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Evidence for the thiamine biosynthetic pathway in higher-plant plastids and its developmental regulation

Faith C. Belanger[1,*], Thomas Leustek[1], Boyang Chu[1] and Alan L. Kriz[2]
[1] *Plant Science Department, Rutgers University New Brunswick, NJ 08903, USA (*author of correspondence);* [2] *DeKalb Genetics Corp., Mystic, CT 06355, USA*

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Abstract

Thiamine or vitamin B-1, is an essential constituent of all cells since it is a cofactor for two enzyme complexes involved in the citric acid cycle, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. Thiamine is synthesized by plants, but it is a dietary requirement for humans and other animals. The biosynthetic pathway for thiamine in plants has not been well characterized and none of the enzymes involved have been isolated. Here we report the cloning and characterization of two cDNAs representing members of the maize *thi1* gene family encoding an enzyme of the thiamine biosynthetic pathway. This assignment was made based on sequence homology to a yeast thiamine biosynthetic gene and by functional complementation of a yeast strain in which the endogenous gene was inactivated. Using immunoblot analysis, the *thi1* gene product was found to be located in a plastid membrane fraction. RNA gel blot analysis of various tissues and developmental stages indicated *thi1* expression was differentially regulated in a manner consistent with what is known about thiamine synthesis in plants. This is the first report of cDNAs encoding proteins involved in thiamine biosynthesis for any plant species.

Introduction

For man and for other non-ruminant animals, thiamine, or vitamin B-1, is a dietary requirement [for a review see 12]. Both yeasts and cereal grains are rich, biological sources of thiamine. Under most circumstances the thiamine requirement of ruminant species is satisfied via synthesis by microorganisms in the rumen [1]. Thiamine pyrophosphate is the active form of the vitamin and is a cofactor for the pentose phosphate pathway enzyme transketolase, and for the citric acid cycle enzyme complexes, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. In plant cells thiamine is also a cofactor for the plastid-localized isozymes pyruvate dehydrogenase and transketolase.

In cereal grains thiamine is concentrated in the aleurone layer and in the embryo [38]. The starchy endosperm has only low levels of thia-

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers U17350 and U17351.

mine so individuals consuming only white flours and polished grains can be susceptible to thiamine deficiency. A lack of dietary thiamine results in disturbance of carbohydrate metabolism and increases in the blood concentrations of pyruvate. The thiamine deficiency disease beriberi is characterized by disturbances of the central nervous system and has been a major health problem in many rice-consuming nations [12].

Plants and many microorganisms can synthesize thiamine *de novo*. The biosynthesis of thiamine has been most extensively studied in yeasts and bacteria but the pathway has not been completely characterized in any organism [for a review see 41]. In the later steps in the pathway, the precursors hydroxymethyl-pyrimidine and hydroxyethyl thiazole are each phosphorylated and then combined to form thiamine monophosphate which is then converted to thiamine pyrophosphate (Fig. 1). The steps producing the pyrimidine and thiazole precursors are not completely known and appear to differ in *Escherichia coli* and yeasts [41].

The study of thiamine biosynthesis in plants is more limited but mutants defective in different steps in thiamine synthesis have been reported [6, 21, 18, 31]. From biochemical studies [25, 26] and mutant feeding studies [21, 18, 31] it appears that hydroxymethylpyrimidine and hydroxyethylthiazole are also thiamine precursors in plants. The biosynthetic pathways leading to the pyrimidine and thiazole precursors in plants has not been determined.

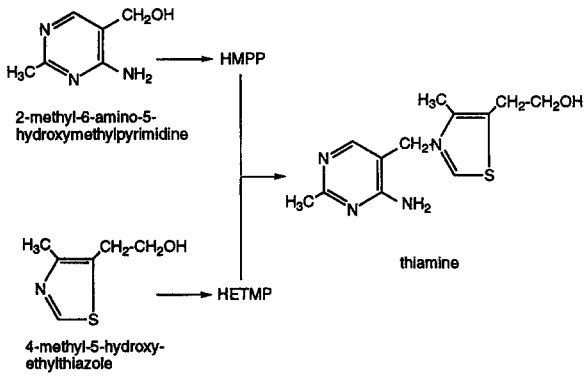


Fig. 1. Later steps in the biosynthetic pathway of thiamine. The pyrimidine and thiazole precursors are each phosphorylated and combined to form thiamine monophosphate. Thiamine is produced through the action of a phosphatase. Thiamine pyrophosphokinase then converts thiamine to the active form of the vitamin, thiamine pyrophosphate. HMPP, 2-methyl-6-amino-5-hydroxyxmethyl-pyrimidine pyrophosphate; HETMP, 4-methyl-5-hydroxy-ethylthiazole monophosphate.

Recently a gene encoding an enzyme involved in the synthesis of the thiazole precursor of thiamine has been identified in the yeast *Saccharomyces cerevisiae* [30]. The yeast gene, designate *TH14*, was originally identified by its induction during early stationary phase growth on molasses medium in response to thiamine depletion [29, 30]. Disruption of the yeast gene resulted in cells which were auxotrophic for thiamine but which did grow when supplied with the thiamine precursor hydroxyethylthiazole [30]. The biosynthetic pathway leading to the thiazole precursor of thiamine is not yet known so the specific enzymatic function of the yeast *TH14* gene product has not been determined. A highly homologous gene isolated from *Schizosaccharomyces pombe* has also been shown to be involved in thiamine biosynthesis [24].

