMP-KDO synthetase encoded by the E. coli kdsB gene (L-
CKS) was previously reported to have, at pH 9.5, an enzymatic activity with UTP nearly half of that obtained with CTP, whereas at pH 7 no activity was detected with UTP (24). In contrast, the maize protein showed a less marked preference for CTP over UTP. At pH 9.5 the enzymatic activity of ΔZmCKS was nearly the same with either CTP or UTP, and only at pH 7 was the maize enzyme significantly more active with CTP than with UTP (Fig. 5c). However, the comparison of the apparent $K_m$ values for CTP and UTP indicates that CTP is also the preferred substrate for the maize enzyme. The apparent $K_m$ for UTP of ΔZmCKS was 1.7 × 10$^{-4}$ M when measured at pH 9.5 and in the presence of 10 mM Mg$^{2+}$, whereas the apparent $K_m$ for CTP was significantly lower at these conditions, 6.9 × 10$^{-5}$ M. These values are similar to the previously reported apparent $K_m$ for CTP of the E. coli L-CKS enzyme, 2.4 × 10$^{-4}$ M (24), and significantly lower than that of the E. coli K-CKS enzyme (product of the kpsU gene), 2.5 × 10$^{-3}$ M (9). No data are available for the apparent $K_m$ for UTP of any bacterial enzyme. The differences observed in the substrate preferences between the E. coli and maize proteins further support the idea that the activity measured was due to the plant protein rather than to a contamination from the endogenous bacterial activity.

Expression of the ΔZmCKS protein in E. coli pREP4-
SG13009 cells after IPTG induction had a drastically detrimental effect on their growth (data not shown). The transformation of the pQE42-ΔZmCKS plasmid into the E. coli XL1-Blue strain, which expresses normal levels of the lac repressor and consequently controls less firmly the expression of ΔZmCKS,
than pREP4-SG13009, could not be achieved. These observations suggest that the ΔZmCKS protein expressed at high levels is toxic to E. coli cells.

**ZmCKS Complements a S. typhimurium Thermosensitive kdsB Mutant**—In Gram-negative bacteria, mutants of the kdsB gene coding for the CMP-KDO synthetase involved in the biosynthesis of lipopolysaccharides do not survive (7). The S. typhimurium RG103 strain is a thermosensitive mutant of the kdsB gene that, because of its low reversion frequency (approximately $2 \times 10^{-7}$) and high transformation efficiency (26), has been very useful in the molecular cloning of the homologous E. coli gene by functional complementation. To confirm the identification of the ZmCKS protein as an eukaryotic counterpart of bacterial CMP-KDO synthetases, its ability to complement the kdsB mutation in RG103 was tested.

To avoid the toxic effect of the overexpression of ZmCKS, the plasmid pREP4 coding for the lac repressor protein (23) was co-transformed with either pQE42-ΔZmCKS or the empty pQE42 plasmid into the S. typhimurium strain RG103. Transformed bacteria were selected by growing overnight at 30 °C on LB agar plates containing ampicillin (resistance provided by the pQE42 plasmids) and kanamycin (resistance provided by pREP4).

Six independent RG103 isolates co-transformed with the pQE42-ΔZmCKS and pREP4 plasmids were able to grow at the restrictive temperature (42 °C; Fig. 6, a and c), whereas none of two control isolates, which carried the intact pQE42 expression plasmid and pREP4, were able to do so. The eight isolates grew normally at the permissive temperature (30 °C; Fig. 6, b and d). CMP-KDO synthetase protein levels are generally very low in bacteria (26), and in these complementation experiments it was observed that the low level basal expression of recombinant ΔZmCKS in the absence of IPTG was enough to allow growth at the restrictive temperature (Fig. 6a). In the presence of the IPTG inducer, the transformed bacteria grew to a lower density, both at the permissive and the restrictive temperature (Fig. 6, c and d). This further confirms that high level expression of the ΔZmCKS protein is toxic to bacteria and probably results in selection against clones expressing that protein at the highest level. Nevertheless, it is clear from the present data that even in the presence of IPTG, plasmid pQE42-ΔZmCKS complements the S. typhimurium RG103 mutation for the kdsB gene (Fig. 6c).

To confirm that temperature resistance was conferred to RG103 cells by the presence of a pQE42-ΔZmCKS plasmid, plasmid DNA was extracted from the temperature-resistant and control isolates. The restriction patterns obtained indicated that only the temperature-resistant isolates carried ΔZmCKS inserts in the pQE42 plasmid (not shown). In addition, analysis of the protein profiles obtained after passage of bacterial crude extracts of the same isolates through a Ni-NTA
agaro-se resin showed that temperature resistance was linked to the expression of the ΔZmCKS recombinant protein (not shown).

