**ZmCTLP1** is required for the maintenance of lipid homeostasis and the basal endosperm transfer layer in maize kernels

**Mingjian Hu**, **Haiming Zhao**, **Bo Yang**, **Shuang Yang**, **Haihong Liu**, **He Tian**, **Guanghou Shui**, **Zongliang Chen**, **Lizhu Li**, **Jinsheng Lai** and **Weibin Song**

1 State Key Laboratory of Plant Physiology and Biochemistry and National Maize Improvement Center, Department of Plant Genetics and Breeding, China Agricultural University, Beijing 100193, China; 2 State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China; 3 State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; 4 Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ 08854-8020, USA; 5 Center for Crop Functional Genomics and Molecular Breeding, China Agricultural University, Beijing 100193, China

**Summary**

- Maize kernel weight is influenced by the unloading of nutrients from the maternal placenta and their passage through the transfer tissue of the basal endosperm transfer layer (BETL) and the basal intermediate zone (BIZ) to the upper part of the endosperm.
- Here, we show that **Small kernel 10** (**Smk10**) encodes a choline transporter-like protein 1 (**ZmCTLP1**) that facilitates choline uptake and is located in the trans-Golgi network (TGN). Its loss of function results in reduced choline content, leading to smaller kernels with a lower starch content.
- Mutation of **ZmCTLP1** disrupts membrane lipid homeostasis and the normal development of wall in-growths. Expression levels of **Mn1** and **ZmSWEET4c**, two kernel filling-related genes, are downregulated in the **smk10** mutant, which is likely to be one of the major causes of incompletely differentiated transfer cells. Mutation of **ZmCTLP1** also reduces the number of plasmodesmata (PD) in transfer cells, indicating that the **smk10** mutant is impaired in PD formation. Intriguingly, we also observed premature cell death in the BETL and BIZ of the **smk10** mutant.
- Together, our results suggest that **ZmCTLP1**-mediated choline transport affects kernel development, highlighting its important role in lipid homeostasis, wall in-growth formation and PD development in transfer cells.

**Key words:** choline transporter, kernel development, maize, plasmodesmata, **ZmCTLP1**.

**Introduction**

Kernel weight, a key determinant of grain yield, depends on the effective transport of carbohydrates from the maternal phloem to the filial tissues (Gui et al., 2014; Sosso et al., 2015). Nutrients pass through four cell types from the maternal tissues to the central endosperm: the pedicel (PED), the placento-chalazal region (PC), the basal endosperm transfer layer (BETL), and the basal intermediate zone (BIZ) (Porter et al., 1987; Lalonde et al., 2004; Royo et al., 2007; Zhang et al., 2007; Becraft & Gutierrez-Marcos, 2012; Bihmidine et al., 2013; Leroux et al., 2014; Larkins, 2019; Song et al., 2019). Assimilates are unloaded through maternal vascular terminals in the PED and pass symoplastically and apoplastically into the PC, then apoplastically through the BETL into the central endosperm (Muhitch, 1989; Miller & Chourey, 1992; Santandrea et al., 2002). The PED and PC are two key maternal tissues that control the supply of nutrients to filial tissues (Muhitch, 1993; Santandrea et al., 2002; Kladnik et al., 2004).

Adjacent to the PC are the BETL and BIZ, two elongated cell types located at the bottom of the endosperm (Shannon, 1972; Becraft & Gutierrez-Marcos, 2012; Leroux et al., 2014; Larkins, 2019). The BETL, a unique endosperm cell layer with wall in-growths that increase the surface area of the plasma membrane and cell wall, plays a major part in the transfer of nutrient solutes from the maternal placenta to the developing endosperm (Davis et al., 1990; Wang et al., 1994). Several genes have been reported to influence nutrient uptake and allocation in this region, thereby regulating kernel development. **Mn1** (Miniature1), located in the BETL and PED and catalyses the cleavage of sucrose into hexoses to generate a physiological gradient of photosynthate that passes symplastically and apoplastically into the PC, then apoplastically through the BETL to the endosperm (Miller & Chourey, 1992). Loss of **Mn1** function results in smaller kernel size (Miller & Chourey, 1992; Cheng et al., 1996; Cheng & Chourey, 1999; Kang et al., 2009; Sosso et al., 2015), whereas **Mn1** overexpression improves kernel filling and markedly increases kernel size and weight (Wang et al., 2008; Li et al., 2013). Its rice homologue **GIF1** (Grain Incomplete Filling 1) is also recognised as a positive regulator of seed filling (Wang et al., 2008).

A second gene, **ZmSWEET4c** (**Sugars Will Eventually be Exported Transporter 4c**), encodes a sugar transporter reported to transport hexoses produced by **Mn1** to the endosperm (Sosso et al., 2014).
et al., 2015). Its soybean homologue GmSWEET10 regulates oil content and seed size by transporting sucrose and hexose from the seed coat to the developing embryo (Wang et al., 2020). The development of fully differentiated BETL with wall in-growths requires the normal functions of Mn1 and ZmSWEET1c (Kang et al., 2009; Sosso et al., 2015). Wall in-growth formation enlarges the surface area of the plasma membrane and therefore requires abundant lipid precursors such as phosphatidic acid, triglyceride, acetyl-CoA and choline (van Greevenbroek et al., 1995; Michel et al., 2006; Correia et al., 2020).

Choline is a quaternary amine that plays a fundamental role in cell membrane phospholipid metabolism and the synthesis of the neurotransmitters acetylcholine and betaine (Michel et al., 2006). Disorders of choline uptake, transport and metabolism affect cell proliferation and differentiation in animals, thereby contributing to neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease (Michel et al., 2006; Ueland, 2011). Choline transport is mediated by three different systems: high-affinity choline transporters (CHT1s), intermediate-affinity choline transporter-like (CTL) proteins, and low-affinity polyspecific organic cation transporters (OCTs) (Michel et al., 2006). CTL1 is widely expressed in animal tissues, and its encoded protein specifically regulates choline supply for phospholipid and sphingolipid synthesis (Mačková et al., 2009; Wang et al., 2017). In plants, the first choline transporter AtCTL1 was isolated in Arabidopsis thaliana, and it has been shown to have choline transport activity (Dettmer et al., 2014). AtCTL1 also regulates auxin distribution by promoting the trafficking of auxin efflux transporters during plant growth and development (Wang et al., 2017). AtCTL1 is essential for the formation and development of PD and plays vital roles in the formation of sieve tubes; mutation of AtCTL1 alters ion homeostasis in rosette leaves from one 5-wk-old plant, ultimately causing a severely stunted growth phenotype (Dettmer et al., 2014; Gao et al., 2017; Kranner et al., 2017; Wang et al., 2017). Nonetheless, information on the choline transporter in plants is very limited, and its roles in kernel development and kernel biology remain largely unknown.

In this study, we identified the maize CTL1 choline transporter homologue ZmCTLP1 through map-based cloning of smk10. Mutation of ZmCTLP1 disrupts kernel development, dramatically decreasing kernel size and weight. In smk10, the nutrient transfer cells of the BETL and BIZ exhibit typical features of dysfunction, including irregular cell shape, decreased PD number, premature cell death and impaired lipid homeostasis. RNA-seq and immunoblot analysis revealed that the mutant shows altered expression of many genes involved in nutrient metabolism and transport. Collectively, these findings demonstrate that ZmCTLP1 is required for kernel development and participates in lipid homeostasis, wall in-growth formation and PD development in transfer tissues.