Here we report the cloning and characterization from *Zea mays* (maize) of two cDNAs which are homologous to the yeast genes. Functional complementation of the *S. cerevisiae THI4* deletion strain confirmed the assignment of a thiamine biosynthetic function for the protein encoded by these cDNAs. Immunoblot analysis indicated the maize protein is located in plastid membranes. This is the first report of plant cDNAs encoding proteins involved in thiamine biosynthesis and the first report of the cellular localization of the biosynthetic pathway.

Materials and methods

Plant materials

Developing embryos, endosperm, immature ears, and immature tassels of the maize inbred line Va26 were obtained from field-grown plants. Green leaves, roots, and etiolated shoot were ob-

tained from Va26 seedlings germinated in the laboratory and greenhouse.

Nucleic acid isolation, gel blot analysis and sequencing

Maize seedling total DNA was isolated as described by Dellaporta *et al.* [11] followed by further purification on CsCl gradients. Total RNA was isolated by using the guanidine-HCl method described by Cox [10]. Polyadenylated RNA was fractionated from total RNA by oligo(dT)-cellulose chromatography [2].

For DNA gel blot analysis 10 μg of restriction enzyme-digested total genomic DNA were electrophoresed in a 0.8% agarose gel and transferred to Magnagraph nylon membrane (Micron Separations, Westborough, MA). For RNA gel blot analysis, total or poly(A)$^+$ RNA was subjected to electrophoresis in formaldehyde agarose gels and transferred onto Magnagraph nylon membranes as descibed by Selden [36]. Equal sample loading of the RNA gel blots was assesed by ethidium bromide staining. Radiolabelling and hybridization of the blots were as previously described [9].

For nucleotide sequence analysis the cDNA clones were subcloned into M13mp18 and mp19 [40] to obtain inserts in opposite directions. Overlapping unidirectional deletions corresponding to either strand were prepared from the appropriate M13 clone RF by using a commercial exonuclease III/mung bean nuclease deletion kit (Stratagene, La Jolla, CA). Dideoxynucleotide sequencing [34] of single-stranded templates with T7 DNA polymerase was performed by using a commercial kit (United States Biochemical Corp., Cleveland, OH).

Expression of maize thi1-1 *in a yeast disruption strain*

The maize *thi1 Eco* RI fragment containing the coding sequence was subcloned into pBluescript and recombinants in both orientations were obtained. The *thi1-1* fragments in both orientations were each excised from pBluescript with *Bam* HI and *Sal* I and subcloned into the yeast expression vector pG-1 [35]. The resultant recombinant pG-1 plasmids were then used to transform a *S. cerevisiae* thi4::URA3 disruption strain, 842Δthi4 (provided by P.A. Meacock). The genotype of this strain is *MATa, leu2-3, ade 2-1, trp1-1, his3-11, ura3, thi4::URA3*.

Synthetic minimal media (SD) for growth of yeast were prepared as described by Sherman [37] with all nutrient supplements required by strain 842, unless noted. The recombinant pG-1 plasmids containing the maize *thi1-1*-coding sequence in both orientations were transformed into yeast strain 842 by electroporation [3]. Transformants were recovered at 30 °C on SD medium lacking tryptophan. The colonies were then replica plated onto SD lacking tryptophan and thiamine and then incubated at 24 °C.

Protein purification and antibody production

Maize THI1-1 was expressed in *E. coli* is a fusion protein with the maltose-binding protein in the plasmid pMAL-c supplied with a commercial kit (New England Biolabs, Beverly, MA). Partial purification of the expressed protein, including cleavage from the maltose binding protein portion of the fusion protein, was as described by the kit manufacturer. THI1-1 was further purified by SDS-PAGE. The gel was stained with cold 0.25 M KCl, 1 mM DTT [15] and the THI1-1 band excised. In order to concentrate the protein and to facilitate elution from the gel matrix, the acrylamide gel strips were then applied to a ProSieve gel (FMC BioProducts, Rockland, ME) and subjected to electrophoresis until the dye front was near the middle of the stacking gel. The protein band, which migrated with the dye front, was excised from the gel. The protein was recovered from the gel as described by the manufacturer. The purified protein was injected into mice. Three injections of 40–50 μg protein each were given at 3-week intervals and the ascites fluid collected.

Antibody specific to the fusion protein was pre-

pared through affinity purification. Bacterial extracts from cells expressing the fusion protein were subjected to SDS-PAGE, stained with cold 0.25 M KCl, 1 mm DTT [15], and the fusion protein band excised. Protein in the excised band was transferred to nitrocellulose [7]. The bound fusion protein was then used in affinity purification of the antibody as described previously [22].

Gels for immunoblots were transferred to nitrocellulose (Nitro-Bind, Micron Separations) [7]. Membranes were blocked in 5% nonfat dry milk in TTBS for 1 h, then incubated overnight in the affinity-purified antibody in TTBS. Membranes were washed four times in TTBS for 15 min each wash and incubated in a 1:10000 dilution of goat anti-mouse IgG horseradish peroxidase conjugate (Promega, Madison, WI) for 1 h. Membranes were washed three times in TTBS prior to chemiluminescent detection using a commercial kit (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL).

To demonstrate the specificity of the antibody, the transformed bacterial cells were grown to an OD of 0.55. IPTG was then added to a final concentration of 0.5 mM to induce expression of the fusion protein and the cells were grown for an additional 2 h. The cells (0.75 ml) were collected by centrifugation and extracted by the addition of 1 ml of 2× SDS sample buffer [20]. Duplicate aliquots were subjected to SDS-PAGE and either stained with Coomassie Brilliant Blue or transferred to nitrocellulose for immunoblot analysis.