**DISCUSSION**

**ZmCKS Is a Novel Plant Gene**—This paper reports the isolation of a maize cDNA clone, ZmCKS (Fig. 1), which codes for a protein sharing very significant structural and functional properties with bacterial CMP-KDO synthetases (Fig. 2), the enzymes involved in the activation step of the KDO sugar prior to its incorporation into the bacterial cell wall.

Genes corresponding to KDO-8-phosphate synthetase, the enzyme catalyzing the synthesis of KDO from phosphonod-pyruvate and n-arabinose 5-phosphate, have also been reported recently in pea (EMBL accession number Y14272), A. thaliana (EMBL accession number AC007202), and Brassica campestris (EMBL accession number L47850). In addition, an EST sequence coding for a homologue of the prokaryotic KDO transferase, the enzyme responsible for the incorporation of CMP-KDO into the cell wall lipopolysaccharide, has been identified in Glycine max (EMBL accession number AI495894). The identification of these plant genes implies that despite their ancient divergence, plant, and bacterial cells might use a similar pathway for the incorporation of KDO into their very different cell walls.

One way to explain the high similarity observed between ZmCKS and the bacterial CMP-KDO synthetase genes would be to think of ZmCKS as a contaminant incorporated into the cDNA library during its construction. Three lines of evidence support the idea that ZmCKS is indeed a maize gene. First, the cDNA clone showed a poly(dA) tail and no signals of a possible artifact origin occurred during the construction of the cDNA library. Second, when used as a probe in Southern (Fig. 3) and Northern hybridizations (Fig. 4), ZmCKS gave hybridization signals consistent with its identification as a maize nuclear gene. Third, sequence analysis of a PCR amplified genomic fragment corresponding to ZmCKS indicated the presence of a single copy gene in the maize nuclear genome (Fig. 3). RT-PCR and Northern analysis demonstrated that the gene is ubiquitously and moderately expressed in the plant genome. Sequence analysis of a PCR amplified genomic fragment corresponding to ZmCKS and the bacterial CMP-KDO synthetase was identified in a partial EST from the gymnosperm P. taeda (Fig. 2). The two plant genes are more closely related to each other than to any of the other bacterial genes (71% amino acid similarity between ZmCKS and the P. taeda EST, compared with 35–41% between the plant and bacterial proteins).

ZmCKS is a single copy gene in the maize nuclear genome (Fig. 3). RT-PCR and Northern analysis demonstrated that the gene is ubiquitously and moderately expressed in the plant (Fig. 4). The level of accumulation of ZmCKS mRNA seems to be regulated in response to the cell wall biosynthetic activity of the cell.

**ZmCKS Encodes a CMP-KDO Synthetase with an Activity Similar to, but Not Identical to, the Bacterial Enzymes**—Bacterial CMP-KDO synthetases catalyze the rather unusual synthesis of a nucleoside monophosphate activated sugar. In plants, sugar activation prior to incorporation into polysaccharides usually involves the synthesis of a nucleoside-diphosphate derivative (30, 31). Similar reactions are seen in animals and prokaryotes. KDO and N-acetylneuraminic acid (6) are the only two known examples of sugars activated via NMP derivatives, and the CMP-KDO synthetase reaction has been traditionally considered exclusive to Gram-negative bacteria. The current in vitro enzymatic data clearly indicate that the maize ZmCKS protein has a CMP-KDO synthetase activity (Fig. 5b) very similar to that of the bacterial enzymes (24). We have shown that pyrophosphate is produced in the KDO activation reaction, excluding the possibility that the maize enzyme catalyzes the formation of CDP-KDO. At present, however, and in the absence of data about the intracellular concentrations of UTP and CTP in plant cells, the activation of KDO to UMP-KDO by ZmCKS cannot be completely excluded (Fig. 5c).

Additional experimental data, including those from kinetic assays using ZmCKS purified from plant tissues, are required before the true pathway can be determined.

Further evidence of the functional similarity between ZmCKS and the bacterial CMP-KDO synthetases was provided by the complementation of a S. typhimurium thermosensitive mutant at the kdoB gene by the maize ZmCKS gene. The maize gene completely restored the virulence of the Salmonella mutant strain (Fig. 6). Interestingly, when expressed at high levels, ZmCKS showed a toxic effect both in E. coli and S. typhimurium. This effect could be a consequence of the above mentioned differences between the enzymatic activities of ZmCKS and its bacterial counterparts.

