Identification of mitochondrial coenzyme A transporters from maize and Arabidopsis¹

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One-sentence summary: Coenzyme A made in the cytosol is imported into plant mitochondria by twin transporters from the mitochondrial carrier family that are cognates of coenzyme A transporters of animals and yeast and can functionally replace the yeast transporter.

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Plants make coenzyme A (CoA) in the cytoplasm but use it for reactions in mitochondria, chloroplasts, and peroxisomes, implying that these organelles have CoA transporters. A plant peroxisomal CoA transporter is already known, but plant mitochondrial or chloroplastic CoA transporters are not. Mitochondrial CoA transporters belonging to the mitochondrial carrier family (MCF) have, however, been identified in *Saccharomyces cerevisiae* (Leu5p) and mammals (SLC25A42). Comparative genomic analysis indicated that angiosperms have two distinct homologs of these mitochondrial CoA transporters whereas non-flowering plants have only one. The homologs from maize (GRMZM2G161299, GRMZM2G420119) and Arabidopsis (At1g14560, At4g26180) all complemented the growth defect of the *S. cerevisiae leu5*Δ mitochondrial CoA carrier mutant and substantially restored its mitochondrial CoA level, confirming that these proteins have CoA transport activity. Dual import assays with purified pea mitochondria and chloroplasts, and subcellular localization of green fluorescent protein fusions in transiently-transformed tobacco BY-2 cells, showed that the maize and Arabidopsis proteins are targeted to mitochondria. Consistent with the ubiquitous importance of CoA, the maize and Arabidopsis mitochondrial CoA transporter genes are expressed at similar levels throughout the plant. These data show that representatives of both monocotyledons and eudicotyledons have twin, mitochondrially located MCF carriers for CoA. The highly conserved nature of these carriers makes possible their reliable annotation in other angiosperm genomes.
INTRODUCTION

Coenzyme A (CoA) acts as an acyl carrier in many reactions of primary and secondary metabolism, and some 8% of the nearly 4,900 enzymes described in the Enzyme Commission database are CoA-dependent (Bairoch, 2000). CoA occupies a central position in lipid metabolism, respiration, gluconeogenesis, and other pathways (Leonardi et al., 2005). It is present in all forms of life, but while all organisms can synthesize it from pantothenate (vitamin B₅), only prokaryotes, plants and fungi are able to synthesize pantothenate; animals obtain pantothenate from the diet (Daugherty et al., 2002; Leonardi et al., 2005; Webb and Smith, 2011).

In plants, the steps that convert pantothenate to CoA are almost certainly cytosolic (Webb and Smith, 2011; Gerdes et al., 2012). CoA is, however, required in mitochondria for the citric acid cycle, in chloroplasts for fatty acid synthesis, and in peroxisomes for β-oxidation. CoA must therefore be imported into these organelles from the cytosol, and indeed early work demonstrated a CoA transport system in potato mitochondria (Neuburger et al., 1984). Yeast (Saccharomyces cerevisiae) and mammalian mitochondria and peroxisomes likewise import CoA because they cannot make it (Fiermonte et al., 2009; Agrimi et al., 2012b). The compartmentation of CoA in all eukaryotes appears to be closely regulated, with cytosol and organelles maintaining separate CoA pools whose levels can modulate fluxes through CoA-dependent reactions (Hunt and Alexson, 2002; Leonardi et al., 2005; De Marcos Lousa et al., 2013).

Mitochondrial CoA transporters belonging to the mitochondrial carrier family (MCF) have been identified in yeast (Leu5p) (Prohl et al., 2001) and humans (SLC25A42) (Fiermonte et al., 2009). Furthermore, peroxisomal CoA carriers from humans (SLC25A17) (Agrimi et al., 2012b) and Arabidopsis (PXN) (Agrimi et al., 2012a) have also been identified. However, no transporters for CoA are known for plant mitochondria or chloroplasts (Palmieri et al., 2011; Gerdes et al., 2012).

In this study, a comparative genomic analysis first identified close Arabidopsis and maize (Zea mays) homologs of the yeast and mammalian mitochondrial CoA carriers as candidates for the missing plant mitochondrial or chloroplast transporters. Experimental evidence then demonstrated that the candidate proteins transport CoA when expressed in yeast, that they are targeted to mitochondria in vitro and in planta, and that they are expressed throughout the plant.