Materials and Methods

Plant materials

The ethyl methanesulfonate (EMS)-generated smk10 mutant was obtained from mutant populations produced in our laboratory on the B73 genetic background. The smk10 mutant was crossed with Mo17 and then self-pollinated to generate F2 and F3 populations. The F3 population was used for fine mapping of smk10. The wild-type and the smk10 were used for cytological and biochemical analyses. Leaves and roots were collected from at least three 10-d-old B73 seedlings. Leaf, root, internode, node, silk, cob and husk tissues were collected at the R1 stage 70 d after sowing. Immature B73 kernels were harvested at 2, 4, 6, 8, 10, 12, 14, 16 and 20 d after pollination (DAP). Maize plants were grown under natural conditions at the Shang-Zhuang experimental field of China Agricultural University in Beijing.

Light microscopy, fluorescence microscopy and transmission electron microscopy

For light microscopy, 4–12 DAP wild-type and smk10 kernels were harvested. Paraffin sections were prepared using the methods described by Li et al. (2018). The sections were stained with fuchsin or eosin and observed using an Olympus SZX11 microscope. Detection of PD after aniline blue staining (0.1% aniline blue in double-distilled water and 1 M glycine, pH 9.5, at a volume ratio of 2 : 3) was performed using a ZEISS LSM710 confocal microscope (Germany) with scan speed set at ‘Best signal’. The excitation wavelength for PD signal was 440 nm, and the emission wavelengths were 450–511 nm.

For transmission electron microscopy observation, 12 DAP immature kernels from the wild-type and smk10 were cut along the horizontal axis for resin section preparation. Each sample was fixed with glutaraldehyde and osmic acid and then embedded with low viscosity resin (Electron Microscopy Sciences, cat. #14300). Sections were stained with lead citrate and observed using a Hitachi H7600 transmission electron microscope (Japan).

Determination of soluble sugar, starch, protein, choline and lipid contents

Soluble sugars, starch and protein  Mature kernels (15 g) were pulverised and extracted with water. The samples were dispersed with an ultrasonic dispersion instrument for 30 min and then measured using a Chromelon chromatography management system (Dionex, Sunnyvale, CA, USA). Starch and protein contents were measured as previously described (Clegg, 1956; Wang et al., 2011).

Choline and lipids  Samples were prepared and measured using the methods described by Kranner et al. (2017). In brief, c. 30 mg of maize kernel tissue were frozen in liquid N2 and ground to a fine powder in a cold mortar. Metabolites were extracted using a MeOH : CHCl3 : H2O (6 : 2 : 2) extraction buffer. MS analysis for the quantification of choline was performed using an Ultimate 3000 UHPLC system coupled to a Q-Exactive MS instrument (Thermo Scientific, Bremen, Germany). Lipid contents were determined according to a previously described protocol (Shui et al., 2010; Gao et al., 2017). In brief, the sample (100 mg) was combined with 900 μl of chloroform : methanol (1 : 2) extraction buffer and acid-washed glass beads (Sigma),...
vortexed for 10 min, and incubated overnight at 4°C with shaking at 1100 rpm. Next, 600 μl of chloroform: H2O (1:1) was added, and the sample was vortexed for 30 s. The mixtures were centrifuged at 7500 g for 5 min, and the lower organic phase was collected and dried with a Savant SpeedVac vacuum system (Thermo Fisher Scientific, Milford, MA, USA). The samples were stored at −80°C before mass spectrometric analysis.

Map-based cloning of smk10

To clone smk10, an F2 population was produced by crossing smk10 with Mo17 and then self-pollinating their F1 progeny. Thirty-five individual miniature kernels were pooled, and 65 genetic markers were used for genotyping. The causal locus was mapped to a 2.3-Mb genomic region on chromosome 2. To fine map the candidate gene, 3264 F3 individuals were yielded from the cross of smk10 and Mo17. Five genetic markers within the 2.3-Mb genomic region further narrowed the smk10-containing region to 300 kb between markers M096 and M127. The candidate genes within this region were sequenced.

Maize transformation

To obtain ZmCTLP1 CRISPR lines, a 20-bp sgRNA-specific target site in the first exon of ZmCTLP1 was cloned into the backbone of the pCAMBIA3301 vector and transformed into maize by Agrobacterium-mediated transformation as previously described (Zhu et al., 2016). Transgenic plants were identified by PCR, and the targeted fragments were sequenced to detect variable sites. Two independent Cas9-edited knockout lines (cas-1 and cas-2) were obtained.

Expression in Xenopus oocytes and choline uptake assays

Isolation and RNA injection of Xenopus oocytes Coding sequences of ZmCTLP1 and AtCTLI were codon optimised and synthesised. These cDNAs were cloned into the pT7TS expression vector. After linearisation of pT7TS plasmids with BamHI, RNA was transcribed in vitro using an mRNA synthesis kit (mMESSAGE mMACHINE T7 kit, Ambion). Xenopus laevis oocytes were isolated in 25 ml ND96 (Ca2+ free) solution containing 43 mg collagenase and 12.5 mg trypsin inhibitor for 1.5 h and were then recovered in ND96 for 24 h. Oocytes were injected with cRNA (25 ng in 25 nl) after recovery and were incubated at 18°C in MBS solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.71 mM CaCl2, 0.82 mM MgSO4, and 15 mM HEPES, pH 7.5) with gentamycin and 5 μM choline ([methyl-14C]-choline chloride 55 mCi mmol−1; ARC). After incubation at 20°C for 2 h, oocytes were transferred to ice-cold Barth’s medium, washed four times, and solubilised with 100 ml 1% (w/v) SDS. Three oocytes were used as one sample, and the accumulated radioactivity in the oocytes was measured. The experiments were repeated independently using three different batches of oocytes with similar results.

Subcellular localisation

The full-length open reading frame (ORF) of ZmCTLP1 was amplified by PCR from maize B73 and cloned into pCAMBIA1300-35S-green fluorescent protein (GFP) using restriction enzymes and ligases. The fused pCAMBIA1300-35S-ZmCTLP1-GFP construct and RFP-SYP61 (a TGN marker) were transiently transformed into maize mesophyll protoplasts as previously described (Yoo et al., 2007). The GFP signal was observed and merged with the RFP signal using a ZEISS LSM710 confocal microscope (Germany) with scan speed set at ‘Best signal’. The excitation wave lengths for GFP and RFP signals were 488 and 561 nm, and the emission wave lengths were 500–586 and 598–656 nm, respectively.

qPCR and RNA-seq

RNA was extracted from the leaves and roots of 10-d-old seedlings, immature kernels (2–20 DAP at 2-d intervals), and leaf, root, internode, node, silk, cob and husk tissue were harvested at the R1 stage (70 d after sowing) using the TRIzol reagent according to the manufacturer’s instructions (Invitrogen). cDNA was synthesised from the extracted RNA using PrimerScript RT Master Mix (Takara, Dalian, China), and qPCR was performed using the TB Green Premix Ex Taq kit (Takara) following the manufacturer’s protocols. Gene expression levels were calculated using the 2−ΔΔCt relative quantification method with Actin as the endogenous control gene. For RNA-seq analysis, total RNA was extracted from two biological replicates of immature 6 and 12 DAP wild-type and smk10 kernels using TRIzol reagent (Invitrogen). RNA-seq libraries were constructed according to the protocol of the VAHTS mRNA-seq v2 Library Prep Kit (Vazyme, Nanjing, China) and sequenced on the Illumina HiSeq 2500 platform to generate 150-bp paired-end reads. The reads were mapped to the B73 reference genome using HISAT2 2.0.4 (Kim et al., 2015). The unique bam files were acquired using CUFFLINKS (v.2.2.0) (Ghosh & Chan, 2016), and FPKM values were calculated to identify differentially expressed genes (DEGs) (<P<0.05, log2 fold-change > 1.0). GO enrichment analysis of the DEGs was performed using the agriGO singular enrichment analysis tool (http://bioinfo.cau.edu.cn/agriGO), and the DEGs were visualised using MeV (https://sourceforge.net/projects/mev-tm4/).