Plastid purification

Intact mesophyll chloroplasts were isolated from primary and secondary leaves of 20-day old Va26 seedlings by Percoll step-gradient centrifugation [28]. The chloroplasts were resuspended in 50 mM Tris-Cl, pH 8.0, and the stroma separated from membranes by centrifugation at 12000× *g*. Whole leaf extracts were prepared in the same buffer by grinding in a cold mortar with 0.5 mm glass beads. Protein concentrations were determined using the bicinchoninic acid assay from Pierce (Rockford, IL).

Results

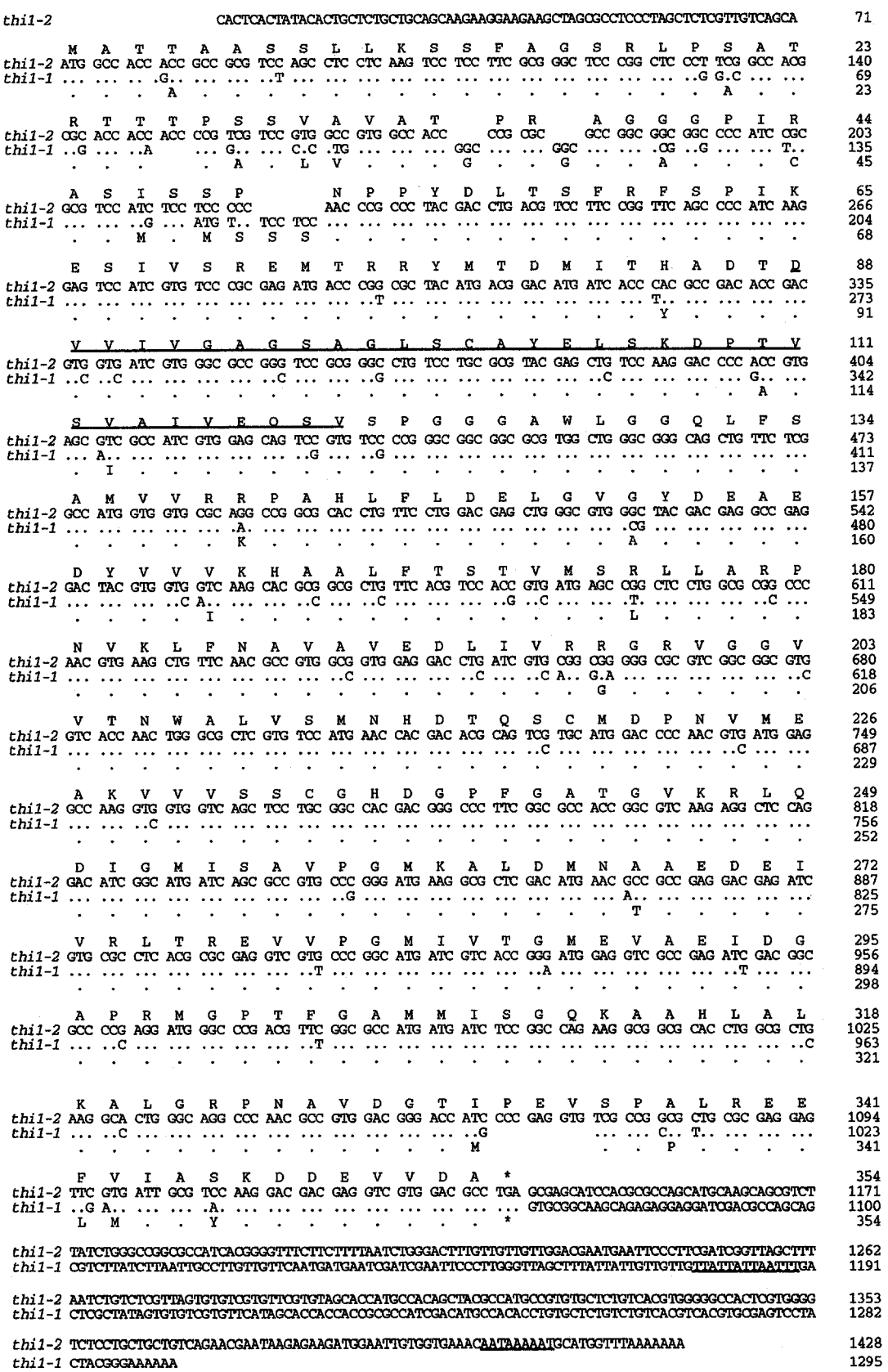
Characterization of maize thi1 *cDNA clones*

In a random screen of a maize embryo cDNA library [5], a cDNA clone was identified that corresponded to transcripts accumulated during embryo development. Sequence analysis of the cDNA revealed it had significant homology to a *S. cerevisiae* gene required for synthesis of the thiazole precursor of thiamine [30]. Based on the strong homology to the yeast sequence, and since this is the first maize clone for a thiamine biosynthetic enzyme, the maize cDNA clone was designated pcThi1-1.

A 5′ 324 bp *Sst* I fragment was used to rescreen the cDNA library for a full-length clone. The sequence of the full-length clone indicated that the ATG sequence beginning at position 1 of the pcThi1-1 clone was the codon for the initial methionine. The sequence of the full-length clone was not identical to that of pcThi1-1, however, indicating it was another member of the gene family. Based on identification of three distinct chromosomal locations for pcThi1 sequences (see below), corresponding gene loci were designated *thi1-1* and *thi1-2*. The nucleotide sequences and deduced amino acid sequences of *thi1*-1 and *thi1*-2 cDNA clones are shown in Fig. 2.

