# *Small kernel2* Encodes a Glutaminase in Vitamin $B_6$ Biosynthesis Essential for Maize Seed Development

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Vitamin $B_6$, an essential cofactor for a range of biochemical reactions and a potent antioxidant, plays important roles in plant growth, development, and stress tolerance. Vitamin $B_6$ deficiency causes embryo lethality in Arabidopsis (*Arabidopsis thaliana*), but the specific role of vitamin $B_6$ biosynthesis in endosperm development has not been fully addressed, especially in monocot crops, where endosperm constitutes the major portion of the grain. Through molecular characterization of a *small kernel2* (*smk2*) mutant in maize, we reveal that vitamin $B_6$ has differential effects on embryogenesis and endosperm development in maize. The $B_6$ vitamer pyridoxal 5′-phosphate (PLP) is drastically reduced in both the *smk2* embryo and the endosperm. However, whereas embryogenesis of the *smk2* mutant is arrested at the transition stage, endosperm formation is nearly normal. Cloning reveals that *Smk2* encodes the glutaminase subunit of the PLP synthase complex involved in vitamin $B_6$ biosynthesis de novo. *Smk2* partially complements the Arabidopsis vitamin $B_6$-deficient mutant *pdx2.1* and *Saccharomyces cerevisiae* pyridoxine auxotrophic mutant MML21. *Smk2* is constitutively expressed in the maize plant, including developing embryos. Analysis of $B_6$ vitamers indicates that the endosperm accumulates a large amount of pyridoxamine 5′-phosphate (PMP). These results indicate that vitamin $B_6$ is essential to embryogenesis but has a reduced role in endosperm development in maize. The vitamin $B_6$ required for seed development is synthesized in the seed, and the endosperm accumulates PMP probably as a storage form of vitamin $B_6$.

The term vitamin $B_6$ refers to six water-soluble compounds, including pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their 5′-phosphorylated derivatives (Drewke and Leistner, 2001; Mooney et al., 2009). Pyridoxal 5′-phosphate (PLP) is the active form of vitamin $B_6$, which serves as a cofactor for over 170 enzymes that are mostly involved in amino acid, lipid, and carbohydrate metabolism (Percudani and Peracchi, 2003). In addition, vitamin $B_6$ has also been identified as a potent antioxidant that quenches singlet oxygen as efficiently as do vitamins C and E (Ehrenshaft et al., 1999). This function has been implicated in conferring plant resistance to abiotic stresses (Shi et al., 2002; Shi and Zhu, 2002; Chen and Xiong, 2005; Titiz et al., 2006).

Vitamin $B_6$ is synthesized de novo in plants, fungi, archae, and most eubacteria, but not in animals, including humans, which have to obtain it from dietary sources. The biosynthesis of vitamin $B_6$ involves two de novo pathways and a salvage pathway. The seven-step DXP-dependent de novo pathway, which occurs exclusively in the γ-subdivision of proteobacteria, uses 1-deoxy-D-xylulose-5-phosphate (DXP) and 4-phosphohydroxy-L-Thr to synthesize pyridoxine 5′-phosphate (PNP; Kennedy et al., 1995; Zhao and Winkler, 1996; Cane et al., 1998; Laber et al., 1999). In contrast, plants, fungi, and most eubacteria use a DXP-independent pathway that synthesizes PLP from Gln, ribose 5-phosphate (or ribulose 5-phosphate), and glyceraldehyde 3-phosphate (or dihydroxyacetone phosphate; Tambasco-Studart et al., 2005). This pathway consists of two enzymes that form a Gln amidotransferase (PLP synthase) comprised of a synthase subunit (PDX1) and a glutaminase subunit (PDX2; Tambasco-Studart et al., 2005). PDX2 catalyzes release of an ammonia from Gln that is combined by PDX1 with ribose 5-phosphate and glyceraldehyde 3-phosphate to synthesize PLP (Burns et al., 2005; Tambasco-Studart et al., 2005). The PLP synthase complex has a dodecameric cogwheel structure that includes 12 PDX1-PDX2 heterodimers (Strohmeier et al., 2006). Channeling of ammonia between the active centers of PDX1 and PDX2 is mediated by a Met-rich hydrophobic tunnel (Strohmeier et al., 2006).

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In addition to the de novo pathways, PLP can be derived through a salvage pathway. As illustrated in Supplemental Figure S1, $B_6$ vitamers are interconvertible. Interconversions of PN, PL, PM, and corresponding 5′-phosphate derivatives are mediated by kinase and phosphatase reactions (Yang et al., 1996, 1998). PL can be reduced to PN by a pyridoxal reductase (Guirard and Snell, 1988; Nakano et al., 1999), whereas PNP and PMP can be converted to PLP by a PMP/PNP oxidase (Zhao and Winkler, 1995). *Salt-Overly-Sensitive4* (*SOS4*) encodes the PN/PL/PM kinase (Shi et al., 2002; Shi and Zhu, 2002) and *PDX3* encodes the PNP/PMP oxidase (Sang et al., 2007), and PLR1 the PL reductase in Arabidopsis (*Arabidopsis thaliana*; Herrero et al., 2011). The salvage pathway is universally present in all kingdoms, including those that cannot synthesize vitamin $B_6$ de novo.

Vitamin $B_6$ plays important roles in plant growth, development, and stress responses. A complete deficiency of vitamin $B_6$ causes embryo lethality (Tambasco-Studart et al., 2005; Titiz et al., 2006), whereas attenuated vitamin $B_6$ biosynthesis negatively affects plant growth and development and reduces stress tolerance (Chen and Xiong, 2005; Wagner et al., 2006; Titiz et al., 2006). The Arabidopsis genome contains three *PDX1* homologs (*AtPDX1.1*, *AtPDX1.2*, and *AtPDX1.3*) and one *PDX2* (Tambasco-Studart et al., 2005). AtPDX1.1 and AtPDX1.3 have the PLP synthase activity (Titiz et al., 2006; Tambasco-Studart et al., 2007), and AtPDX1.2 functions by enhancing the catalytic activity of PLP synthase (Moccand et al., 2014). The Arabidopsis *pdx2* knockout mutants and *pdx1.1*:*pdx1.3* double mutants blocking the vitamin $B_6$ biosynthesis are embryo lethal (Tambasco-Studart et al., 2005; Titiz et al., 2006). The *atpdx1.1* and *atpdx1.3* single mutants have reduced growth (particularly root growth in *pdx1.3*) and are hypersensitive to oxidative stresses (Chen and Xiong, 2005; Wagner et al., 2006; Titiz et al., 2006). A recent study revealed that differential expression of *AtPDX1.1* and *AtPDX1.3* in developing roots affects homeostasis of auxin and ethylene (Boycheva et al., 2015). Biosynthetic pathways of both hormones involve PLP-dependent enzymes (Boycheva et al., 2015). The stress tolerance function is partially attributed to the antioxidant properties of vitamin $B_6$. Stress-induced reactive oxygen species can be quenched by vitamin $B_6$ (Ehrenshaft et al., 1999; Bilski et al., 2000, Mittler, 2002). In addition to total vitamin $B_6$ levels, proper balancing of $B_6$ vitamers by salvage reactions is essential for normal growth and development (Colinas et al., 2016). In the PMP/PNP oxidase-deficient *pdx3* mutant of Arabidopsis, increased levels of PMP and reduced levels of PLP and PL are associated with impaired growth and development (Colinas et al., 2016).