Antibodies

For production of monoclonal antibody against Mn1, a specific cDNA fragment (129–450 sequence site, representing 43–150 amino acids) of Mn1 was cloned into pET-28a expression vector...
(Amersham Biosciences, Piscataway, NJ, USA) and transformed into BL21 cells (TransGen Biotech, Beijing, China). Mn1-His fusion protein was purified using Ni Sepharose™ 6 Fast Flow (GE Healthcare, Uppsala, Sweden), and production of antibodies in mice was performed according to standard protocol of Abmart Inc. of China. Anti-α-tubulin (anti-α-tubulin mouse monoclonal antibody, BE0031; Easybio) was purchased from EASYBIO Inc. of China.

Immunoblot analysis

Total proteins extracted from immature wild-type and smk10 kernels at 4, 8 and 12 DAP were used for western blot using the methods described by Wang et al. (2018). The nitrocellulose membranes with the protein samples attached were incubated with anti-Mn1 and anti-α-tubulin in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) with 5% milk for 2.5 h at 25°C and then washed three times using TBST for 10 min. The nitrocellulose membrane and attached protein were then incubated with the secondary antibody (anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody; Proteintech, Rosemont, IL, USA). HRP was detected using the Immobilon Crescendo Western HRP substrate kit (Millipore), and the signals were visualised with a C300 imaging system (Azure Biosystems, USA).

In situ hybridisation

Paraffin sections were prepared as described above, and gene-specific ZmSWEET4c and ZmCTLP1 probes with locked nucleic acid modification was generated (Supporting Information Table S2, see later). In situ hybridisation was performed according to previously described protocols (Ding et al., 2015).

TUNEL assay

Sections from wild-type and smk10 mutant kernels collected at 8 and 12 DAP were dewaxed using the HistoChoice Clearing Agent (Sigma) and hydrated through an ethanol series (95%, 90%, 80%, 70%). The sections were treated with proteinase K in phosphate buffer (pH 7.4), and the terminal deoxynucleotidyl transferase (TdT)-mediated dNTP nick end labelling (TUNEL) assay was performed using the DeadEnd Fluorometric TUNEL Kit (Promega) according to the manufacturer’s protocol. Cell nuclei were stained using propidium iodide (PI) (Beyotime Biotechnology, Shanghai, China). The green fluorescence (the TUNEL signal) and the red fluorescence of PI were observed using a ZEISS LSM710 confocal microscope (Germany) with the parameters as described in the section of ‘Subcellular localisation’.

Phylogenetic tree

ZmCTLP1 and its orthologous protein sequences were downloaded using MaizeGDB (https://www.maizegdb.org/) and NCBI (https://www.ncbi.nlm.nih.gov/) databases. The obtained orthologous full-length proteins grouped together and their sequences were aligned to compare equivalent residues using the program CLUSTALW. (https://www.megasoftware.net/). Neighbour-joining phylogenetic trees were constructed from full-length protein sequences, and the numbers at the nodes represent the percentage support from 1000 bootstrap replicates.

Results

Phenotypic and genetic characterisation of smk10

We screened a mutant library generated by EMS mutagenesis on the B73 background, aiming to identify novel regulators of kernel development based on the phenotype of small kernel size. We screened one mutant, designated smk10 and performed three continuous backcrosses with B73 to clean the smk10 background. We confirmed that smk10 harboured a recessive mutation by genetic analysis, as the F2 ears segregated at a 3:1 ratio (χ² = 0.16–0.92 < χ²ₚ = 3.84) of wild-type vs smk10 kernels. The average hundred-kernel weight of smk10 was significantly lower than that of the wild-type (Fig. S1a). On the B73 genetic background, smk10 exhibited reduced size of both endosperm and embryo (Figs 1a–c, S1b,c). The smk10 mutant had a higher embryo:endosperm ratio than that of wild-type (Fig. S1d), and its kernel development was slower (Fig. S1e). Soluble sugar and storage protein contents were much higher in mature smk10 kernels, whereas total starch content was much lower (Fig. S2), suggesting that the kernel development process was attenuated in smk10. The growth of seedling leaves and roots was slower in smk10 than that of the wild-type, which could mainly be due to the smaller endosperm and embryo. However, there were no obvious differences between the wild-type and smk10 in important agronomic traits such as kernel row number, ear length, plant height, and tassel branch number at the mature stage (Figs 1d,e, S3–S5).

Paraffin sections of immature wild-type and smk10 kernels collected 4–12 DAP were prepared to observe morphological features during kernel development (Fig. 1f,g). The mutant kernel phenotype was distinguishable at 6 DAP, when the endosperm and pericarp showed an irregular periphery. Phenotypic differences in the embryo could be discerned at 8 DAP: the wild-type embryo showed a visible scutellum, whereas the smk10 embryo was still in the proembryo stage. Clear phenotypic differences were observed at 12 DAP: smk10 exhibited an empty pericarp and a small, irregular endosperm and embryo (Figs 1f,g, S6a,b). Starch granules were smaller in smk10 than in the wild-type at 12 DAP and in mature kernels (Fig. S6c–h).

These phenotypic changes motivated us to investigate the morphological differences in different endosperm compartments. At 12 DAP, the wild-type conducting zone (CZ) and central starchy endosperm (CSE) cells were fairly uniform in size and shape, whereas the smk10 CZ and CSE cells were smaller and nonuniform with highly variable sizes (Figs 2a,b, S7a–d). The wild-type AL cells were similar to those of smk10 (Fig. 2c,d). In the wild-type, the BETL displayed a characteristic slightly elongated shape with labyrinth-like wall in-growths (Figs 2e, S7e). By contrast, the mutant exhibited misshapen BETL cells with few thickenings or wall in-growths (Figs 2f, S7f). The wild-type had extremely elongated BIZ cells with wall in-growths that decreased gradually.
that contained 10 candidate genes following by sequencing (Fig. 3a; Tables S1, S2). RNA-seq analysis indicated that there was a single nucleotide transition (G→A) in the 5’ splicing site of the fourth intron of the candidate Zm00001d001803, encoding a putative choline transporter with 10 transmembrane helices (Fig. S8a,b). Consistent with this nucleotide transition in the intron splicing site, PCR analysis with a gene-specific primer pair (F, R) based on the Zm00001d001803 sequence revealed at least four different transcripts in smk10 kernels, visualised by agarose gel electrophoresis (Fig. S8c). A primer set (D1–J1) was designed to amplify the full-length cDNA. Subsequent cloning and sequencing of the products indicated that five alternative spliced variants with premature termination codons (PTCs) generated in upstream regions of the intact mRNA were found (Fig. S8d).

Interestingly, the five alternative splicing transcripts were at least partially escaped from nonsense-mediated mRNA decay (NMD), a surveillance pathway that degrades mRNAs containing PTCs. Although the cause of the escape remains unclear, several studies had also reported examples of NMD escapes (Ruiz-Echevarria et al., 1998; Nyikó et al., 2009; Lindeboom et al., 2019).