RESULTS

Identification of candidate plant CoA carriers

An earlier analysis of Arabidopsis, human, and yeast MCF proteins identified At1g14560 and At4g26180 as potential CoA carriers, based on the presence of sequence motifs (symmetry-related
amino acid triplets) similar to those in known, mitochondrially localized CoA carriers (Palmieri et al., 2011). At1g14560 and At4g26180 share only 59% amino acid identity, and so are substantially diverged.

Blast searches of the maize genome using each Arabidopsis sequence as query detected GRMZM2G420119 and GRMZM2G161299 (henceforth ZM420119 and ZM161299) as the closest homologs (66-67% identity) of At1g14560 and At4g26180, respectively. All four proteins have the tripartite structure that characterizes the MCF family (Palmieri et al., 2011) and are predicted to contain the usual six transmembrane domains (Supplemental Fig. S1). Phylogenetic analyses of these proteins, the human and yeast mitochondrial CoA carriers, and all Arabidopsis, yeast, or human MCF proteins consistently placed all four candidates and the yeast and human CoA carriers in the same clade. Thus, among all 58 Arabidopsis MCF proteins, those closest to the known mitochondrially localized CoA carriers are the candidates (Fig. 1A) and, among all yeast or human MCF proteins, the candidates are closest to the known mitochondrial CoA carriers (Supplemental Fig. S2). The recently identified peroxisomal CoA carriers from human (Agrimi et al., 2012b) and Arabidopsis (Agrimi et al., 2012a) occupy a very different clade (Fig. 1A).

The phylogenetic trees all separate the four candidate genes into two types: type 1 comprised of At1g14560 and ZM420119, and type 2 comprised of At4g26180 and ZM161299 (Fig. 1A, Supplemental Fig. S2). One or two genes of each type were found in all other eudicotyledon and monocotyledon genomes and transcriptomes examined, as well as in that of the basal angiosperm Amborella trichopoda (Fig. 1B; Supplemental Fig. S3). In contrast, gymnosperms had type 1 but not type 2 sequences, whereas lower plants each had a single sequence that could not be confidently assigned to either type (Fig. 1B; Supplemental Fig. S3).

**Functional complementation of the yeast leu5Δ mutant**

Deletion of the yeast LEU5 gene, encoding a mitochondrial CoA transporter, results in strongly retarded growth on rich media containing a nonfermentable carbon source, and expression of a functional CoA carrier protein in the deletant (leu5Δ) strain restores normal growth (Prohl et al., 2001). The human Leu5p homolog, SLC25A42, used as a positive control, restored normal growth on YP medium plates containing 3% glycerol as expected (Fierrmonte et al., 2009). Similarly, all four candidate plant CoA transporters, At1g14560, At4g26180, ZM420119, and ZM161299, restored normal growth, as opposed to little or no growth for cells transformed with vector alone or a negative control (the Arabidopsis NAD⁺ carrier AtNDT1) (Fig. 2). Similar results were obtained in liquid medium (Supplemental Fig. S4). These data indicate that the plant type 1 and type 2 proteins have CoA transport activity, and that they are targeted to mitochondria in yeast. Furthermore, because the yeast Leu5p protein is known to be in the mitochondrial inner membrane (Prohl et al., 2001), the
observed complementation by the plant proteins indicates that they are likewise, at least in part, localized in the inner membrane when expressed in yeast.

**Complementation restores mitochondrial CoA levels in the leu5Δ strain**

The CoA content in mitochondria of the leu5Δ strain is severely reduced compared to wild type, reflecting the role of Leu5p in mitochondrial CoA import (Prohl et al., 2001). The candidate proteins from maize and Arabidopsis complemented this biochemical phenotype of the leu5Δ strain: expression of the type 1 candidates At1g14560 and ZM420119 fully restored mitochondrial CoA levels to that of the wild type; expression of the type 2 candidates At4g26180 and ZM161299 restored levels to over half that of wild type (Fig. 3). These data reinforce the conclusion that the plant proteins mediate CoA transport into yeast mitochondria.

**Subcellular localization**

Organellar targeting of the four CoA carrier candidates was investigated using dual import assays, in which labeled proteins are incubated with a mixture of purified pea mitochondria and chloroplasts and then re-isolated (Rudhe et al., 2002). Soybean alternative oxidase (Rudhe et al., 2002) and Arabidopsis YgfZ protein (Waller et al., 2012) were used as positive controls for mitochondrial and chloroplast import, respectively. All four candidates behaved similarly to the mitochondria-targeted control protein, i.e. the re-isolated mitochondria treated with thermolysin contained a labeled band corresponding to the imported protein (Fig. 4), which indicates an intramitochondrial location and is consistent with the complementation evidence above for an inner membrane location. The observed lack of difference between the molecular mass of the precursors and the imported proteins is expected because MCF family proteins typically lack cleavable targeting peptides (Palmisano et al., 1998; Zara et al., 2009). None of the candidates mirrored the behavior of the chloroplast-targeted control protein (Fig. 4).