Although vitamin $B_6$ biosynthesis is essential for embryogenesis in Arabidopsis (Tambasco-Studart et al., 2005), the roles of vitamin $B_6$ in embryogenesis and endosperm development in grasses has not been addressed. Whereas Arabidopsis seeds develop a transient endosperm that is quickly consumed by the developing embryo leaving a single layer of aleurone cells in mature seeds, endosperm is a major component of the grass seed. Here, we report a detailed characterization of *small kernel2* (*smk2*), a vitamin $B_6$ biosynthetic mutant of maize. Molecular analysis indicates that *Smk2* encodes the glutaminase subunit of the PLP synthase. Null mutations of *Smk2* arrest embryogenesis at the transition stage, whereas endosperm development is only moderately affected. Vitamin $B_6$ levels are decreased dramatically in both the endosperm and embryo of the *smk2* mutant, indicating that embryogenesis is more sensitive to reduced vitamin $B_6$ levels than endosperm development. Furthermore, our results also indicate that the developing endosperm accumulates vitamin $B_6$ in the form of PMP, probably as a storage form of the vitamin.

## RESULTS

### Phenotypic Characterization of *smk2-1*

The recessive embryo-lethal *small kernel2-1* (*smk2-1*) mutant was isolated from the UniformMu transposon mutagenesis population (McCarty et al., 2005). Self-pollinated *Smk2-1* heterozygous plants segregate *smk* and wild-type kernels in ratios that range from 1:3 to 1:6.5, depending on the season. PCR genotyping enabled by cloning of the gene indicated that the underrepresentation of mutant homozygote is caused by reduced transmission of the mutant allele through pollen, suggesting that environmental factors affect pollen transmission. The *smk2-1* kernels were smaller than wild-type siblings throughout kernel development (Fig. 1, A and B). At maturity, the mutant kernels contained normal-appearing but slightly smaller endosperms, whereas the embryos were aborted (Fig. 1C). Histological sections showed that at 18 days after pollination (DAP), wild-type embryos had reached late stage embryogenesis as indicated by formation of scutellum (SC), leaf primordia, shoot apical meristem (SAM), and root apical meristem (Fig. 1, D and F). By contrast, the *smk2-1* mutant embryos were arrested at an early transition stage (Fig. 1, E and G). Although morphologically normal, endosperms of *smk2-1* mutant seeds contained fewer cells than in endosperms of wild-type siblings (Fig. 1, D and E). Development of basal endosperm transfer cells was not visibly affected in the mutant (Supplemental Fig. S2). Overall, these results suggest that the *smk2-1* mutation arrests embryogenesis at the transition stage and causes a moderate reduction in endosperm size in maize. Consistent with the early block in embryogenesis, mature mutant seed invariably failed to germinate. In addition, attempts to rescue the *smk2-1* mutants on Murashige and Skoog medium were not successful. Hence, the *smk2-1* mutation was maintained as a heterozygote.

### Cloning of *Smk2*

*Smk2* was cloned by transposon tagging based on the Southern-blot cosegregation analysis. A 5.1 kb *Eco*RI

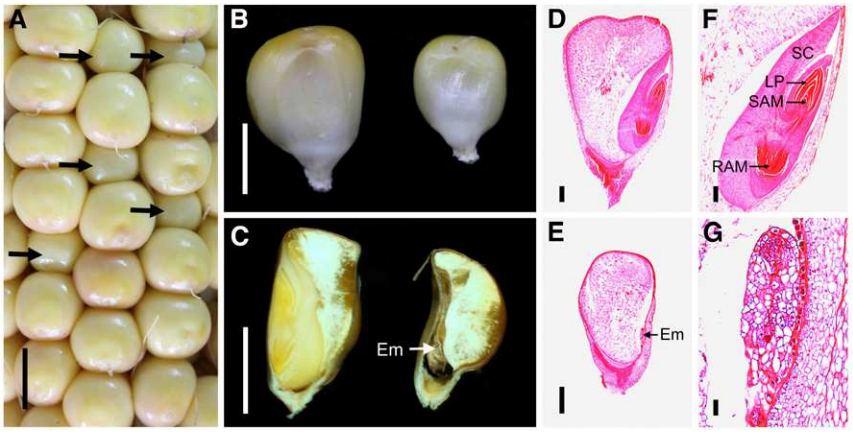


**Figure 1.** Seed development is affected in the *smk2-1* mutant. A, An ear segregating *smk2-1* mutants (arrows). B, Germinal face of the wild-type and *smk2-1* mutant kernels at 18 DAP. C, Section of mature wild-type and *smk2-1* kernels. D to G, Cytological sections of wild-type (D and F) and *smk2-1* mutant kernels (E and G) at 18 DAP. LP, Leaf primordia; RAM, root apical meristem; Em, embryo. Scale bars = 0.5 cm in A to C, 1 mm in D and E, 500 $\mu$m in F, and 100 $\mu$m in G.

fragment that hybridized to the *Mutator-Don Robertson* (*MuDR*)-specific probe was found to cosegregate with the *smk2-1* mutant in a 24-plant population (Fig. 2A). Further analysis in a 70-plant population did not recover any recombinations, indicating a tight linkage between the *smk2* phenotype and the *MuDR* insertion. This 5.1 kb *Eco*RI fragment was cloned by screening a size-selected $\lambda$-phage library. Sequencing of the fragment revealed that the *MuDR* was inserted in GRMZM2G023528 (accession AY109859). This gene contains seven exons, and the *MuDR* element is inserted in the first intron (Fig. 2B). To confirm that this candidate is the causal gene for the *smk2-1* phenotype, we isolated alleles of this gene from the Trait Utility System in Corn population (Bensen et al., 1995). Eleven independent alleles were identified, named from *smk2-2* to *smk2-12*, respectively (Fig. 2B). Crosses of each allele to the *smk2-1* allele showed noncomplementation of the *smk* phenotype in F1 seeds, thus confirming that GRMZM2G023528 is the causal gene for the *smk2* mutation.