CRISPR-Cas9 knockouts were created to confirm that Zm00001d001803 was responsible for the small kernel phenotype. A guide RNA was designed to target the first exon, and two knockout events with framingshifts in the first exon were identified (Figs 3b, 9, S10a). The kernels of knockout plants exhibited a serious defective kernel phenotype with empty pericarps at the crown kernels, very similar to smk10. An allelism test was performed by crossing smk10 with heterozygous cas-1 (cas-1/+), heterozygous cas-2 (cas-2/+ and cas-1; neither cas-1 nor cas-2 complemented smk10 (Fig. 3b; Table S3). In addition, cas-1 developed defective basal endosperm cells that resembled those of smk10 (Fig. 3c). To investigate whether there is maternal effect of ZmCTLP1 or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT) or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT) or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT) or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT) or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT) or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT) or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT) or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT) or not, we performed phenotypic analysis by using kernels of reciprocal-crossed ears derived from wild-type (WT)

Smk10 encodes the choline transporter ZmCTLP1 that facilitates choline transport

Smk10 and its homologous proteins were used to construct a neighbour-joining phylogenetic tree to investigate their evolutionary relationships (Fig. S12). The sequences were divided into three clades: dicots (clade I), monocots (clade II), and human–mouse (clade III). Smk10 was grouped with the monocots, and AtCTL1 was grouped with the dicots. A phylogenetic tree containing Smk10 and its paralogues indicated that Smk10 and Zm00001d052748 were highly similar to AtCTL1 (Fig. 3c), which is a CTL protein located at the TGN (Fig. S13) (Dettmer et al., 2014). However, the expression of Zm00001d052748 (named ZmCTLP2) was much lower than that of ZmCTLP1 (Fig. S14a). Therefore, Smk10 was named ZmCTLP1. ZmCTLP1, like other CTL proteins, is a transmembrane protein

Map-based cloning of smk10

To identify the causal gene of smk10, an F2 mapping population of 70 individuals was obtained from a cross between heterozygous smk10 and Mo17. The smk10 mutant locus was mapped to a 2.3-Mb physical interval on chromosome 2 that was flanked by the two InDel markers Chr2-M018 and Chr2-M248. To narrow down the interval, a high-resolution map was obtained from 3264 F3 individuals, and smk10 was fine mapped to a 300-kb region containing Smk10 and its paralogues indicated that Smk10 and Zm00001d052748 were highly similar to AtCTL1 (Fig. 3c), which is a CTL protein located at the TGN (Fig. S13) (Dettmer et al., 2014). However, the expression of Zm00001d052748 (named ZmCTLP2) was much lower than that of ZmCTLP1 (Fig. S14a). Therefore, Smk10 was named ZmCTLP1. ZmCTLP1, like other CTL proteins, is a transmembrane protein.
with 10 highly conserved transmembrane helices and a large extracellular loop at the N terminus, as predicted by TOPO2 (http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py). In maize, five genes containing a conserved plasma-membrane choline transporter domain were identified (Fig. S15). They contain four conserved amino acids (V422G500R516N560), and these sites may be functionally important. The other three maize genes with lower expression levels were less similar (<26%) to AtCTL1 (Fig. S14a). ZmCTLP1 is closest in homology to animal and Arabidopsis thaliana CTL1 proteins (Fig. S12), which have been shown to have choline transport activity (O’Regan et al., 2000; O’Regan & Meunier, 2003; Kommareddi et al., 2010; Dettmer et al., 2014). To examine the choline transport activity of ZmCTLP1, we expressed both ZmCTLP1 and AtCTL1 in Xenopus laevis oocytes and measured their choline uptake capacity at two pH levels (pH 5.5 and pH 7.5). The results showed that the choline uptake capacity of ZmCTLP1 was similar to that of AtCTL1 (Fig. 3d).

Subcellular localisation and expression patterns of ZmCTLP1

In previous studies, AtCTL1 was localised to the TGN (Dettmer et al., 2014; Kramer et al., 2017). To examine the subcellular localisation of ZmCTLP1, the full-length ORF of ZmCTLP1 from the wild-type was fused to the N terminus of GFP driven by the CaMV 35S promoter. The construct was co-transformed into maize protoplasts with SYP61-RFP (a TGN marker; Poulsen et al., 2014; Rosquete et al., 2018). Transient expression of ZmCTLP1-GFP showed that it co-localised with SYP61 (Fig. 3e), suggesting that ZmCTLP1 was located at the TGN in maize. ZmCTLP1 was constitutively expressed in various tissues, with higher expression levels in kernels and lower expression levels in other examined tissues (Fig. 3f). During kernel development, the expression of ZmCTLP1 was higher at 2–6 DAP and slowly decreased after 8 DAP (Figs 3f, S14a). At the protein level, the ZmCTLP1 content was higher in endosperms at 10–12 DAP (Fig. S14b). To accurately delineate the expression patterns of ZmCTLP1 within the kernel, we performed RNA in situ hybridisation on 12 DAP kernels. ZmCTLP1 expression was higher in the BETL, BIZ, aleurone layer (Al), PC, and embryo (EMB), but lower in the CSE and in the endosperm adjacent to scutellum (EAS) (Fig. S16).

ZmCTLP1 is involved in lipid homeostasis during kernel development

ZmCTLP1 functions in choline transport, and we therefore measured choline content in 12 DAP kernels using high-performance liquid chromatography (HPLC). The smk10 mutant had significantly lower choline content than the wild-type (Fig. 4a), implying that ZmCTLP1 was required for normal choline homeostasis. Choline serves as a precursor for phosphatidylcholines and sphingolipids, which are the main components of the cell membrane (Wang et al., 2017), and we therefore investigated the contents of 24 classes of membrane lipids using HPLC-mass spectrometry (HPLC-MS). Content of total lipids was higher in the smk10 mutant (Fig. 4b), and free fatty acid (FFA) levels were also greater.

© 2021 The Authors
New Phytologist published by John Wiley & Sons Ltd on behalf of New Phytologist Foundation

Fig. 2 Longitudinal sections of wild-type (WT) and smk10 kernels in maize. (a, b) Comparison of the developing CZ in WT (a) and smk10 (b) kernels at 12 d after pollination (DAP). CZ, conducting zone. Bars, 100 µm. (c, d) Comparison of the developing aleurone layer (AL) in WT (c) and smk10 (d) kernels at 12 DAP. The AL is indicated by a red arrow. EN, endosperm; PE, pericarp. Bars, 20 µm. (e, f) Comparison of the developing basal endosperm transfer layer (BETL) and basal intermediate zone (BIZ) in WT and smk10 kernels at 12 DAP. Black arrows indicate wall in-growths (WIG) of reticulate and flange BETL cells and BIZ cells in the WT (e). Wall in-growths (WIG) were stunted in smk10 (f). PC, placentochalazal region. Bars, 50 µm. (g) The lengths of developing BIZ cells in wild-type and smk10 longitudinal sections. Values are means ± SE, n = 200 cells (**, P < 0.01, Student’s t-test).
Fig. 3 Positional cloning and identification of maize smk10 mutant. (a) The smk10 locus was mapped to a 300-kb region on chromosome 2 using a high-resolution map consisting of 3264 F3 individuals derived from a cross between smk10 and Mo17. The red triangle denotes the site of a mutation in the predicted intron-exon splicing site. (b) Zm00001d001803 was targeted with a specific gRNA located in the first exon. Two independent events with different fragment deletions were generated with the CRISPR/Cas9 system. (c) Neighbour-joining phylogenetic tree using Arabidopsis thaliana paralogues of AtCTL1 and maize paralogues of ZmCTLP1 (with four human and mouse protein sequences as an outgroup). Red and green triangles indicate ZmCTLP1 and AtCTL1, respectively. (d) pH-dependent choline uptake mediated by ZmCTLP1 in Xenopus oocytes. The means ± SE from five independent experiments are presented (**, P < 0.01, Student’s t-test). (e) Subcellular localisation of ZmCTLP1 in maize protoplasts. Bar, 10 μm. (f) Expression pattern of ZmCTLP1 in various tissues measured by qRT-PCR. Actin was used as the endogenous control gene. Three biological replicates of each tissue type were analysed, and the values are reported as means ± SE.
in \textit{smk10} than that of wild-type. A third of the phospholipids, including phosphatidylserines (PS), lyso-PS (LPS), and lyso-PA (LPA), and more than half of the sphingolipids, including phytoceramides (PhytoCer), phyto-glucosylceramides (Phyto-GluCer), phytosphingosines (PhytoSph), sphingosines (Sph), and sphingosine-1-phosphate (S1P) showed significant alteration in \textit{smk10}. Glycolipid profiles did not differ between \textit{smk10} and the wild-type (Fig. 4c).