The sequence surrounding the presumed initiator methionine in the *thi1-2* cDNA differs in only one position from the consensus sequence of AACAAUGGC for plant mRNAs [23]. An AT-rich

Fig. 2. Nucleotide and deduced amino acid sequences of the *Z. mays thi1-1* and *thi1-2* cDNAS. Throughout the coding sequence only the complete sequence of *thi1-2* is shown. The sequence of *thi1-1* is shown where it differs from *thi1-2*. Dots indicate identity to *thi1-2*. Gaps were introduced to maintain the alignment. The putative dinucleotide binding sequence is underlined. The termination codons are indicated by asterisks. AT-rich regions in the 3′-untranslated sequences which may be polyadenylation signals are underlined.



region between nucleotide positions 1404 and 1412 in the *thi1-2* cDNA may be a polyadenylation signal and contains the consensus sequence AATAAA [32]. There is no consensus polyadenylation signal in the 3′ untranslated region of the *thi1-1* cDNA. There is, however, an AT-rich region between nucleotide positions 1177 and 1189. The *thi1-1* and *thi1-2* cDNA clones are not homologous in their 3′-untranslated regions. Both cDNAs have *Eco* RI restriction sites in the 3′-untranslated region, which were presumably protected during the library construction. The nucleotide sequence predicts a polypeptide of 354 amino acids for both cDNAs and calculated molecular weights of 37104 for *thi1-1* and 37233 for *thi1-2*.

The deduced amino acid sequence of maize *thi1-1* exhibits a considerably hydrophobic character (Fig. 3). Non-polar amino acids account for 49.7% of the total. The predicted polypeptide also has a relatively high methionine content of 5.9%.

Both *thi1-1* and *thi1-2* encode polypeptides with amino-terminal sequences, not found in the yeast enzyme, that resemble transit peptides for targeting to plastids. The first 40 amino acids of both sequences are rich in hydroxylated and small hydrophobic amino acids and have a net positive charge (Fig. 2), features that are typical of plastid transit peptides [17]. Most transit peptides are proteolytically removed after import into plastids by a specific protease that recognizes a consensus sequence, V/I-X-A/C-A, where X is frequently an R [13]. THI1-2 contains such a sequence between residues 43 and 46 (sequence IRAS) with a potential cleavage site after amino acid 45. A protease recognition sequence is less apparent for THI1-1 although, based on sequence alignment with THI1-2, it may exist between residues 44 and 47 (sequence ICAS) with a potential cleavage site after amino acid 46. If THI1-1 and THI1-2 are proteolytically processed at these sites, the sizes of the mature proteins would be 32770 and 32956, respectively. Consistent with an overall hydrophobic character and a likely plastid transit sequence, THI1 has been localized to a plastid membrane fraction (see below).

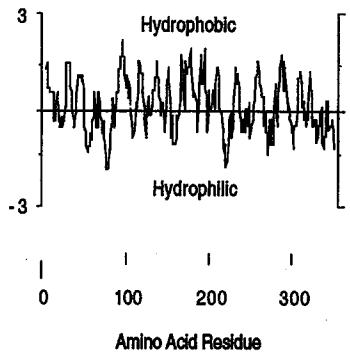


Fig. 3. Hydropathy plot of deduced protein sequence of THI1-1. This plot was generated by using the algorithm of Kyte and Doolittle [19], as supplied with the Genetics Computer Group sequence analysis software package, using a window of nine amino acids.

A comparison of the deduced amino acid sequences of the maize *thi1* genes with homologous sequences from other organisms is shown in Fig. 4. The only other complete sequence of a plant gene homologous to the maize *thi1* gene is that from *Arabidopsis thaliana*. A partial cDNA sequence from *Ricinus communis* has also been reported (GenBank accession number T24355). Since the sequence is not complete, however, it is not included in Fig. 4.

The central portions of the proteins show considerable amino acid conservation, whereas there is much less overall homology in the amino and carboxyl terminal domains. From the first conserved phenylalanine to the final conserved threonine, there is a 46% identity among all 7 sequences, based on the maize sequence length of 243 amino acids. Among the nonidentical amino acids there is a high degree of conserved substitutions. Overall, these proteins show a striking degree of evolutionary conservation. Table 1 shows the amino acid identities of the sequences in pairwise comparisons.

The deduced amino acid sequences of the maize *thi1* clones and their homologues from other organisms all contain a conserved dinucleotide binding site. Figure 5 depicts the putative dinucleotide binding regions and the consensus sequence developed from several known NAD and FAD binding enzymes [39]. All seven amino acid sequences match perfectly with the proposed con-