### SMK2 Shows a High Similarity to the Glutaminase Subunit of PLP Synthase and Is Localized in Cytosol

BLAST analysis identified a single copy of the *Smk2* gene in the B73 genome (RefGen_v3, Maize GDB; Schnable et al., 2009). *Smk2* encodes a 255-amino acid protein that shows a high similarity to the glutaminase subunit of PLP synthases. The SMK2 protein sequence is 66% identical and 85% similar to AtPDX2 of Arabidopsis (Tambasco-Studart et al., 2007); 46% identical and 60% similar to YaaE of *Bacillus subtilis* (Raschle et al.,

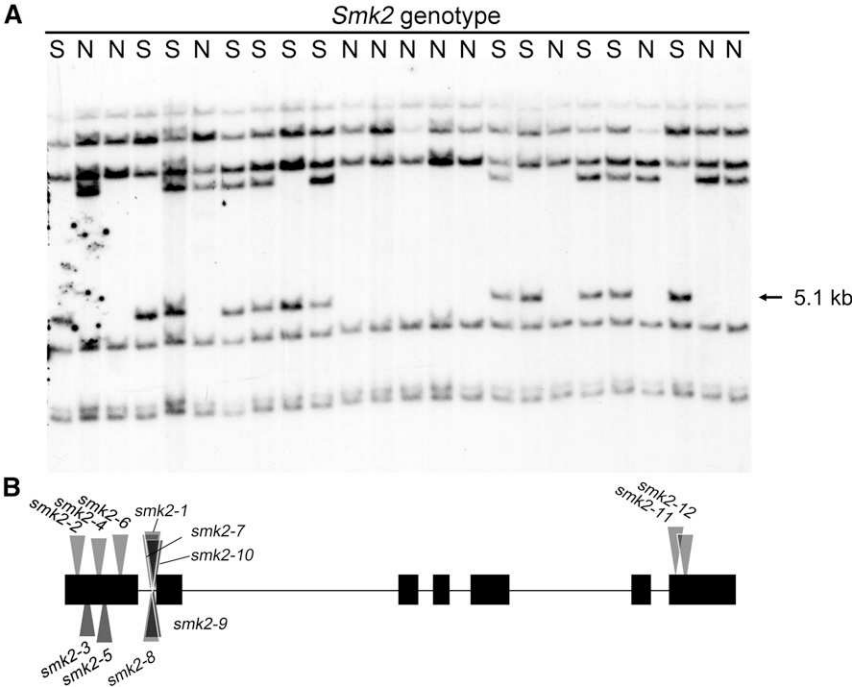


**Figure 2.** Cloning of *Smk2*. A, Southern hybridization of an F2 segregating family of *smk2-1* mutants. DNAs were digested with *Eco*RI and probed with the 1.3-kb *Eco*RI-*Bam*HI fragment of the *MuDR* element. A 5.1-kb fragment cosegregating with *smk2-1* mutants is indicated by the arrow. The genotype N (nonsegregating, i.e. wild type) and S (segregating, i.e. heterozygote) was determined by phenotyping the selfed progeny of these individuals. B, Gene structure of *Smk2* and *Mu* insertion sites. Boxes represent exons and lines introns. Triangles denote *Mu* insertions.

2005), and 38% identical and 53% similar to SNZ (snooze)-proximal open reading frame 1 (SNO1) of *Saccharomyces cerevisiae* (Rodríguez-Navarro et al., 2002; Dong et al., 2004). In addition, the highly conserved signature sequence of glutaminase subunits ([G/A]LI[L/I/V]PGGEST[S/T/A]; Zalkin and Smith, 1998), and the catalytic triad sites Glu-His-Cys (Bauer et al., 2004) are present in SMK2 (Fig. 3A). The two plant proteins, SMK2 and AtPDX2, are similar in size, whereas the bacterial and yeast, YaaE and SNO1, are slightly shorter in the C terminus. The sequence similarity indicates that *Smk2* encodes the glutaminase subunit of PLP synthase in the vitamin $B_6$ biosynthesis pathway.

To determine the subcellular localization, SMK2 was fused with GFP at the N or C terminus and transiently expressed in tobacco leaves via *Agrobacterium* infiltration. The alcohol dehydrogenase-RFP fusion (ADH-RFP) was coexpressed as the cytosolic marker (Denyer et al., 1996; Heazlewood et al., 2004; Giegé et al., 2003). Confocal microscopy analysis indicated that the green signals from both SMK2-GFP and GFP-SMK2 were merged with the red signals from ADH-RFP (Fig. 3B), indicating that SMK2 is localized in the cytosol. To test whether SMK2 carries an N-terminal signal peptide that could be cleaved after localization, we compared the size of SMK2-GFP and GFP-SMK2 in these leaves by western blot. As shown in Figure 3C, the anti-GFP antibody detected a single 56-kD band that appeared to be indistinguishable in size between SMK2-GFP and GFP-SMK2, suggesting that SMK2 is unlikely to have a cleavable signal peptide.