As a second approach for investigating subcellular localization, tobacco BY-2 cells, serving as a model plant cell system for studying protein localization (Brandizzi et al., 2003; Miao et al., 2007), were transiently transformed with each of the four candidates linked at their N- or C-termini to GFP, and examined by epifluorescence microscopy. The expressed C-terminal fusions of both maize proteins and both Arabidopsis proteins showed a fluorescence pattern that co-localized with that attributable to the endogenous mitochondrial inner membrane marker, the β-ATPase subunit (Fig. 5). Notably, the fusion proteins often yielded a torus-like fluorescence pattern that circled the more punctate fluorescence attributable to the β-ATPase (Fig. 5, high-magnified images), suggesting that they may localize to two distinct subdomains of the inner mitochondrial membrane, namely the inner boundary membrane and cristae membrane, respectively (reviewed in Logan, 2006; Mannella,
Thus, the toroid fluorescence pattern exhibited by the candidate fusion proteins in BY-2 cells resembles that of several yeast GFP-tagged proteins that preferentially localize to the inner boundary membrane, which lies parallel to the mitochondrial outer membrane (Wurm and Jakobs, 2006; Suppanz et al., 2009). The punctate fluorescence pattern exhibited by endogenous β-ATPase in BY-2 cells, on the other hand, is consistent with the localization of its yeast counterpart in the cristae membrane, which extends into the matrix (Wurm and Jakobs, 2006). There are, however, other possible explanations for the concentration of GFP-tagged inner membrane proteins at the mitochondrial rim (e.g. Donzeau et al., 2000), so that this question needs to be studied in more detail.

Unlike the C-terminal fusions, the N-terminal GFP fusions for all four proteins localized only partially to mitochondria and mostly to the cytosol (not shown), suggesting that the N-terminally-appended GFP moiety interfered with the mitochondrial targeting information in the CoA carriers. Similar interference with mitochondrial targeting was observed for N-terminal fusions of GFP to thiamin diphosphate carriers (Frelin et al., 2012). Finally, there was no apparent targeting of either the C- or the N-terminal GFP fusion proteins to plastids or peroxisomes. Together, the results of dual import assays and of cell imaging demonstrate targeting of all four candidate proteins to plant mitochondria and not other organelles.

**Expression patterns of CoA transporter genes**

The relative levels of the ZM161299 and ZM420119 mRNAs were determined by qRT-PCR in ten tissues representing the whole adult maize plant (Fig. 6). Both genes are expressed in all tissues tested, with fairly little variation (maximum 4.2-fold). Their expression patterns are similar, except that the type 1 gene ZM420119 predominates in silks and the type 2 gene ZM161299 predominates in tassels. Analysis of expression data for both maize genes from genome-wide RNA-seq datasets gave a similar picture (Supplemental Fig. S5A). Publicly available microarray data for the Arabidopsis genes likewise show expression throughout the plant, with relatively little variation except in stamens and pollen, and with type 1 gene At1g14560 always predominant (Supplemental Fig. S5B). The ubiquitous expression of both types of CoA carrier gene in both species is consistent with the universal requirement of mitochondria for CoA, which is essential, among other things, for oxidation of pyruvate via the tricarboxylic acid cycle.

**DISCUSSION**

The bioinformatic prediction that the Arabidopsis and maize homologs of the yeast and human mitochondrial CoA carriers themselves transport CoA is validated by the evidence that they functionally complement the growth defect of the yeast *leu5Δ* mutant and restore its mitochondrial
CoA level. We therefore propose that these proteins be designated AtCoAc1 (At1g14560), AtCoAc2 (At4g26180), ZmCoAc1 (GRMZM2G420119), and ZmCoAc2 (GRMZM2G161299).