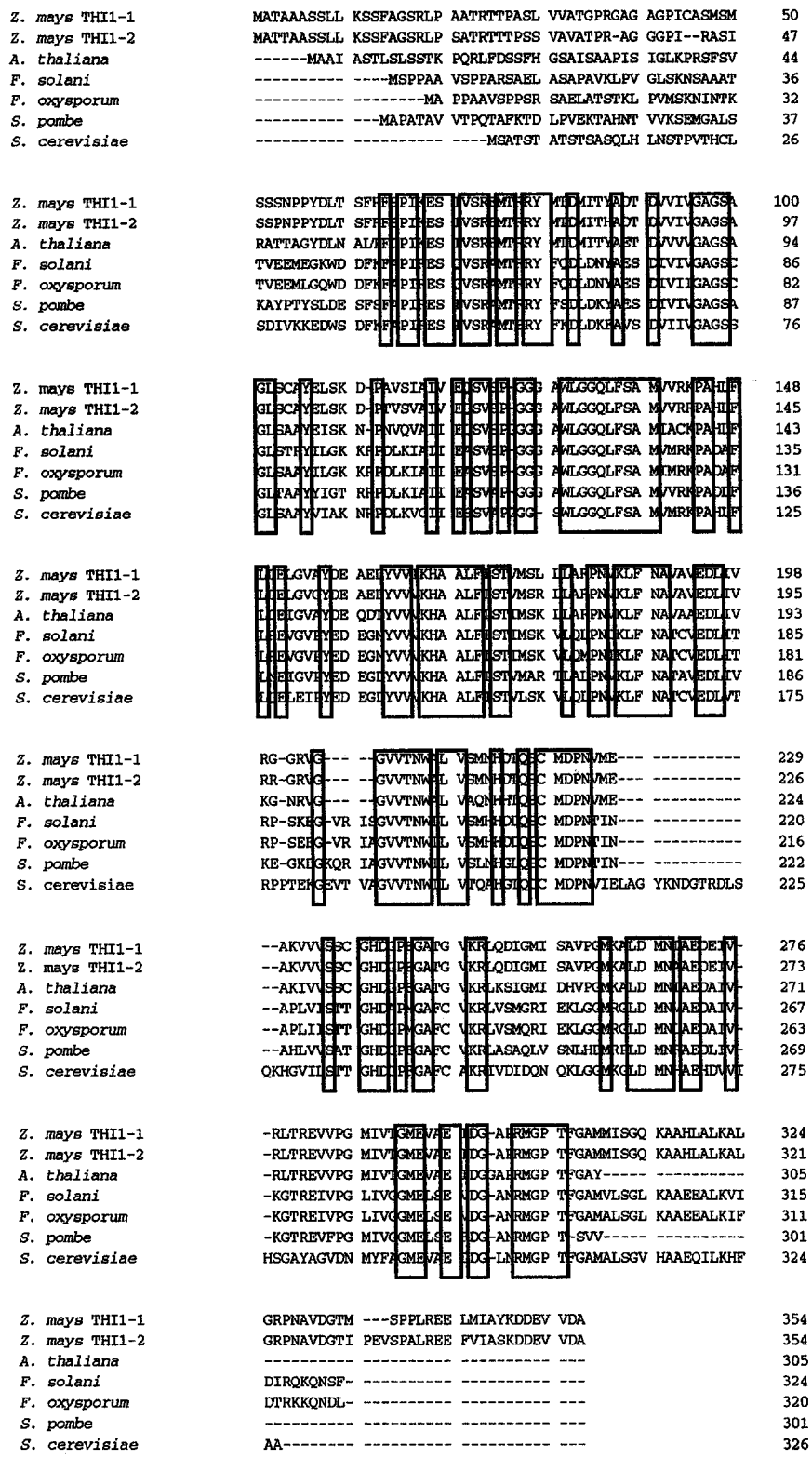


Fig. 4. Comparison of the deduced amino acid sequences of the *Z. mays thi1* cDNA clones and the *A. thaliana*, *Fusarium* spp., *S. pombe*, and *S. cerevisiae* homologues. Boxes enclose identical amino acids. GenBank accession numbers for the corresponding DNA sequences are: *Z. mays*, U17350 and U17351; *A. thaliana*, U17589; *F. solani*, M33642; *F. oxysporum*, M33643; *S. pombe*, X82363; and *S. cerevisiae*, X61669.

Table 1. The extent of amino acid identities of THI1 homologues.

	Z.m. 1	Z.m. 2	A.t.	F.s.	F.o.	S.p.	S.c.
Z.m. 1[a]	*	90	65	51	51	50	43
Z.m. 2[a]		*	63	50	49	49	44
A.t.[b]			*	49	50	54	45
F.s.[c]				*	89	61	55
F.o.[d]					*	61	56
S.p.[e]						*	46
S.c.[f]							*

[a] *Z. mays* THI1-1 and THI1-2 sequences from this study.
[b] *A. thaliana* sequence from an unpublished GenBank submission (accession number U17589).
[c] *F. solani* sequence from Choi *et al.* [8].
[d] *F. oxysporum* sequence from Choi *et al.* [8].
[e] *S. pombe* sequence from Manetti *et al.* [24].
[f] *S. cerevisiae* sequence from Praekelt and Meacock [29].

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A. thaliana      85   DVVVVGAGSAGLSAAYEISKN PNVQVAIIE
F. oxysporum     73   DIVIIGAGSCGLSAAYILGKKRPDLKIAIIE
F. solani        77   DIVIVGAGSCGLSTRYILGKKRPDLKIAIIE
S. cerevisiae    67   DVIIVGAGSSGLSAATVIAKNRPDLKVCIIE
S. pombe         78   DIVIVGAGSAGLTAAYYIGTRRPDLKIAIIE
Z. mays,1        91   DVVIVGAGSAGLSCAYELSKD PAVSIAIVE
Z. mays,2        88   DVVIVGAGSAGLSCAYELSKD PTVSVAIVE

CONSENSUS             PH H G G  G   H  H        H H *
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Fig. 5. Comparison of the putative dinucleotide binding regions of the *Z. mays* THI1 sequences and their homologues. The consensus sequence [39] is symbolized at the bottom: P, invariant hydrophilic residue; H, neutral or hydrophobic residues; G, invariant glycine; *, invariant negatively charged residue.

sensus with the single exception of the Arg at position 91 of the *Fusarium solani* sequence. That these sequences are so highly conserved strongly supports the identification of a dinucleotide binding site.