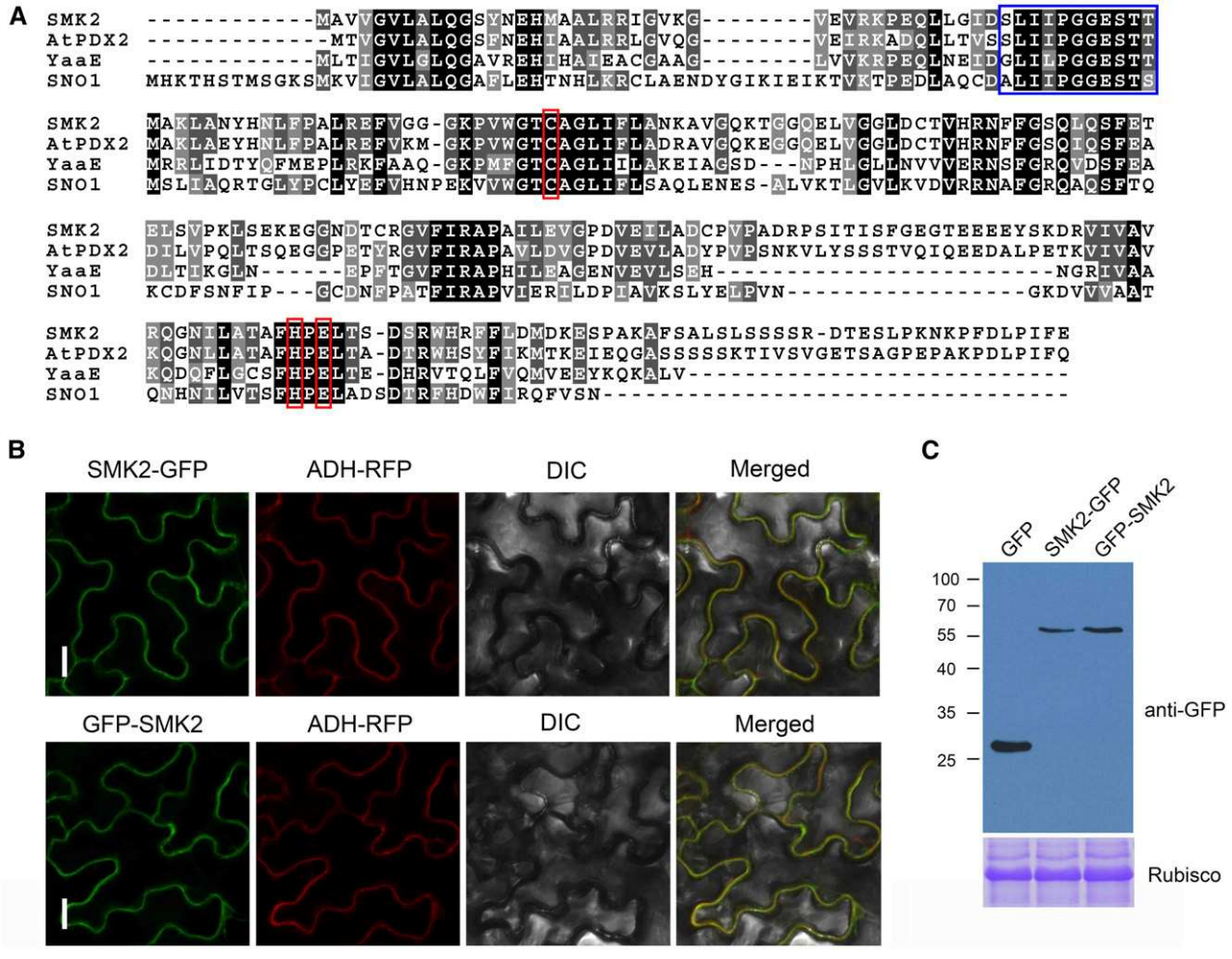


**Figure 3.** *Smk2* encodes a cytosolic protein that shows significant similarity to the glutaminase subunits of PLP synthases. A, Alignment of SMK2 with characterized glutaminase subunits from *S. cerevisiae* (SNO1), Arabidopsis (AtPDX2), and *B. subtilis* (YaaE). The blue box indicates the signature motif of glutaminase subunits ([G/A]LI[L/I/V]PGGEST[S/T/A]). The red boxes highlight the conserved catalytic triad sites (Glu-His-Cys). B, SMK2-GFP or GFP-SMK2 fusion protein was transiently expressed in tobacco leaf epidermal cells with ADH-RFP as a cytosolic marker. Fluorescent signals were observed under confocal laser-scanning microscopy at 36 h after infiltration. C, Western-blotting analysis of tobacco leaves transiently expressing GFP, SMK2-GFP, or GFP-SMK2 with anti-GFP antibody. Rubisco large subunit stained with Coomassie Blue serves as a loading control. DIC, differential interference contrast. Scale bars = 10 $\mu$m.

### Vitamin $B_6$ Deficiency Arrests Embryogenesis But Has Reduced Effects on Endosperm Development

As wild-type *Smk2* transcripts were not detected in the embryo and endosperm of *smk2-1* mutants by reverse transcription (RT)-PCR analyses (Supplemental Fig. S3), *smk2-1* is probably a null allele. Hence, *smk2-1* was subjected to further analyses. To test whether the *smk* phenotype is associated with a vitamin $B_6$ deficiency, we measured the vitamin $B_6$ contents in the embryo and endosperm of the wild-type and *smk2-1* kernels by high-performance liquid chromatography (HPLC). The five $B_6$ vitamers were identified and quantified based on the standards (Supplemental Fig. S4). To further aid the identification and quantification of PMP and PLP, the embryo and endosperm extracts were treated with alkaline phosphatase, which converts PMP to PM and PLP to PL, respectively. As a result, the treatment caused a complete disappearance of the PMP and PLP peaks and increases in the PM and PL peaks (Supplemental Fig. S4). The decreased amounts of PMP and PLP were in a good agreement with the increased amount of PM and PL in both the embryo and endosperm. Noted in the standard injection is the much stronger emission signal of PL than that of PLP at 395 nm and such that the PL signal converted from PLP was amplified. The results showed that the content of total vitamin $B_6$ was drastically reduced in the *smk2-1* embryo and endosperm in comparison to the wild type (Fig. 4A). This reduction was found in the *smk2-1* mutants from multiple ears, confirming that the mutation in *Smk2* causes the vitamin $B_6$ deficiency. In wild-type kernels, PMP and PLP are the major $B_6$ vitamers, together accounting for about 90% of total vitamin $B_6$ in both embryos and endosperms at 18 DAP (Fig. 4B). PMP accounted for ~50% of vitamers in the embryo and 75% of vitamers in the endosperm (Fig. 4B). During seed development, PLP content increased in the embryo but remained relatively unchanged in the endosperm. In contrast, PMP levels increased dramatically in the endosperm from 6 to 18 DAP (Fig. 4B).

In the mutant, the level of PLP was comparable in embryo and endosperm (Fig. 4C). As in wild type, PMP accumulated to higher levels than PLP in the endosperm, although the levels of each vitamer are drastically reduced. These results suggest that embryogenesis is more sensitive to vitamin $B_6$ deficiency than endosperm development in maize.

### The Maize *Smk2* Partially Complements the Arabidopsis *pdx2.1* and the Yeast MML21 Mutant Phenotypes

The maize SMK2 shares a 66% amino acid identity with the Arabidopsis AtPDX2, which is implicated in vitamin $B_6$ biosynthesis (Tambasco-Studart et al., 2005). To address the functional relationship between these two genes, we tested whether *Smk2* could complement the Arabidopsis *pdx2* mutant. The Arabidopsis T-DNA insertion mutant *pdx2.1* (SALK_072168) was obtained from the Arabidopsis Biological Resource Center. Development analysis indicated that embryogenesis of the *pdx2.1* mutant was arrested at the globular stage, producing albino seeds (Supplemental Fig. S5; Tambasco-Studart et al., 2005). The mutant seeds could not germinate and were hence completely embryo lethal. When heterozygous *pdx2.1* plants were transformed with the maize *Smk2* driven by the CaMV 35S promoter, we were able to identify six viable plants that were homozygous for *pdx2.1*. All six plants were confirmed to contain a *Smk2* transgene that was expressed (Supplemental Fig. S6, A and B). While these plants