Furthermore, we also examined the global lipid profiles of wild-type and \textit{smk10} kernels by measuring 423 molecular species from 24 classes (Fig. 4d; Table S4). Over 30\% of the individual lipids were markedly higher in the \textit{smk10} mutant, and 6.9\% were markedly lower. All FFA and most phospholipid molecular species showed significant alteration in \textit{smk10}. Although the total contents of lyso-PC (LPC), phosphatidylethanolamines (PE), and lyso-PE (LPE) were similar between \textit{smk10} and the wild-type, more than...
half of the individual molecular species were significantly altered in smk10 (Fig. 4d; Table S4). Phosphatidylcholines are the major phospholipid components of the plasma membrane. There was no significant difference in total phosphatidylcholine content between the genotypes, but 29% of the individual phosphatidylcholine species differed markedly between the wild-type and smk10 (Fig. S17).

Interestingly, although the absolute lipid contents were higher in smk10, the relative proportions of some lipid classes were significantly lower. The phospholipids including LPC and phosphatidylinositol (PI), glycolipids including monogalactosyl diacylglycerols (MGDG) and digalactosyl diacylglycerols (DGDG) etc., were markedly lower in the smk10 than in the wild-type (Fig. S17).

ZmCTLP1 is crucial for PD formation in endosperm transfer cells

Because the transfer cells in smk10 were misshapen, we further investigated whether the symplastic pathway of nutrient transport among transfer cells was affected. To this end, we performed aniline blue staining of paraffin sections followed by confocal microscopy to identify the location of callose deposition around the neck regions of the PD. Signals at the BIZ cell walls were significantly diminished in smk10 compared with the wild-type at 12 DAP (Fig. 5a–d). The number of PD in the BIZ region at 12 DAP was dramatically lower in smk10, but there were no obvious differences in PD morphology between the wild-type and smk10 (Fig. 5e–m). These observations are consistent with previous reports in Arabidopsis thaliana (Kraner et al., 2017) and show that ZmCTLP1 is required for PD formation in endosperm transfer cells.

Loss of ZmCTLP1 function alters the expression of key kernel development genes

To better understand the impacts of smk10 on global gene expression, RNA-seq analysis was performed on 6 and 12 DAP
Fig. 6 Gene Ontology analysis of the DEGs between 6 and 12 d after pollination (DAP) wild-type (WT) and smk10 maize kernels based on RNA-seq data. (a, b) The most significantly enriched GO terms in the DEGs of 6 DAP kernels (a) and 12 DAP kernels (b) and their associated P-values are shown. Lower x-axis, $-\log_{10}(P$-value); upper x-axis, the number of genes with a given GO term. (c) Expression of basal endosperm transfer layer (BETL) and basal intermediate zone (BIZ)-specific genes quantified by RNA-seq analysis. BIZ-specific genes were obtained from Li et al. (2014). (d) Tissue-specific expression patterns of transport-related genes associated with kernel development quantified by RNA-seq analysis. PC (purple), placento-chalazal region; PED (green), pedicel. The log₂ (fold-change) values between smk10 and the WT were calculated from RNA-seq data and are shown as a heat map (right). (e) Immunoblot analysis showing Mn1 protein accumulation in 4, 8, and 12 DAP WT and smk10 kernels. α-Tubulin served as the loading control. (f–h) In situ hybridization of ZmSWEET4c using antisense probes in sections of smk10 and WT kernels at 12 DAP. (f, g) ZmSWEET4c-antisense probes show the expression of ZmSWEET4c in the BETL of smk10 and wild-type kernels. (h) The ZmSWEET4c-sense probe served as the negative control. The BETL is indicated by a black arrow. Bars, 200 μm.
**smk10** and wild-type kernels. In total, we identified 578 DEGs (97 upregulated and 481 downregulated in **smk10**) in 6 DAP kernels and 3477 DEGs (1322 upregulated and 2155 downregulated) in 12 DAP kernels ($P < 0.05$, log$_2$fold-change $> 1.0$) (Table S5). Here, 10 DEGs were selected for qRT-PCR validation of the RNA-seq results, and both methods showed similar expression differences between the wild-type and **smk10** (Fig. S19). Gene Ontology (GO) analysis of DEGs in 6 DAP kernels revealed enrichment of the GO terms ‘response to abiotic stimulus’, ‘nutrient reservoir activity’, ‘response to lipid’, ‘cell wall macromolecule metabolic process’ and ‘killing of cells of other organism’ (Fig. 6a). The DEGs at 12 DAP were enriched in GO terms related to nutrient metabolism and transport such as ‘nutrient reservoir activity’, ‘carbohydrate metabolic process’, ‘transmembrane transporter activity’, and ‘cell–cell junction (PD)’ (Fig. 6b). To further explore GO terms enriched in DEGs from different endosperm compartments, we analysed available data from a laser-capture microdissection experiment (Zhan et al., 2015). Enriched GO terms included ‘cell killing’ in the BETL at 6 and 12 DAP, ‘hydrolase activity, acting on glycosyl bonds’ in the PE, and ‘ion homeostasis’ and ‘ion transport’ in the PED at 12 DAP (Fig. S20; Table S6).

Eight BETL-specific genes, including **Mn1** and **ZmSWEET4c**, were significantly downregulated in **smk10** (Fig. 6c). Tissue-specific transporters for ions, amino acids and sugars located in the PED, PC, and BETL were also differentially expressed in **smk10** at 12 DAP (Fig. 6d). In fact, c. 11.5% (90/784) of all transporters in **smk10** were differentially expressed at 12 DAP; these included 27 ion transporters, 13 sugar transporters, nine amino acid transporters, and two lipid transporters (Table S7). **Mn1** and **ZmSWEET4c** are essential for the development of fully differentiated transfer cells. To measure the abundance of Mn1 protein, total proteins were isolated from immature wild-type and **smk10** kernels at 4, 8 and 12 DAP and analysed by western blotting with anti-Mn1 mouse monoclonal antibody. Mn1 protein accumulation was significantly lower in **smk10** than in the wild-type (Fig. 6e). To accurately determine the expression level of **ZmSWEET4c** in **smk10** and the wild-type, we performed...
RNA in situ hybridisation with 12 DAP kernels. Consistent with the RNA-seq results, ZmSWEET4c expression was again significantly lower in smk10 than in the wild-type (Fig. 6f–h). These results indicated that ZmCTLP1 influenced the expression of key kernel development genes.