Maize thi1-1 *functionally complements a yeast disruption strain*

To test for the function of maize THI1-1, the *thi1-1* cDNA was cloned into an *S. cerevisiae* expression plasmid and then transformed into a thi4::URA3 deletion strain, 846 [30]. The results of this complementation test are shown in Fig. 6. The cells carrying the recombinant pG-1 plasmids which had the *thi1-1* sequence inserted in

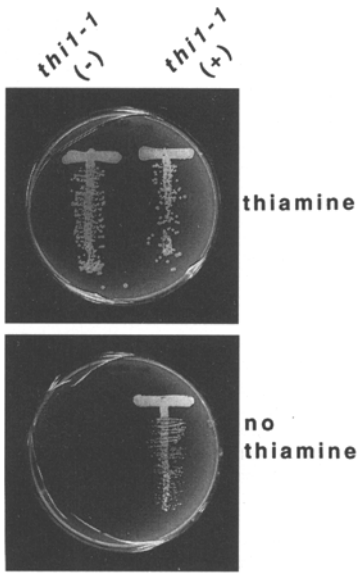

Fig. 6. Functional complementation of an *S. cerevisiae* disruption strain by maize *thi1-1*. The maize *thi1-1* coding sequence, in both the sense (+) and antisense (−) orientations for expression, was cloned into the yeast expression vector pG-1. The yeast thi4::URA3 disruption strain, 846, was then transformed with both plasmids. The resulting transformants carrying either the + or − *thi1-1* construct were inoculated onto media supplemented with, or lacking thiamine. The cultures were incubated at 24 °C for 3 days (thiamine-supplemented plate) or 20 days (thiamine-deficient plate) before being photographed.

either the correct or reverse orientation for expression could both grow on thiamine-supplemented medium. Only the strain carrying *thi1-1* in the correct orientation for expression could grow on thiamine deficient medium. These results confirm the thiamine requirement of strain 846 and that maize *thi1-1* encodes the functional homolog of the yeast *THI4* gene.

It should be noted that the strain carrying *thi1-1* required at least 10 to 14 days for growth to become evident on thiamine-deficient medium, compared to 1–2 days on thiamine-supplemented medium. We believe that *thi1-1* truly complements strain 846 because similar growth results were obtained when a different yeast expression vector was used (not shown). One possible explanation for this delayed-growth phenotype is that the yeast enzyme may be localized within mitochondria, a cellular compartment that may not be readily ac-

cessible to the plastid localized maize enzyme. Indeed, the yeast amino acid sequence (Fig. 4) shows a short amino-terminal domain that resembles a mitochondrial targeting signal sequence [4].

Representation of thi1 *sequences in the maize genome*

In DNA gel blot analysis of maize DNA from the inbred line Va26, the *thi1-1* insert hybridized with three restriction fragments after cleavage with several different enzymes (Fig. 7). This suggested that THI1 is encoded by a small gene family. A gene family was confirmed by mapping the *thi1* genes [as UIU(pog1a), UIU(pog1b), and UIU(pog1c), respectively] to 3 different chromosomal locations: the long arm of chromosome 3, the short arm of chromosome 6, and the long arm of chromosome 8.

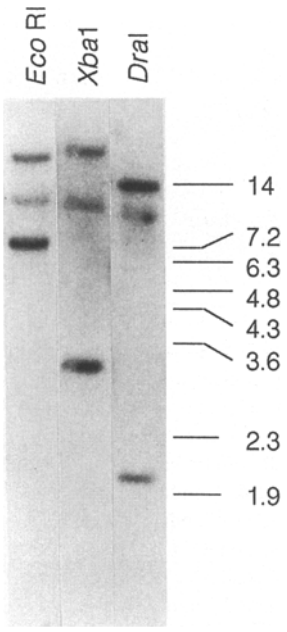

Fig. 7. DNA gel blot analysis of maize DNA from the inbred line Va26. Ten μg of total DNA per sample was digested with the indicated restriction enzyme, fractionated by electrophoresis on an agarose gel, transferred onto a nylon membrane and hybridized with the radiolabelled *thi1-1* cDNA. Sizes of the DNA markers are in kb.

Developmental and tissue specific expression of maize thi1

The accumulation pattern of *thi1* transcripts during embryo development and seed germination is depicted in Fig. 8. Total RNA from Va26 embryos at 15 to 36 days after pollination and the embryo portions of mature and germinating kernels was subjected to RNA gel blot analysis in which the *thi1-1* insert was used as a radiolabelled probe. The level of *thi1* transcripts increased from 15 to 21 days then slightly decreased at 24 days. The 24 day level was maintained through 36 days. There were no detectable transcripts in the embryo portions of dry mature kernels, overnight imbibed kernels, or in 1 or 3 day germinated kernels. From these results it is clear that *thi1* expression is developmentally regulated in maize embryos.