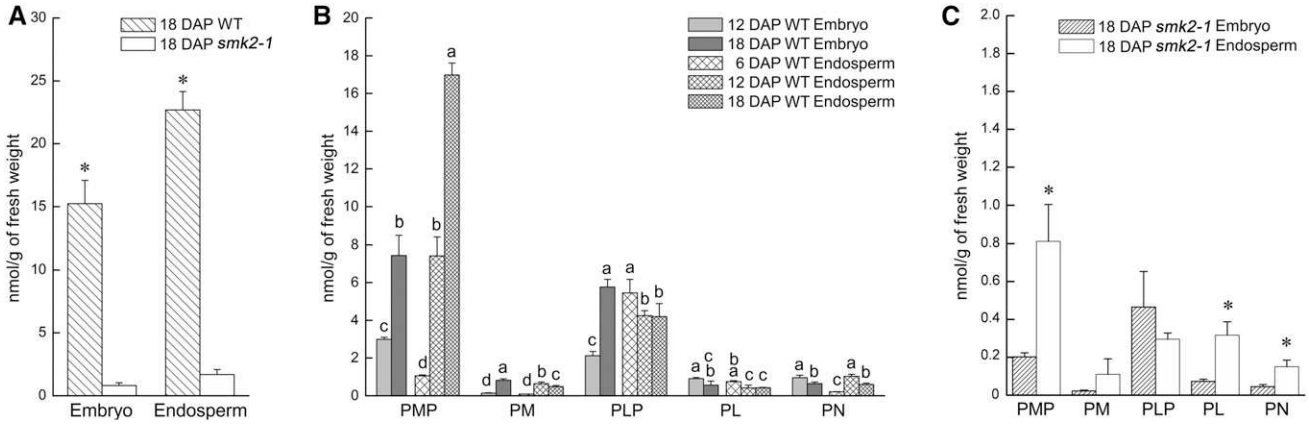


**Figure 4.** HPLC analysis of vitamin $B_6$ content in embryo and endosperm of wild-type (WT) and *smk2-1* kernels. A, Total vitamin $B_6$ content in embryo and endosperm of WT and *smk2-1* kernels. Total vitamin $B_6$ value represents the sum of five $B_6$ vitamers. B, $B_6$ vitamer levels in embryo and endosperm of WT kernels at different developmental stages. C, $B_6$ vitamer levels in embryo and endosperm of 18 DAP *smk2-1* kernels. Error bars represent the SE of three independent experiments. For each vitamer, different letters indicate significant differences, according to the Waller-Duncan *k*-ratio *t* test with $P = 0.05$. Asterisks represent significant differences ($P < 0.01$). PLP, Pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine.

were viable, they grew slower than the wild type and the seeds produced showed a high percentage of lethality (Fig. 5, A–D). Phenotype analysis of developing transgenic embryos revealed varying degrees of rescue ranging from partial to complete complementation (Supplemental Fig. S6, C–E). Thus, *Smk2* can only partially rescue the *pdx2.1* mutant. HPLC analysis indicated that vitamin $B_6$ levels in *Smk2* transgenic plants were about one-third of the wild-type level (Fig. 5I). We speculate that the partial rescue may be due to suboptimal function and/or expression of the *Smk2* transgene in the heterologous system. Partial rescues are common in complementation tests, as the transgene cannot completely mimic the endogenous gene expression.

We tested the possibility that partial rescue is due to incompatibility between maize SMK2 and Arabidopsis AtPDX1 subunits of the PLP synthase complex. PDX1 and PDX2 have to form a complex where PDX2 catalyzes the removal of the ammonia group from a Gln molecule and PDX1 transfers it to a specific substrate (Raschle et al., 2005). The activity requires proper channeling of the cell-damaging free ammonia from PDX2 to PDX1 without leakage; thus, accurate association between PDX1 and PDX2 is necessary. X-ray structural analysis of the PDX1:PDX2 complex showed that the N terminus of PDX1 interacts with PDX2 (Strohmeier et al., 2006), and interestingly the N-terminal sequences of maize PDX1s are divergent from the Arabidopsis PDX1 (Supplemental Fig. S7). For this reason, we created transgenic plants expressing *ZmPDX1.1* (GRMZM2G120652) and *Smk2* in the *pdx2.1* mutant in Arabidopsis. The transgenic lines were verified by PCR genotyping (Supplemental Fig. S6G). The *pdx2.1* plants coexpressing both maize genes showed a slight improvement in growth over the plants expressing only *Smk2*. However, growth was still slower than the wild type (Fig. 5, F–H). Consistent with the growth phenotype, HPLC analysis detected an increase in the total level of vitamin $B_6$ in these plants that was still lower than wild type (Fig. 5I). These results indicate that the cause of partial rescue of the *pdx2.1* mutant likely involves transgene expression as well as subunit compatibility. The CaMV 35S promoter confers relatively low expression during the early stages of embryogenesis (Sunilkumar et al., 2002).

As indicated in Figure 3A, SMK2 has a moderate similarity to SNO1 of *S. cerevisiae*, which encodes the glutaminase subunit of PLP synthase (Rodríguez-Navarro et al., 2002; Dong et al., 2004). To further explore the function of SMK2, we tested whether SMK2 could complement the *SNO1* knockout mutant in *S. cerevisiae*. Yeast has three *PDX2* genes, *SNO1*, *SNO2*, and *SNO3*, of which *SNO1* plays a predominant role in PLP biosynthesis (Rodríguez-Navarro et al., 2002). The MML21 mutant strain, which was generated by disrupting *SNO1* with the *KanMX4* cassette, showed slow growth in medium lacking vitamin $B_6$ (Rodríguez-Navarro et al., 2002). To test whether *Smk2* can rescue the *sno1* mutant, we placed *Smk2* into a yeast expression vector, pESC-HIS, which is under control of the GAL10 promoter. The *SNO1* gene cloned in the same vector was used as a positive control, and the empty vector was used as a negative control. When cultured in medium supplemented with pyridoxine, all strains grew equally well (Fig. 6A). When cultured in medium deficient of pyridoxine, however, the MML21 mutant strains transformed with *Smk2* grew faster than the strains with empty vector and slower than the strains with *SNO1* (Fig. 6, A and B). These results indicate that *Smk2* can partially rescue the pyridoxine auxotrophic phenotype of MML21 in yeast. Taken together, these results support the conclusion that SMK2 functions as the glutaminase subunit of PLP synthase in vitamin $B_6$ biosynthesis in maize.

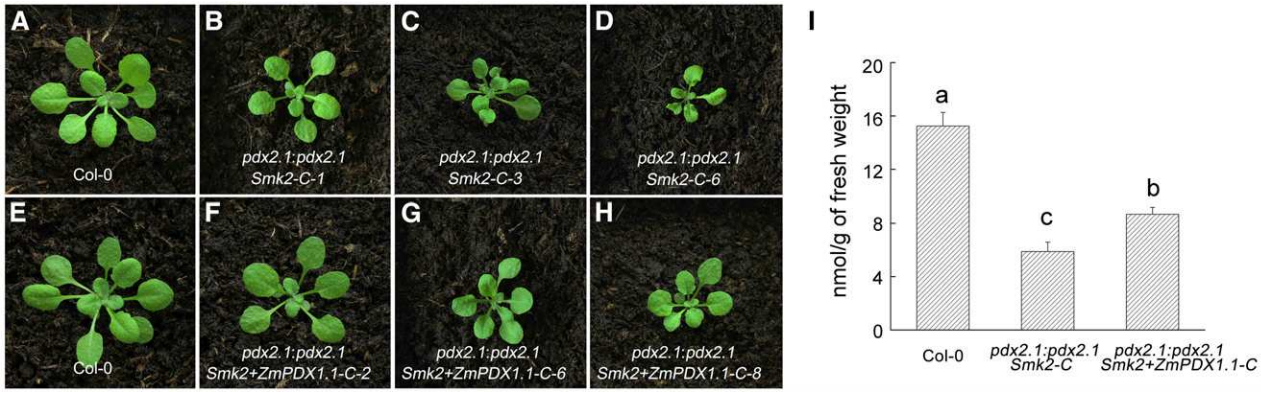


**Figure 5.** *Smk2* partially complements the embryo-lethal phenotype of Arabidopsis *pdx2.1* mutant. A to D, Three-week-old plants of Col-0 (A) and *pdx2.1* mutants carrying *Smk2* transgene (B–D). Different transgenic lines show different extents of restored growth of *pdx2.1* mutants. E to H, Three-week-old plants of Col-0 (E) and *pdx2.1* mutants carrying *Smk2* and *ZmPDX1.1* transgene (F–H). I, The total vitamin $B_6$ level of wild-type, *pdx2.1*/*Smk2*-C, and *pdx2.1*/*Smk2*+*ZmPDX1.1*-C plants. Error bars represent the SE of three independent experiments. Different letters indicate significant differences, according to the Waller-Duncan *k*-ratio *t* test with $P = 0.05$. C, Complementation.