That these carriers belong to the MCF family (Palmieri, 2013) does not necessarily mean that they are targeted to mitochondria, as several cases of plastid (Bedhomme et al., 2005; Kirchberger et al., 2008) or peroxisomal (Agrimi et al., 2012a; Agrimi et al., 2012b; Bernhardt et al., 2012) targeting are known. Nor does the fact that the plant proteins are targeted to mitochondria in yeast (i.e. replace the missing mitochondrial CoA carrier in the leu5Δ mutant) prove that they are also targeted to mitochondria in planta. However, the results of in vitro dual import assays and of in vivo localization of GFP fusion proteins provide strong evidence for mitochondrial (inner membrane) localization for both type 1 and type 2 transporters. As neither type showed any plastid targeting in vitro or in vivo, they are most probably not responsible for CoA import into plastids. Plastidial CoA transporters thus still remain to be identified.

That homologs of both types of mitochondrial CoA transporter occur in all the sequenced angiosperm genomes that were analyzed, but not in those of gymnosperms or lower plants, implies that type 1 and 2 genes are ancient paralogs that diverged not only before the split between eudicotyledons and monocotyledons, but also before the Amborella lineage (Soltis et al., 2008) diverged from the lineage that gave rise to other angiosperms. Furthermore, the presence in gymnosperms of type 1 but not type 2 genes implies that type 1 is the ancestral gene and that type 2 arose from it in the lineage that gave rise to angiosperms.

The existence of two similarly expressed genes in angiosperms but only one in other plants could imply either subfunctionalization or neofunctionalization, i.e. that gene duplication was followed either by (i) subdivision of an originally broad function, or (ii) acquisition of a novel function by the duplicated gene. The available data rule out neither possibility. However, assuming CoA transport to be an ancestral function (because it is shared with other eukaryotes), and that the type 2 gene is of quite recent origin (see above), it is reasonable to speculate that type 2 carriers have acquired a new function without losing the original one (Khersonsky and Tawfik, 2010). This new function is presumably a capacity to transport a substrate or substrates other than CoA, and in this regard it is noteworthy that the Arabidopsis peroxisomal CoA carrier PXN accepts NAD⁺, NADH, AMP, ADP, and adenosine 3′,5′-phosphate as well as CoA (Agrimi et al., 2012a; Bernhardt et al., 2012). It is also noteworthy that type 1 carriers restored mitochondrial CoA levels in the yeast leu5Δ mutant more effectively than type 2 carriers, for this is consistent with some differentiation of function. It will therefore be valuable to assess the transport activities of type 1 and type 2 proteins in reconstituted liposomes; attempts to do this have so far been unsuccessful.
Lastly, from a practical standpoint, the conservation of the type 1 and type 2 proteins throughout the angiosperms, and the presence of close homologs in other plants, means that the functional annotation ‘mitochondrial CoA carrier’ can be confidently propagated from maize and Arabidopsis to any plant genome.

MATERIALS AND METHODS

Bioinformatics
Sequences of Arabidopsis, human, and yeast MCF carriers were taken from Palmieri et al. (2011). Maize carrier sequences were from Maizesequence.org (http://maizesequence.org/index.html); most other plant carrier sequences were from genomes at NCBI, the Joint Genome Institute (http://www.jgi.doe.gov/), or the Amborella genome database (http://www.amborella.org/). Gymnosperm sequences were based on expressed sequence tag contigs at the Gene Index Project (http://compbio.dfci.harvard.edu/tgi/plant.html). Sequence alignments were made with ClustalW and phylogenetic trees were constructed using MEGA5 (Tamura et al., 2011). Arabidopsis gene expression was analyzed at BAR http://bar.utoronto.ca/welcome.htm (Winter et al., 2007); maize gene expression data were from qTeller http://qteller.com/.

Constructs
All PCR reactions were performed using high fidelity polymerase Phusion TAQ (NEB). Maize ORFs GRMZM2G420119 and GRMZM2G161299 were amplified from the plasmids ZM_BFb0285D11 and ZM_BFb0063E21 obtained from the Arizona Genomics Institute. Arabidopsis ORFs At1g14560 and At4g26180 were amplified from a leaf cDNA library. For complementation of the yeast leu5\∆\ strain, coding sequences were cloned in pYES2/CT (Invitrogen). For dual import assays, the coding sequences were cloned into pGEM-4Z (Promega) with addition of a Kozak sequence ACC just upstream of the start codon. The plasmids (Frelin et al., 2012) used for in vivo localization studies were pRTL2\∆\NS/mGFP-MCS for N-terminal green fluorescent protein (GFP) fusions of candidate proteins and pUC18/Ncol-mGFP for C-terminal GFP fusions. The Kozak sequence AACA was added just upstream of the transporter sequence start codon for C-terminal GFP fusions, to match that in the pRTL2\∆\NS/mGFP-MCS plasmid. The primers used to build these constructs are listed in Supplemental Table S1.