The accumulation of *thi1* transcripts in other tissues was also investigated by RNA gel blot analysis. An RNA gel blot of total RNA from 27-day developing embryos, 27-day developing endosperm, etiolated shoots, roots, green leaves, immature ears and immature tassels was probed with the *thi1-1* insert (Fig. 9A). The highest level of *thi1* transcripts was found in the developing embryo sample. Most of the signal observed in the sample from green leaves was actually due to hybridization to 16S rRNA (see below). Imma-

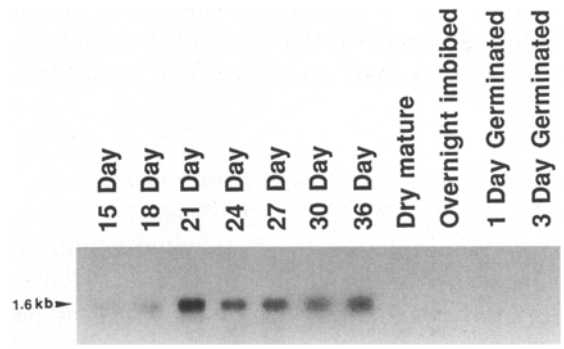

Fig. 8. Z. mays thi1 expression is developmentally regulated. Total RNA (10 μg per sample) from VA26 embryos from 15–36 days after pollination and from the embryo portion of mature and germinating kernels was subjected to RNA gel blot analysis. The radiolabelled insert from *thi1-1* cDNA was used as a probe.

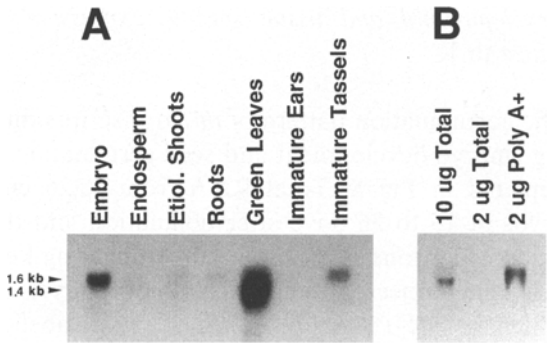

Fig. 9. Tissue-specific expression of *Z. mays thil*. A. RNA gel blot analysis of RNA from various maize tissues probed with the *thil-1* cDNA insert. B. Comparison of the leaf total RNA and leaf poly(A)$^+$ RNA probed with the *thil-1* cDNA insert.

ture tassels also had detectable levels of *thil* transcripts. Only very low levels of *thil* transcripts were detected in endosperm, roots, etiolated shoots, or immature ears. From these results it is clear that expression of *thil* is also regulated at the tissue level.

Since there appeared to be two different-sized transcripts in the RNA sample from green leaves, we investigated the possibility that the lower 1.4 kb strongly hybridizing band was due to hybridization of the *thil-1* insert to 16S rRNA which was abundant in the leaf total RNA. In Fig. 9B the hybridization of the *thil-1* insert to total and poly(A)$^+$ RNA from green leaves is compared. Since only the 1.6 kb band was seen in the poly(A)$^+$ RNA sample we concluded that the 1.4 kb band seen in the total RNA sample was indeed due to hybridization to the 16S RNA.

Maize THI1 *is localized in plastid membranes*

As discussed above, the N-terminal amino acid sequences of the maize and *A. thaliana* THI1 proteins have nearly ideal chloroplast transit peptides suggesting a plastid location for the pathway in plants. In order to determine if THI1 is indeed localized in plastids, immunoblot analysis was carried out on leaf extracts using antiserum prepared against THI1-1 expressed as a fusion protein in *E. coli*. Detection of *thil* mRNA in green leaves (Fig. 9) suggested the protein was expressed in leaf tissue, in addition to developing embryos.

Fig. 10, A and B, demonstrates the specificity of the antibody for THI1. There was no antibody reaction in the absence of IPTG induction of expression of the fusion protein (Fig. 10B, lane 4).

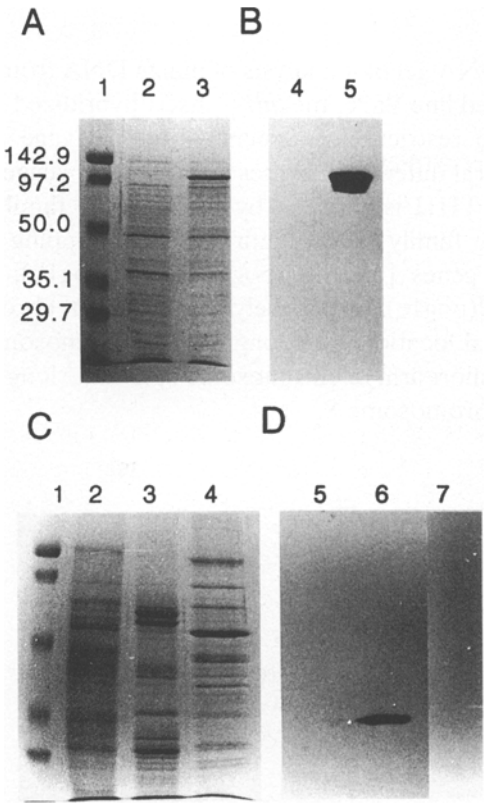

Fig. 10. Specificity of antibody to maize THI1-1 fusion protein and immunological detection of THI1 in plastid membranes. A and B. Coomassie stained gel (A) and immunoblot (B) of extracts from bacterial cells containing the recombinant pMAL-c plasmid with the maize *thil-1*-coding sequence. Lane 1, prestained protein standards; lanes 2 and 4, cells grown in the absence of IPTG; lanes 3 and 5, cells grown in the presence of IPTG. C and D. Coomassie stained gel (C) and immunoblot (B) of maize leaf extracts. Lane 1, prestained protein standards; lanes 2 and 5, leaf crude extract; lanes 3 and 6, membrane fraction from purified chloroplasts; lanes 4 and 7; stroma fraction from purified chloroplasts. A 9 μg and a 90 μg of each sample were applied to the stained gel and immunoblot, respectively. The prestained protein standards and their apparent molecular masses (Da) are: phosphorylase B, 142900; bovine serum albumin, 97200; ovalbumin, 50000; carbonic anhydrase, 35100; soybean trypsin inhibitor, 29700.