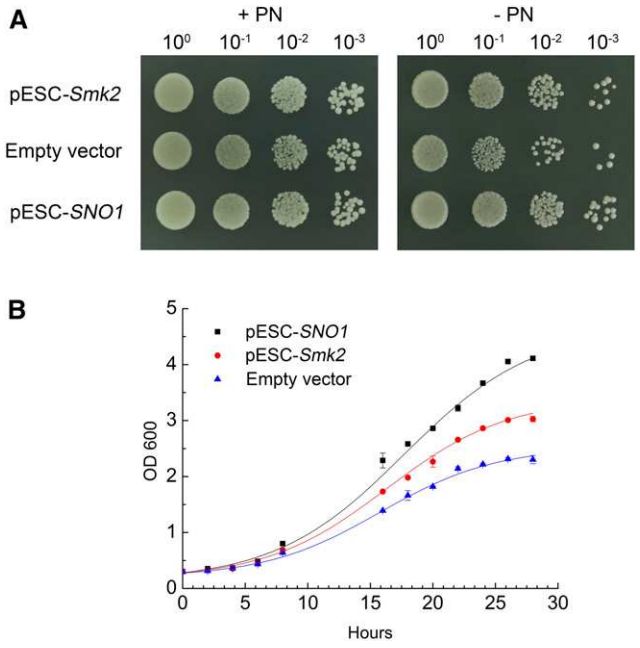


**Figure 6.** *Smk2* partially restores the pyridoxine auxotrophy of the yeast mutant MML21. A, Growth of yeast strains on plates with or without pyridoxine. The strain MML21 is a *SNO1* knockout mutant and shows slow growth in medium lacking vitamin $B_6$. MML21 was transformed with pESC-HIS-*SNO1*, pESC-HIS-*Smk2*, or empty pESC-HIS vector. The transformants were spotted on plates supplemented with or without pyridoxine. Each horizontal row represents a serial 10-fold dilution of each strain starting at an $OD_{600}$ of 0.5. B, Growth of yeast strains in liquid medium with or without pyridoxine. The strains grew in liquid medium starting at an $OD_{600}$ of 0.3. PN, pyridoxine. Values represent the mean OD values from three independent experiments. Error bars represent SE.

### Vitamin $B_6$ Rescues the *smk2-1* Embryo-Lethal Phenotype

To further test whether the arrested embryogenesis is due to vitamin $B_6$ deficiency, we attempted to rescue the embryo-lethal *smk2-1* mutants by applying vitamin $B_6$ to plants. Ears from self-pollinated *Smk2-1* heterozygotes were sprayed with 2 mM vitamin $B_6$ (PN) daily after pollination. After 18 d, the unsprayed ears showed clear segregation of *smk* kernels as shown in Figure 1A, whereas the treated ears did not. Dissection indicated that mutant embryos from treated ears developed coleoptilar, SC, and SAM structures (Fig. 7B), whereas mutant embryos from untreated ears were uniformly blocked at the early transition stage (Fig. 7A). Notably, the structures of coleoptilar, SC, and SAM in the treated mutant embryos were not identical to those of wild-type siblings, suggesting that application of vitamin $B_6$ in plants can only partially rescue mutant embryogenesis. When the untreated mutant embryos were cultured on medium without vitamin $B_6$, they did not grow after 20 d in culture (Fig. 7C). By contrast, when the treated *smk2-1* embryos (18 DAP) were cultured on medium containing vitamin $B_6$, they could grow and develop into seedlings (Fig. 7D). These rescued seedlings were confirmed to be homozygous for *smk2-1* by PCR genotyping, and no expression of the wild-type *Smk2* transcripts was detected by RT-PCR (Fig. 7, F and G). When the rescued *smk2-1* seedlings were transplanted to soil and continuously watered with vitamin $B_6$, they grew slowly and died at the five-leaf stage. These results indicate that exogenous application of vitamin $B_6$ partially rescues the arrested embryogenesis of the *smk2-1* mutant. However, watering the *Smk2-1* heterozygous plants with vitamin $B_6$ did not rescue the *smk* kernels, suggesting that the capacity of transporting vitamin $B_6$ from maternal tissues to developing kernels is limited.

### Spatial and Temporal Expression Pattern of *Smk2*

To determine the temporal expression of *Smk2*, total RNA from different tissues was subjected to regular and quantitative RT-PCR (qRT-PCR) analyses. *Smk2* expression was detected in all major plant organs (Fig. 8, A and B). Relatively high levels of expression were detected in root, stem, leaf, silk, and ear, while expression was low in developing kernels (Fig. 8, A and B). These results suggest that *Smk2* is not seed specific but rather functions in many tissues. This conclusion is consistent with the basic cellular function of vitamin $B_6$.

To gain further insights of *Smk2* expression during embryogenesis, we performed RNA in situ hybridization on sections of wild-type embryos at different developmental stages. *Smk2* antisense and sense probes were used (Fig. 8, C–H). *Smk2* mRNA signals were detected in 5 DAP early transition stage embryos (Fig. 8C). Expression was highest at the late transition stage (7 DAP; Fig. 8D) and then declined at the early coleoptile stage (9 DAP; Fig. 8E). At the leaf stage 1 (15 DAP), the signal appeared to spread over the whole embryo, and strong expression occurred in the SAM and in the epidermis of the SC (Fig. 8F). In 21 DAP embryos, expression was restricted to the top region of leaf primordia (Fig. 8G). As a negative control, the sense probe did not yield significant signals (Fig. 8H). These results suggest that embryogenesis requires high level of vitamin $B_6$ during the late transition stage, conforming to the observation that embryogenesis in *smk2-1* mutant is arrested at the early transition stage.