Complementation of the yeast leu5\∆\ mutant
The leu5\∆\ deletant and corresponding wild type W303-1B strain were grown in rich (YP) medium, containing 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, supplemented with 3% (w/v) glycerol. The final pH was adjusted to 4.8 with HCl. Strains were transformed with pYES2/CT alone or
pYES2/CT constructs, including positive (human SLC25A42) and negative (Arabidopsis AtNDT1; Palmieri et al., 2009 or human SLC25A33; Floyd et al., 2007) controls, as previously described (Fiermonte et al., 2009; Frelin et al., 2012).

Yeast mitochondrial CoA content measurement
The leu5Δ strain transformed with recombinant pYES2/CT vectors was grown at 30°C to mid-log phase in YP medium supplemented with 2% (v/v) ethanol. Mitochondria were isolated as described (Daum et al., 1982) and extracted with phenol/chloroform 1:1 (v/v) for mass spectrometric analysis of CoA. A Quattro Premier mass spectrometer interfaced with an Acquity UPLC system (Waters) was used for LC-MS/MS analysis. Chromatographic resolution was achieved using an HSS T3 column (2.1 × 100 mm, 1.8 µm particle size, Waters). The flow rate was 0.3 mL/min. The reaction monitoring transition selected in the positive ion mode for CoA was m/z 768 > 261. Calibration curves were established using standards, processed under the same conditions as the samples, at five concentrations. The best fit was determined using regression analysis of analyte peak area.

Dual import assays
Dual import assays with purified pea mitochondria and chloroplasts were carried out as described (Rudhe et al., 2002; Frelin et al., 2012). The soybean alternative oxidase protein GmAOX (Rudhe et al., 2002) and the Arabidopsis YgfZ protein At1g60990 (Waller et al., 2012) served as positive controls for mitochondrial and chloroplast import, respectively. Import times used in reactions were 20 min for CoA transporter candidates and GmAOX, and 5 min for Arabidopsis YgfZ.

Expression and visualization of GFP fusion proteins in BY-2 cells
Expression and microscopic analysis of GFP fusion proteins in tobacco Bright Yellow-2 (BY-2) suspension-cultured cells was performed as previously described (Frelin et al., 2012), except that endogenous mitochondrial β-ATPase protein was immunostained using mouse anti-maize mitochondrial β-ATPase E monoclonal antibodies (Luethy et al., 1993) (provided by T. Elthon) and goat anti-mouse Rhodamine Red-X IgGs (Jackson ImmunoResearch Laboratories).

Analysis of maize gene expression
RNA preparations were a gift from J.-C. Guan (University of Florida). Maize (inbred B73) plants were grown at the University of Florida Citra research farm in spring 2010; samples were harvested at tassel emergence. cDNAs were synthesized with SuperScript® III First-Strand Synthesis System (Life Technologies) using oligo(dT)20 primer. Real-time PCR was performed using MyiQ2 Real Time PCR Detection System (Bio-Rad) using iQ™ SYBR® Green Supermix (Bio-Rad). Amplification parameters were 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s with
fluorescence measurement, followed by establishment of a melting curve. Data were analyzed with the Bio-Rad Expression Macro using the \(2^{\Delta\Delta Ct}\) method. Expression was normalized against three reference genes: FPGS (GRMZM2G393334 T01), CUL (GRMZM2G166694 T04), and UBCP (GRMZM2G102471 T01) (Vandesompele et al., 2002; Manoli et al., 2012). Primers used are given in Supplemental Table S2. Primer concentrations were optimized for the lowest threshold cycle values, and amplification efficiency was determined.