Addition of IPTG resulted in induction of a protein migrating close to the 97.2 kDa standard which was recognized by the antibody (Fig. 10B, lane 5). The expected size of the fusion protein is about 79 kDa but THI1-1 migrated in SDS-PAGE slower than would be expected based on its calculated size. Following cleavage of the maltose binding protein portion of the fusion protein, the THI1-1 protein migrated at about 44 kDa, instead of at the expected position (data not shown).

To determine the cellular location of THI1 in plant cells, equal protein amounts from a leaf crude extract and from the membrane and stroma fractions of purified mesophyll chloroplasts were subjected to immunoblot analysis using the THI1 antiserum (Fig. 10C and D). A faint reaction was detectable in the crude extract (Fig. 10D, lane 5) and a strong reaction was detectable in the plastid membrane fraction (Fig. 10D, lane 6). There was no reaction in the chloroplast stroma fraction (Fig. 10D, lane 7). These results indicated that THI1 is located in the plastid membranes, as suggested by the N-terminal sequence. The apparent size of the antibody reactive protein was as would be expected if the proposed plastid targeting sequence were cleaved. Why the fusion protein and the intact THI1-1 protein, following cleavage from the maltose binding protein, migrated slower than expected, yet the mature form migrated as expected, is now known.

Discussion

In this report we have characterized maize *thi1* cDNAs and demonstrated by functional complementation of an *S. cerevisiae* disruption strain that the protein product is an enzyme in the biosynthetic pathway leading to the thiazole precursor of thiamine. The *S. pombe* homologue has also been demonstrated to be involved in thiamine synthesis [24]. The *S. cerevisiae* and *S. pombe* homologues are both highly induced in response to thiamine depletion [30, 24]. *Fusarium* spp. homologues have been reported which were identified as mRNAs which were induced by various stresses, such as ethanol, copper chloride, heat, and phytoalexins [8]. Perhaps the physiological stresses imposed on the *Fusarium* spp. resulted in thiamine depletion and subsequent induction of the thiamine biosynthetic genes. Our data indicate that expression of the maize *thi1* genes is developmentally regulated. Whether expression is also responsive to stress is not known.

Since the specific steps in the thiamine biosynthetic pathway are not yet known, assignment of the particular enzymatic function for maize THI1 and its homologues cannot be made. In both yeast and bacteria, however, pentose sugars are believed to be intermediates in this pathway [41]. The proteins encoded by maize *thi1* genes, and the homologous proteins from other organisms, have a highly conserved dinucleotide binding site. In general, enzymes which bind NAD or FAD function as dehydrogenases, hydroxylases, reductases, or oxidases. The proposed pathways for conversion of pentoses to the thiazole precursor [41] are likely to involve the types of enzymes which would require NAD or FAD.

Cellular localization of the thiamine biosynthetic pathway has not previously been investigated in any organism. Our data indicates that maize THI1 is localized in the plastid membrane fraction, implying a plastid location for thiamine biosynthesis. This result is consistent with the presence of an N-terminal plastid targeting sequence [17] and the overall hydrophobic character of the protein. Expression of *thi1* in developing embryos implies that plastids other than photosynthetically active chloroplasts are also capable of thiamine biosynthesis.

The four fungal sequences all have N-terminal sequences suggestive of mitochondrial targeting sequences. In general, mitochondrial targeting sequences are positively charged and capable of forming an amphiphilic, α-helical secondary structure [4, 33]. Although there are some negatively charged residues in the fungal N-terminal sequences, when they are displayed as helical wheels, the positively charged residues are clustered on one side of the protein.

Although maize THI1-1 clearly complements the *S. cerevisiae* disruption strain, the growth of

the cells is slow. This may be due to the presence of the plastid transit sequence at the amino terminus of the protein. If the thiamine biosynthetic pathway in yeast is indeed localized in the mitochondria, the chloroplast targeting sequence may be inefficient in targeting THI1-1 to the yeast mitochondria. Alternatively, the presence of an uncleaved transit sequence may affect the folding of the protein, resulting in reduced activity. Ultimately, constructs in which the chloroplast targeting sequence has been replaced with a yeast mitochondrial targeting sequence could be tested for complementation.

Our data on expression of maize *thi1* are consistent with what is known regarding thiamine synthesis in plants. The low level of *thi1* transcripts in the mature embryos and during germination is consistent with the report that there was no net synthesis of thiamine during the first ten days of germination of maize seedlings [16]. Expression of *thi1* highest in the developing embryos and in green leaves. Photosynthetic tissues are known to synthesize thiamine while roots do not [42]. Thiamine synthesized in the leaves is translocated to the roots via the phloem [42]. Roots may also obtain thiamine via uptake from the soil of microbial synthesized thiamine [27]. Embryos are known to contain thiamine [38] which has been considered to have been translocated from other parts of the plant [14]. Our data implies the thiamine biosynthetic pathway is also active in developing embryos.

The characterization of the maize *thi1* cDNAs should facilitate future investigations into thiamine biosynthesis in plants. *A. thaliana* and *Pisum sativum* mutants deficient in synthesis of the thiazole precursor of thiamine have been reported [18, 21]. Since an *A. thaliana thi1* homologue has been detected, it would be interesting to determine if any of the mutants have a defective *thi1* gene.

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