## DISCUSSION

In this work, we isolated and characterized the maize *smk2* mutant obtained by transposon tagging. Multiple pieces of evidence indicate that *Smk2* encodes the glutaminase subunit of the PLP synthase complex in vitamin $B_6$ biosynthesis. The evidence includes (1) the *smk2* mutants are vitamin $B_6$–deficient, and exogenous application of vitamin $B_6$ could partially rescue the embryo-lethal phenotype of the *smk2* mutant kernels (Fig. 7); (2) SMK2 shows significant similarity to PDX2 in Arabidopsis and SNO1 in yeast, which were previously characterized as glutaminase subunits (Fig. 3A);

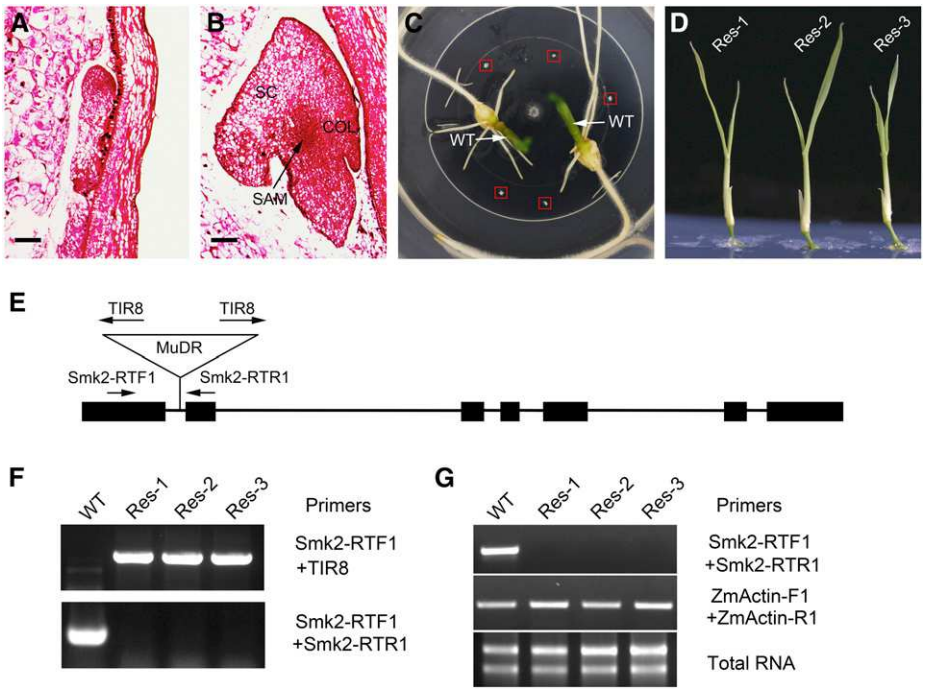


**Figure 7.** Rescue of the homozygous *smk2-1* mutant embryos by exogenous vitamin $B_6$ supplement. A and B, Histological section of *smk2-1* mutant (A) and rescued *smk2-1* (B) kernels at 18 DAP. C, The *smk2-1* and wild-type (WT) embryos cultured on the medium without vitamin $B_6$ supplementation. Arrows indicate the WT embryos grown as a control. Squares indicate *smk2-1* embryos. D, Rescued *smk2-1* embryos grown on the medium with vitamin $B_6$ for 20 d. E, Gene structure of *Smk2* and the location of *MuDR* insertion in the *smk2-1* allele. The positions of primers for RT-PCR and genotype analysis are shown. F and G, The genotype (F) and expression levels (G) of *Smk2* in WT and rescued plants. COL, coleoptile; Res, rescued *smk2-1* plant. Scale bars = 200 $\mu$m in A and B.

(3) the maize *Smk2* partially complemented the Arabidopsis *pdx2.1* and yeast MML21 mutants (Figs. 5 and 6). Furthermore, the SMK2 protein was localized to cytosol, indicating the compartment of vitamin $B_6$ biosynthesis (Fig. 3, B and C). Further biochemical and developmental analyses allowed us to dissect the differential roles of vitamin $B_6$ in embryogenesis and endosperm development in maize, an important monocot model crop.

### Vitamin $B_6$ Biosynthesis Is Essential to Embryogenesis But Less So to Endosperm Development

Loss of *Smk2* results in embryo lethality in maize, suggesting that vitamin $B_6$ is essential to embryogenesis. This essentiality is likely conserved in flowering plants, considering the cofactor function of vitamin $B_6$. In Arabidopsis, loss of *PDX2* reduces vitamin $B_6$ content and causes embryo lethality as well (Tambasco-Studart et al., 2005; Titiz et al., 2006). The maize *smk2-1* embryo is partially rescued by direct application of vitamin $B_6$ to developing ears. Continuous culture on vitamin $B_6$-rich medium allowed the partially rescued embryos to develop into seedlings. The biotin-deficient *bio2* mutant of Arabidopsis can be rescued by watering heterozygous plants with biotin (Patton et al., 1998). But this is not the case for vitamin $B_6$ mutants of Arabidopsis and maize. This observation implies (1) the translocation efficiency of vitamin $B_6$ from maternal tissues or neighboring seeds to developing mutant seeds is limited; (2) embryogenesis requires endogenous biosynthesis of vitamin $B_6$.

In contrast to the strong block during the transition stage of embryogenesis, the *smk2* mutation has less of an effect on endosperm development. The *smk2* mutant kernel can form an endosperm filled with starch, suggesting that vitamin $B_6$ is not as essential to the endosperm development as it is to embryogenesis. One possibility is that endosperm development may not rely on high levels of vitamin $B_6$ for its cofactor function. The Enzyme Commission (http://www.chem.qmul.ac.uk/iubmb/enzyme/) has cataloged over 170 PLP-dependent activities mainly involving in amino acid metabolism. Endosperm, as the storage organ mainly for starch and proteins, may be less dependent on these activities since amino acids and Suc are supplied maternally. Starch biosynthesis appears to not involve PLP-dependent enzymes. Starch biosynthesis requires a concerted action of ADP-Glc pyrophosphorylase, starch synthase, and starch-branching and debranching enzymes (Sabelli and Larkins, 2009). Based on the vitamin $B_6$ database (http://bioinformatics.unipr.it/cgi-bin/bioinformatics/B6db/home.pl; Percudani and Peracchi, 2003), these four enzymes are not PLP dependent. An alternative possibility is that the endosperm has priority access to maternal PLP over the embryo because the maize endosperm is positioned between the maternal vasculature and the embryo. Indeed, a 7% of wild-type level PLP was detected in the *smk2* mutant endosperms (Fig. 4), which may be near the threshold for normal endosperm metabolism. In that scenario, it is possible that the maternal vitamin $B_6$ is sufficient to maintain a basal level of amino acid metabolism in the *smk2* endosperm.

During the seed development, the endosperm maintains a roughly constant level of PLP but accumulates significantly high levels of PMP, reaching more than three times the amount of PLP at 18 DAP (Fig. 4B).