Loss of ZmCTLP1 causes premature cell death in transfer tissues

Disruption of choline and lipid homeostasis can trigger cell death in plants and animals (Li et al., 1998, 2020; Hirasawa et al., 2004; Lin et al., 2008), and we were therefore motivated to investigate cell death in the BETL and BIZ of smk10. We performed a TUNEL assay to examine this cell death signal in situ at 8 and 12 DAP. Intense positive TUNEL signals that co-localised with cell nuclei were detected at the BETL and BIZ positions in smk10, whereas no TUNEL signals were detected in the wild-type (Fig. 7). We also observed that both the PC and EAS exhibited similar cell death in the wild-type and the mutant, as reported by Kladnik et al. (2004) and Doll et al. (2020) (Figs 7, S21). The transfer cells are extremely active and do not undergo programmed cell death before maturity in normal maize kernels (Gao et al., 1998). Therefore, these findings indicated that smk10 exhibited premature BETL and BIZ cell death, which presumably attenuates nutrient transport from the maternal placenta to the filial tissues.

Discussion

Choline and its metabolism have been studied extensively in animals and humans. Despite its important physiological functions, little information is known about the role of choline in plants (Michel et al., 2006; Dettmer et al., 2014; Iwao et al., 2016). In Arabidopsis thaliana, choline deficiency impairs phloem development and conductivity by reducing the number of sieve pores and altering the pore structure in sieve areas (Dettmer et al., 2014). In this study, the smk10 mutant contained less choline than the wild-type and exhibited an irregular and small endosperm, a small embryo and significantly lower kernel weight.
BETL and BIZ cells in the basal endosperm region of the mutant displayed irregular shapes and had few wall thickenings and wall in-growths. We therefore concluded that loss of ZmCTLP1 function disrupted choline and lipid homeostasis of transfer cells, thereby reducing nutrient transport from the maternal placenta to the developing endosperm.

ZmCTLP1 is required for the normal expression of Mn1 and other nutrient transporters

Membrane lipid composition is crucial for maintaining the correct structure and function of membrane proteins (Sanders & Mitten-dorf, 2011). Choline, an essential membrane lipid component, is predominantly used for the synthesis of phosphatidylcholine and sphingomyelin (Michel et al., 2006; Wang et al., 2017). HPC1 (High Phosphatidylcholine 1) encodes a phospholipase A1 enzyme; when introgressed from teosinte Mexicana, it induces early maize flowering by modulating phosphatidylcholine levels (Rodriguez-Zapata et al., 2021). Here, we showed that ZmCTLP1 is required for kernel development and that it appears to regulate choline transport to maintain the homeostasis of membrane lipids. Nutrient transport tissues such as the BETL and BIZ have convoluted wall in-growths that increase the surface area of the plasma membrane, thereby enhancing nutrient transport efficiency (Kang et al., 2009). The formation of cell wall architecture is a membrane-related process that relies on the supply of lipids, proteins and other components (Liu et al., 2015; Rosquete et al., 2018). Here, loss of ZmCTLP1 function significantly disrupted choline and lipid homeostasis, and this appeared to block the formation of wall in-growths. Mn1 is synthesised on the endoplasmic reticulum and delivered to the wall in-growths by the TGN (Kang et al., 2009). Mn1 and wall in-growths are interdependent, reinforcing each other (Kang et al., 2009). Our results suggested that ZmCTLP1-mediated regulation of membrane lipid homeostasis is required for the normal accumulation of Mn1 (Figs 6e, 8).

Lipid-dependent protein sorting plays a central role in cargo sorting and trafficking to the cell surface (Surma et al., 2012). Disruption of membrane lipid homeostasis in the smk10 mutant appeared to strongly influence membrane transport capacity, as 11.5% of all transporter genes were differentially expressed in the mutant, including ion transporters, amino acid transporters and sugar transporters (Table S7). In situ hybridisation suggested that ZmSWEET4c-mediated hexose import may have been reduced in smk10 (Fig. 6f–h). It has been proposed that ZmSWEET4c-mediated sugar accumulation in the BETL induces hexose import by increasing membrane area of the basal cells in developing endosperm, therefore the capacity to hold transporters (including ZmSWEET4c) exists as a result of increased ZmSWEET4c activity. In our study, loss of function of ZmCTLP1 disrupted lipid homeostasis and led to incomplete wall in-growth of BETL, which could decrease the membrane area in transfer cells, resulting in reduced accumulations of Mn1 and ZmSWEET4c in BETL. Thereby, the overall activities of hydrolysing sucrose by Mn1 and importing hexose into BETL by ZmSWEET4c were decreased, which might be one of the major causes for small kernel phenotype in smk10.

ZmCTLP1 is essential for PD formation and development

PD are membrane-lined channels between neighbouring cells that provide a direct route for the symplastic movement of proteins, RNAs, sugars and other small molecules (Wu et al., 2016; Sager & Lee, 2018). During the development of primary PD, materials derived from ER tubules (desmotubules) are enclosed by cytoplasmic strands, and TGN secretory vesicles provide wall materials and membrane lipids (Ehlers & Kollmann, 2001). Sphingolipids that are enriched in the TGN perform essential functions to regulate PD permeability (Albright et al., 1996). Our results showed that sphingolipid levels differed significantly between smk10 and the wild-type, and it is plausible that disrupted membrane lipid homeostasis in smk10 may have caused the defects in PD formation. There were fewer PDs in the smk10 mutant than in the wild-type, indicating that the mutant was impaired in PD formation and development. The morphological abnormalities of the BETL cells in smk10 were partly attributable to reduced numbers of PD, which would have disrupted cell-to-cell movement of nutrients and other signals (Fig. 8). PD numbers are also reduced in the shoot apical meristems and developing leaves of the atctl1 mutant, causing a severely stunted growth phenotype. Soluble sugar and starch contents are significantly increased in the atctl1 mutant, suggesting that nutrient homeostasis is disrupted in the leaves (Kraner et al., 2017). Our results showed that starch, sugar and protein contents were significantly altered in smk10 kernels, and this may have resulted from defects in cell–cell symplastic transport mediated by PD.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (91935303; 31971957; 31971959; 91935305) and National Key Research and Development Programme of China (2016YFD0100404, 2016YFD0101803).

Author contributions

WS, JL and MH designed the experiments. MH, HZ, BY, SY, HL, HT, GS, ZC and LE performed the experiments. MH and WS, analysed the data. MH, WS and JL wrote the article. MH, HZ and BY contributed equally to this work.

ORCID

Zongliang Chen https://orcid.org/0000-0003-1469-3699
Jinsheng Lai https://orcid.org/0000-0001-9202-9641
Guanghou Shui https://orcid.org/0000-0002-1621-9643
Weibin Song https://orcid.org/0000-0003-3486-2585
He Tian https://orcid.org/0000-0002-5388-0297
Haiming Zhao https://orcid.org/0000-0003-1600-9619

Data availability

Sequence data from this article can be found in the MaizeGDB database under the following accession numbers:
Gao YQ, Chen JG, Chen ZR, An D, Lv QY, Han ML, Wang YL, Salt DE. Analysis of RNA-seq data using tophat and cufflinks. Grain setting defect1, encoding a remorin and are available under the accession number PRJNA720421.

References

Albright CD, Liu R, Bethca TC, Da Costa KA, Salganik RI, Zeisel SH. 1996. Choline deficiency induces apoptosis in SV40-immortalized CWSV-1 rat hepatocytes in culture. *FASEB Journal* 10: 510–516.

Becraft PW, Gutierrez-Marcos J. 2012. Endosperm development: dynamic processes and cellular innovations underlying sibling altruism. *Wiley Interdisciplinary Reviews Developmental Biology* 1: 579–593.

Bhmländer S, Hunter CT, Johns CE, Koch KE, Braun DM. 2013. Regulation of assimilate import into sink organs: update on molecular drivers of sink frontiers. *Frontiers in Plant Science* 4: 177.