A significant difference between ZmCKS and its bacterial homologues is the presence in the maize gene of a leader signal peptide. This suggests that the enzyme is located in the endomembranous system of the plant cell. In bacteria, CMP-KDO synthetases are cytoplasmic enzymes, and the incorporation of the resulting activated KDO into the wall lipopolysaccharides occurs at the cytoplasmic side of the membrane (1). In plant cells, pectins are synthesized in the Golgi apparatus before being secreted into the apoplastic space (30, 19). However, the reported plant KDO-8-phosphate synthetases have no signal peptide and are probably cytoplasmic enzymes. Consequently, the activation of KDO by ZmCKS in the endomembranous system would provide a link between the synthesis of the sugar in the cytoplasm and its incorporation into pectins in the Golgi apparatus.

**The Evolution of ZmCKS**—Until now, KDO has never been reported as a constituent of any yeast or animal polysaccharide. No homologues of the enzymes involved in its synthesis and activation (KDO-8-phosphate synthetase and CMP-KDO synthetase) were found after examining the complete genomic sequences of the yeast S. cerevisiae and the worm C. elegans. KDO is, however, a common component of some plant and algal polysaccharides, but an extensive data base search for homologous genes (including the already advanced Arabidopsis genome data base) produced only one eukaryotic representative, the EST sequence from P. taeda (Fig. 2). While this manuscript was in preparation, a second plant CKS gene, from tomato, was reported as an EST (EMBL accession number AI489926). The Southern analyses presented in this work (Fig. 3) suggest that homologous sequences are present in cereals, but the tomato gene could not be detected under experimental conditions that allowed the detection of the E. coli homologue. This could be a consequence of the added effect of sequence divergence and dilution into the large eukaryotic genomes, which could also have rendered a possible Arabidopsis CKS undetectable. In any case, these findings suggest that CKS genes are not evenly distributed in eukaryotes and that their scattered distribution could derive from an anomalous (i.e. nonvertical) pattern of evolution for these genes.

Consistent with this suggestion, ZmCKS is very poorly adapted to the general pattern of codon usage of maize as measured using the CAI index (0.64) (28). In fact, the E. coli gene KpsU has a higher CAI value (0.68) than ZmCKS using the same maize table. The CAI assesses the extent to which selection has been effective in molding the pattern of codon usage. The level of expression of the gene therefore influences the efficiency of the process. Indeed, there is considerable divergence in the CAI indices between individual genes within an
organism (32, 33). In higher organisms codon usage is thought to be influenced by the chromosomal position of the gene and the overall G+C content rather than by natural selection (34). In the case of ZmCKS, however, a remarkable finding is that the codon usage, as measured by CAI, only deviates significantly from the maize pattern in that part of the coding sequence which shows homology to the prokaryotic genes. The exclusively eukaryotic part of the coding sequence, the signal peptide coding region, shows a rather high CAI value (0.78). When the gene was compared against a maize codon usage table constructed from highly expressed genes, even more divergent CAI values were obtained for the mature protein (0.25) and signal peptide (0.71) coding sequences. Unfortunately, neither the P. taeda (Fig. 2) nor the tomato ESTs contain the complete coding sequence of the corresponding CKS, and therefore no comparisons can be made between their signal peptides (if they have them) and mature protein coding sequences.

These results do not provide conclusive evidence, but they strongly suggest a chimerical origin for ZmCKS. It is possible that some taxonomic groups had acquired a bacterial-like enzyme by horizontal transfer. Integration of this gene into eukaryotic metabolism would have required subsequent adaptation of the protein, for example the incorporation of a signal peptide and the modification of its substrate preference. Horizontal transfer seems to be an important evolutionary force in bacteria, where it might explain the origin of a substantial portion of the genome (up to 18% in E. coli; Ref. 35). In eukaryotes, the best documented cases include two transposons, the P element (36) and mariner (37). It has been argued that horizontal transfer might explain the anomalous phylogenies of some multigene family genes such as rubisco (38) and the cyclophilins (39).

Concluding Remarks—The plant cell wall acts not only as an exoskeleton to give the plant cell its shape, but it also plays a pivotal role in numerous cell-cell signaling, defense, and differentiation processes (40, 41). Assessing the function of its different components in these processes is an important research goal. The rhamnogalacturonan II pectin fraction, in particular, is usually considered too scarce to be a major structural cell wall polymer, but its complex structure and widespread occurrence in plants suggest that it could participate in signaling processes (42). The cloned ZmCKS gene could be used as a tool to specifically alter the biosynthesis of the rhamnogalacturonan II pectin fraction of the primary cell wall. This may serve to help us better understand its role in plant cell function. The pattern of distribution of ZmCKS homologues within the plant kingdom and the evolutionary mechanisms responsible for the presence of these genes in plants such as cereals is an attractive subject for further studies.

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