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LITERATURE CITED


FIGURE LEGENDS

Figure 1. Phylogenetic analysis of known and predicted CoA transporters. A, Phylogenetic tree of the experimentally validated CoA transporters from yeast, human, and Arabidopsis, the candidate CoA transporters from Arabidopsis (At1g14560, At4g26180) and maize (GRMZM2G420119, GRMZM2G161299, abbreviated to ZM420119, ZM161299), and all other Arabidopsis MCF family proteins. Sequences were aligned with ClustalW; the tree was constructed by the neighbor-joining method with MEGA5. Bootstrap values (%) for 1000 replicates are shown next to nodes; those <50% are omitted. Only the tree topology is shown. The yellow highlighted sector contains the validated human and yeast mitochondrial CoA transporters and their Arabidopsis and maize homologs; the type 1 and type 2 plant carriers are indicated. The green highlighted sector contains the validated human and Arabidopsis peroxisomal CoA transporters. B, Phylogenetic distribution of type 1 and type 2 predicted CoA transporters among angiosperms, gymnosperms, and lower plants. Red boxes and blue boxes respectively indicate the number of type 1 and type 2 sequences represented in each genome or transcriptome. Piebald red/blue boxes indicate homologs in lower plants that could not be assigned to either type 1 or type 2.

Figure 2. Complementation of the growth phenotype of the yeast CoA carrier deletant leu5Δ by expression of candidate plant CoA carriers. Serial dilutions of wild-type (WT) cells harboring pYES2/CT (vector) or leu5Δ cells harboring pYES/CT alone or encoding maize (ZM420119, ZM161299) or Arabidopsis (At4g26180, At1g14560) proteins were plated on YP medium containing 3% glycerol and incubated for 72 h at 30°C. pYES2/CT constructs encoding the human CoA transporter SLC25A42 or the Arabidopsis NAD⁺ transporter AtNDT1 were included as positive and negative controls, respectively.

Figure 3. Effect of the expression of candidate plant CoA carriers on mitochondrial CoA contents of leu5Δ yeast cells. Cells were cultured in liquid YP medium containing 2% ethanol. Mitochondria were isolated from wild-type (WT) cells harboring pYES/CT (vector) or from leu5Δ cells harboring pYES/CT alone or encoding At1g14560, At4g26180, ZM420119, or ZM161299. pYES2/CT constructs encoding the human CoA transporter SLC25A42 or the human pyrimidine nucleotide transporter SLC25A33 were included as positive and negative controls, respectively. Data are means and SE of at least three independent experiments, and were subjected to one-way analysis of variance followed by Bonferroni post hoc tests. Differences in CoA content between leu5Δ cells harboring pYES/CT and other strains that are significant at *P<0.05, **P<0.01 or ***P<0.001; ns, not significant.
Figure 4. Protein uptake by purified pea mitochondria and chloroplasts in dual import assays. The four CoA carrier cDNAs, plus the Arabidopsis YgfZ (AtYgfZ) and soybean alternative oxidase (GmAox) cDNAs as positive controls for chloroplast and mitochondrial import, respectively, were transcribed and translated in vitro in the presence of [3H]leucine. The translation products (TP) were incubated in the light with mixed pea chloroplasts (C) and mitochondria (M). The organelles were mock treated (−) or thermolysin treated (+) to remove adsorbed proteins, then re-isolated on a Percoll gradient. Proteins were separated by SDS-PAGE and visualized by fluorography. Samples were loaded on the basis of equal chlorophyll or mitochondrial protein content next to an aliquot of the translation product. Exposure times were adjusted to give comparable band intensities in all tracks. Molecular masses are indicated on the right.

Figure 5. Representative epifluorescence images of tobacco BY-2 cells transiently expressing maize or Arabidopsis CoA carrier candidates C-terminally fused to GFP. Cells were biolistically bombarded with plasmid DNA encoding a GFP-tagged candidate. Approximately 4 h later, cells were formaldehyde fixed, permeabilized with pectolyase and Triton X-100, immunostained for endogenous mitochondrial β-ATPase, serving as a mitochondrial marker protein, and then examined by epifluorescence microscopy. As indicated by the labels, each row of images corresponds to the fluorescence attributable to the candidate fusion protein and endogenous β-ATPase immunostaining (green and red, respectively), and the corresponding merged image. Boxes represent the portion of the cell shown at higher magnification in the panels to the right. Arrowheads indicate examples of toroidal structures containing a candidate fusion protein enclosing a punctate structure containing β-ATPase. Bar in top row = 10 μm.

Figure 6. Expression patterns of maize genes GRMZM2G161299 and GRMZM2G420119 (abbreviated to ZM161299 and ZM420119). Relative mRNA levels were determined by qRT-PCR using the $2^{-ΔCt}$ method against three reference genes (FPGS, CUL, and UBCP). Error bars represent the SE for two technical replicates of two biological replicates.
WT

vector

vector

At1g14560

vector

ZM420119

At4g26180

ZM161299

leu5Δ

AtNDT1

SLC25A42

AtNDT1

SLC25A42