Chen J, Zeng B, Xie S, Wang G, Hauck A, Lai J. 2014. Dynamic transcriptome landscape of maize embryo and endosperm development. *Plant Physiology* 166: 252–264.

Cheng WH, Chourey PS. 1999. Genetic evidence that invertase-mediated release of hexoses is critical for appropriate carbon partitioning and normal seed development in maize. *Theoretical and Applied Genetics* 98: 485–495.

Cheng WH, Taliercio EW, Chourey PS. 1996. The miniatures seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. *Plant Cell* 8: 971–983.

Clegg KM. 1956. The application of the anthrone reagent to the estimation of starch in cereals. *Journal of the Science of Food and Agriculture* 7: 40–44.

Correa SM, Fernie AR, Nikolski Z, Brotman Y. 2020. Towards model-driven characterization and manipulation of plant lipid metabolism. *Progress in Lipid Research* 80: 101051.

Davis RW, Smith JD, Cobb BG. 1990. A light and electron microscope investigation of the transfer cell region of maize caryopses. *Canadian Journal of Botany* 68: 471–479.

Dettmer J, Ursache R, Campilho A, Miyashima S, Belechiv I, O’Regan S, Mullendore DL, Yadav SR, Lanz C, Beverina L et al. 2014. CHOLINE TRANSPORTER-LIKE1 is required for sieve plate development to mediate long-distance cell-to-cell communication. *Nature Communications* 5: 4276.

Ding L, Yan S, Jiang L, Zhao W, Ning K, Lu X, Zhang J, Wang Q, Zhang X. 2015. HANABA TARANU (HAN) bridges meristem and organ primordia boundaries through PINHEAD, JAGGED, BLADE-ON-PETIOLE2 and CYTOKININ OXIDASE 3 during flower development in *Arabidopsis*. *PLoS Genetics* 11: e1005479.

Doll NM, Just J, Bruanda V, Caius J, Grimaud A, Depege-Fargeix N, Esteban E, Pasha A, Provat NJ, Ingram GC et al. 2020. Transcriptomics at maize embryo/endosperm interfaces identifies a transcriptionally distinct endosperm subdomain adjacent to the embryo scutellum. *Plant Cell* 32: 833–852.

Ehlers K, Kollmann R. 2001. Primary and secondary plasmodesmata: structure, origin, and functioning. *Protoplasma* 216: 1–30.

Gao R, Dong S, Fan J, Hu C. 1998. Relationship between development of endosperm transfer cells and grain mass in maize. *Biologia Plantarum* 41: 539–546.

Gao YQ, Chen JG, Chen ZR, An D, Lv QY, Han ML, Wang YL, Salt DF. 2017. A new vesicle trafficking regulator CTLP1 plays a crucial role in ion homeostasis. *PLoS Biology* 15: e2002978.

Ghosh S, Chan CK. 2016. Analysis of RNA-seq data using tophat and cufflinks. *Methods in Molecular Biology* 1374: 339–361.

Gui J, Liu C, Shen J, Li L. 2014. Grain setting defect1, encoding a remorin protein, affects the grain setting in rice through regulating plasmodesmatal conductance. *Plant Physiology* 166: 1463–1478.

Hirasawa KI, Amano T, Shioi Y. 2004. Lipid-binding form is a key conformation to induce a programmed cell death initiated in tobacco BY-2 cells by a proteinaceous elicitor of cryptogein. *Physiologia Plantarum* 121: 196–203.

Iwao B, Yara M, Hara N, Kawai Y, Yamakawa T, Nishihara H, Inoue T, Inazu M. 2016. Functional expression of choline transporter like-protein 1 (CTLP1) and CTLP2 in human brain microvascular endothelial cells. *Neurochemistry International* 93: 40–50.

Kang BH, Xiong Y, Williams DS, Ponseta-Rodero D, Choureuy PS. 2009. Microtubule-encoded cell wall invertase is essential for assembly and function of wall-in-growth in the maize endosperm transfer cell. *Plant Physiology* 151: 1366–1376.

Kim D, Langmead B, Salzberg SL. 2015. Hisat: a fast spliced aligner with low memory requirements. *Nature Methods* 12: 357–360.

Kladnik A, Chamuskos K, Dermastia M, Choureuy P. 2004. Evidence of programmed cell death in post-phloem transport cells of the maternal pedicel tissue in developing caryopsis of maize. *Plant Physiology* 136: 3572–3581.

Kommareddy PK, Nair TS, Thang LV, Galano MM, Babu E, Ganapathy V, Kanazawa T, McHugh JB, Carey TE. 2010. Isoforms, expression, glycosylation, and tissue distribution of CTLP2/SLC44A2. *Protein Journal* 29: 417–426.

Kraemer ME, Link K, Melzer M, Ekici AB, Uebe S, Tarazona P, Feussner I, Hofmann J, Sonnewald U. 2017. Choline transporter-like1 (CHER1) is crucial for plasmodesmata maturation in *Arabidopsis thaliana*. *The Plant Journal* 89: 394–406.

Lalonde S, Wipf D, Frommer WB. 2004. Transport mechanisms for organic forms of carbon and nitrogen between source and sink. *Annual Review of Plant Biology* 55: 341–372.

Larkins BA. 2019. Chapter 12 - proteins of the kernel. In: Serna-Saldivar SO, ed. *Corn*, Oxford, UK: AACCC International Press, 319–336.

Leroux BM, Goodyke AJ, Schumacher KJ, Abbott CP, Clore AM, Yadegari R, Larkins BA, Dannenhoffer JM. 2014. Maize early endosperm growth and development: from fertilization through cell type differentiation. *American Journal of Botany* 101: 1259–1274.

Li B, Liu H, Zhang Y, Kang T, Zhang L, Tong J, Xiao L, Zhang H. 2013. Constitutive expression of cell wall invertase genes increases grain yield and starch content in maize. *Plant Biotechnology Journal* 11: 1080–1091.

Li G, Wang D, Yang R, Logan K, Chen H, Zhang S, Skaggs MI, Lloyd A, Burnett WJ, Laurie JD et al. 2014. Temporal patterns of gene expression in developing maize endosperm identified through transcriptome sequencing. *Proceedings of the National Academy of Sciences, USA* 111: 7582–7587.

Li T, He M, Zeng J, Chen Z, Hongxia Q, Duan X, Jiang Y. 2020. Choline chloride alleviates the pericarp browning of harvested litchi fruit by inhibiting energy deficiency mediated programmed cell death. *Postharvest Biology and Technology* 167: 111224.

Li X, Gu W, Sun S, Chen Z, Chen J, Song W, Zhao H, Lai J. 2018. Defective kernel 39 encodes a PPR protein required for seed development in maize. *Journal of Integrative Plant Biology* 60: 45–64.

Li Y, Maher P, Schubert D. 1998. Phosphatidylcholine-specific phospholipase C regulates glutamate-induced nerve cell death. *Proceedings of the National Academy of Sciences, USA* 95: 7748–7753.

Lin SS, Martin R, Mongrand S, Vandenbeecke S, Chen KC, Jang IC, Chua NH. 2008. RING1 E3 ligase localizes to plasma membrane lipid rafts to trigger FBL-induced programmed cell death in arabidopsis. *The Plant Journal* 56: 550–561.

Lindeboom RGH, Vermeulen M, Lehnert B, Supek F. 2019. The impact of nonsense-mediated mRNA decay on genetic disease, gene editing and cancer immunotherapy. *Nature Genetics* 51: 1645–1651.

Liu Z, Persson S, Sanchez-Rodriguez C. 2015. At the border: the plasma membrane-cell wall continuum. *Journal of Experimental Botany* 66: 1553–1563.

Machova E, O’Regan S, Newcombe J, Meunier FM, Prentice J, Dove R, Liśi R, Doležal V. 2009. Detection of choline transporter-like 1 protein CTLP1 in neuroblastsoma x glioma cells and in the CNS, and its role in choline uptake. *Journal of Neurochemistry* 110: 1297–1309.

Michel V, Yuan Z, Ramsurub S, Bakovic M. 2006. Choline transport for phospholipid synthesis. *Experimental Biology and Medicine* 231: 490–504.
Miller ME, Chourey PS. 1992. The maize invertase-deficient miniature-1 seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell 4: 297–305.

Muhitch MJ. 1989. Purification and characterization of two forms of glutamine synthetase from the pedicel region of maize (Zea mays L.) kernels. Plant Physiol. 91: 868–875.

Muhitch MJ. 1993. In vitro metabolism of L-aspartate by maize kernels. Physiochemistry 32: 1125–1130.

Niyaki T, Sonkoly B, Merai Z, Benkovics AH, Silhavy D. 2009. Plant upstream ORFs can trigger nonsense-mediated mRNA decay in a size-dependent manner. Plant Molecular Biology 71: 367–378.

O’Regan S, Meunier FM. 2003. Selection and characterization of the choline transport suppressor from torpedo electric lobe, CTL1. Neurochemical Research 28: 551–555.

O’Regan S, Traiffort E, Ruat M, Cha N, Compère D, Meunier FM. 2000. An electric lobe suppressor for a yeast choline transport mutation belongs to a new family of transporter-like proteins. Proceedings of the National Academy of Sciences, USA 97: 1835–1840.

Porter GA, Knievel DP, Shannon JC. 1987. Asseimulate unloading from maize (Zea mays L.) pedicel tissues. Plant Physiology 85: 558–565.

Poulos CP, Diokopimol A, Mouille G, Burow M, Geshi N. 2014. Arabino/uloc/ulocul glycosyltransferases target to a unique subcellular compartment that may function in unconventional secretion in plants. Traffic 15: 1219–1234.

Rodriguez-Zapata F, Barnes AC, Bilcher-Juarez KA, Gates D, Kur A, Wang L, Janzen GM, Jensen S, Estévez-Palmas JM, Crow T et al. 2021. Teosinte introgression modulates phosphatidylcholine levels and induces early maize flowering time. bioRxiv. doi: 10.1101/2021.01.25.426574.

Rosquete MR, Davis DJ, Drakakaki G. 2018. The plant Trans-Golgi network: not just a matter of distinction. Plant Physiology 176: 187–198.

Royo J, Gómez E. 2007. Transfer cells. In: Olsen OA, ed. Endoderm. Plant cell monographs. Berlin, Heidelberg, Germany: Springer Verlag, 73–89. doi: 10.1007/978_007_2007_110.

Ruiz-Echevarría MJ, Gonzalez CL, Peltz SW. 1998. Identifying the right stop: determining how the surveillance complex recognizes and degrades an aberrant mRNA. EMBO Journal 17: 575–589.

Sager RE, Lee JY. 2018. Plasmodesmata at a glance. Journal of Cell Science 131: jcs209346.

Sanders CR, Mittendorf KF. 2011. Tolerance to changes in membrane lipid composition as a selected trait of membrane proteins. Biochemistry 50: 7858–7867.

Santandrea G, Gao Y, O’Connell T, Thompson RD. 2002. Post-phloem protein trafficking in the maize cytoplast: zMTRXH1, a thioredoxin specifically expressed in the pedicel parenchyma of Zea mays L., is found predominantly in the placentochalaza. Plant Molecular Biology 50: 743–756.

Shannon JC. 1972. Movement of C-labeled assimilates into kernels of Zea mays L.: II. Pattern and rate of sugar movement. Plant Physiology 49: 198–202.

Shu G, Guan XL, Low CP, Chua GH, Goh JS, Yang H, Wenk MR. 2010. Toward one step analysis of cellular lipidoles using liquid chromatography coupled with mass spectrometry: application to saccharomyces cerevisiae and schizosaccharomyces pombe lipidoles. Molecular & Biosystems 6: 1008–1017.

Song W, Zhu J, Zhao H, Li Y, Liu J, Zhang X, Huang L, Lai J. 2019. OSl functions in the allocation of nutrients between the endosperm and embryo in maize seeds. Journal of Integrative Plant Biology 61: 706–727.

Sossi D, Luo D, Li QB, Sasse J, Yang J, Gendrot G, Suzuki M, Koch KE, McCarty DR, Chourey PS et al. 2015. Seed filling in domesticated maize and rice depends on SWEET-mediated hexose transport. Nature Genetics 47: 1489–1493.

Surma MA, Klose C, Simons K. 2012. Lipid-dependent protein sorting at the trans-Golgi network. Biochimica et Biophysica Acta 1821: 1059–1067.

Ueland PM. 2011. Choline and betaine in health and disease. Journal of Inherited Metabolic Disease 34: 3–15.

van Greevenbroek MM, Voorhout WF, Erkelens DW, van Meer G, de Bruin TW. 1995. Palmitic acid and linoleic acid metabolism in Caco-2 cells: different triglyceride synthesis and lipoprotein seeration. Journal of Lipid Research 36: 13–24.
Fig. S7 Statistical analysis of CZ and CSE cell areas and longitudinal sections of wild-type and smk10 kernels.

Fig. S8 Characteristics of the Smk10 gene.

Fig. S9 Sequences of the kernels from WT and CRISPR-generated mutants in the Zm00001d001803 genomic fragment.

Fig. S10 Confirmation that Zm00001d001803 is responsible for the defective kernel phenotype.

Fig. S11 Investigation of the maternal effect for ZmCTLP1.

Fig. S12 A neighbour-joining phylogenetic tree of ZmCTLP1 and its orthologous proteins from other organisms.

Fig. S13 The CTL1 transmembrane helix is conserved among different species.

Fig. S14 Expression patterns of five maize genes (Chen et al., 2014) and the abundance of ZmCTLP1 protein (Walley et al., 2016) in various tissues.

Fig. S15 Comparison of the conserved domains of CTLs in maize, Arabidopsis thaliana and rice.

Fig. S16 In situ hybridisation of ZmCTLP1 using antisense probes in sections of wild-type kernels at 12 DAP.

Fig. S17 Forty-two phosphatidylcholine species were identified in wild-type and smk10 kernels.

Fig. S18 Lipid contents per kernel between smk10 and wild-type at 12 DAP.

Fig. S19 qPCR confirmation of 10 selected DEGs at 12 DAP.

Fig. S20 Gene Ontology analysis of the DEGs between wild-type and smk10 kernels at 6 and 12 DAP.

Fig. S21 Cell death detected by the TUNEL assay in 12 DAP wild-type (a–c) and smk10 (d–f) kernels.

Table S1 The candidate genes predicted by maizeGDB in the 300-kb smk10 interval.

Table S2 Primers used in this study.

Table S3 Chi-squared tests of the mature kernel phenotypes in smk10 × CRISPR-generated mutants ears ($\chi^2_{0.05} = 3.84$).

Table S4 The contents of 423 lipid species in wild-type (B1–B5) and smk10 (A1–A5) kernels.

Table S5 Expressed genes in 6 and 12 DAP smk10 kernels compared with wild-type.

Table S6 Enriched GO terms analysis of compartments-specific genes in smk10 mutant compared with wild-type.

Table S7 Expression of transporter genes in the DEGs quantified by the RNA-seq analysis.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the New Phytologist Central Office.