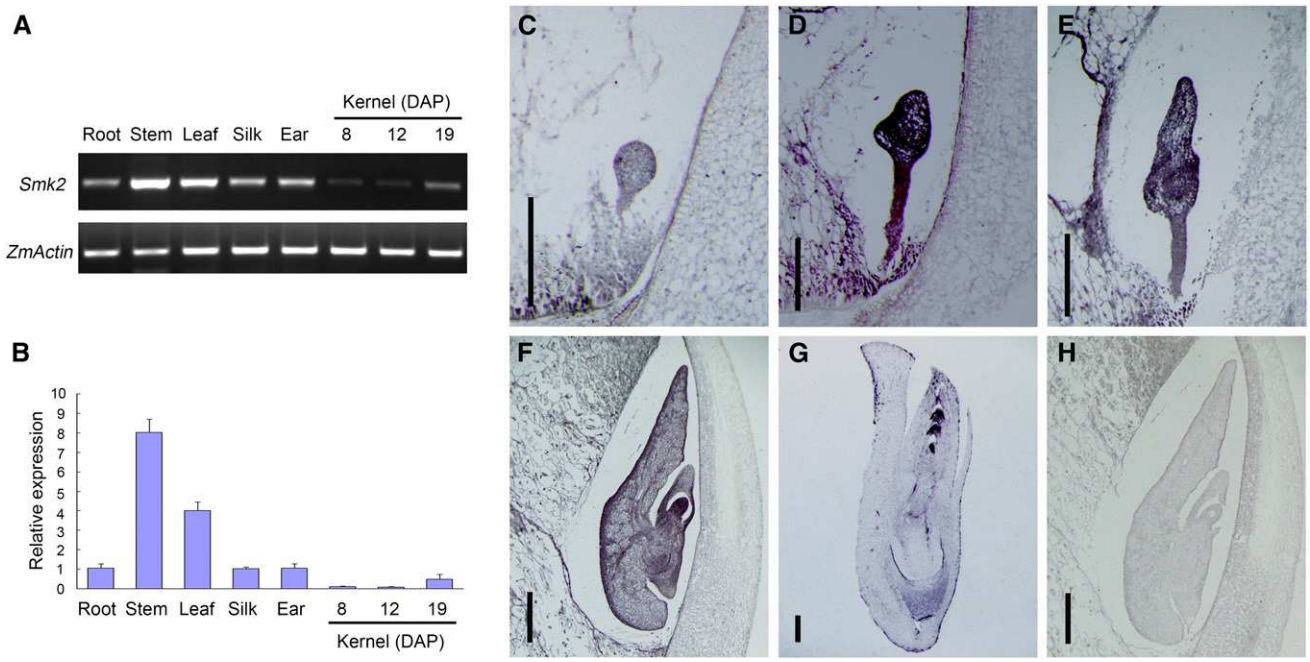


**Figure 8.** Spatial-temporal expression of *Smk2* during plant growth and seed development. A, RT-PCR analysis of *Smk2* expression in major tissues of maize and during seed development. *ZmActin* (GRMZM2G126010) was used as control. B, qRT-PCR determination of the expression levels in the tissues. *ZmActin* was used as an internal control to normalize RNA quantity, and the expression level of *Smk2* in silk was set to 1. Data represents three biological replicates, and each was measured three times. Error bars represent SE. C to H, RNA in situ hybridization of wild-type embryos at 5 (C), 7 (D), 9 (E), 15 (F and H), and 21 (G) DAP. The antisense probe of *Smk2* was hybridized in C to G, and the sense probe in H (refer to "Materials and Methods"). Scale bars = 500 $\mu$m.

PMP serves as a cofactor for only a few enzymes (Agnihotri and Liu, 2001; Adrover et al., 2009). And no evidence indicates that the enzymes involved in starch biosynthesis are PMP dependent (http://bioinformatics.unipr.it/cgi-bin/bioinformatics/B6db/home.pl). PMP can be oxidized by the PMP/PNP oxidase (PDX3) to form PLP. Thus, it is reasonable to propose that the accumulated PMP in endosperms is a storage form that can be converted to PLP during seed germination to nurture the seedling growth.

### Biosynthesis of Vitamin $B_6$ in Developing Seeds and Transportation from Maternal Tissues

Although the disruptive *Mu* insertions occur in various positions of the *Smk2* gene, the mutants are not completely vitamin $B_6$ deficient. A residual 7% of the wild-type vitamin $B_6$ level was consistently detected in the *smk2-1* mutants (Fig. 4). This low level of vitamin $B_6$ raises the question on how developing mutant kernels acquire essential vitamin $B_6$. This result invokes two possibilities: (1) The developing mutant kernels may still retain a limited vitamin $B_6$ biosynthesis capability, and/or 2) the mutant kernels can obtain vitamin $B_6$ from maternal tissues or neighboring wild-type siblings. In both cases, the amount of vitamin $B_6$ synthesized or transported is limited (Fig. 4) and not sufficient to support normal embryogenesis. The first possibility could be realized by either a leaky mutation in *smk2-1* or redundancy for *PDX2*; however, the evidence does not favor either scenario. First, the possibility that *smk2-1* is leaky is not supported by the data. We analyzed multiple independent *smk2* alleles that contain *Mu* insertions in different regions. In these alleles, the SMK2 proteins are predicted to be truncated to various lengths. All of the alleles show a typical *smk* phenotype. These results led us to rule out the possibility of leaky mutations. Secondly, we explored whether a *Smk2* homologous gene might code for glutaminase activity in the maize. BLAST analysis did not uncover a homolog with significant similarity in the maize B73 RefGen_v3 draft, rendering this possibility unlikely. The second possibility for residual vitamin $B_6$ in *smk2-1* mutant kernels is that a small amount of vitamin $B_6$ can be transported to developing kernels from maternal tissues. Although no vascular system exists between maternal tissues and developing kernels, small molecules such as vitamins (Patton et al., 1998), amino acids (Tegeder et al., 2000), hormones (Frey et al., 2004), and Suc (Weschke et al., 2000) are capable of being transported into filial tissue. In maize and several other tropical cereals, assimilates are delivered to the phloem terminals in the pedicel and then diffuse to endosperm transfer cells (Kladnik et al., 2004). The transport efficiency of different molecules varies. In maize kernels,

the efficiency of movement of the vitamins thiamine and nicotinic acid is significantly lower than that of Suc (Shimamoto and Nelson, 1981). Thus, import of vitamin $B_6$ from maternal tissue to mutant *smk2* kernels is likely insufficient to support embryo development. This notion is supported by our experiment where exogenous application of vitamin $B_6$ only caused a partial rescue of the embryo-lethal phenotype (Fig. 7B). This implies that the vitamin $B_6$ required for embryogenesis is synthesized within the developing embryo, whereas vitamin $B_6$ transport from maternal tissues to the embryo is minimal.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

The *smk2-1* allele was isolated from the UniformMu population (McCarty et al., 2005), and other alleles were identified from the Trait Utility System in Corn population by PCR with gene-specific primers (Bensen et al., 1995). The maize plants were grown under natural conditions. The *pdx2.1* T-DNA insertion line (SALK_072168) was obtained from the Arabidopsis Biological Resource Center. Arabidopsis (*Arabidopsis thaliana*) plants were grown under 16 h light/8 h darkness conditions at 22°C.

### Complementation of Arabidopsis *pdx2.1* Mutant

The full-length *Smk2* CDS was amplified by using primers SMK2-*Bgl*II-F and SMK2-*Bst*EII-R, which introduced *Bgl*II and *Bst*EII restriction sites. The resulting PCR product was cloned into pCAMBIA3301 downstream of the CaMV 35S promoter. *ZmPDX1.1* was introduced into pGWB2 vector. The constructs were introduced into *Agrobacterium* strain *EHA105*. The *PDX2.1/pdx2.1* heterozygous plants were transformed by using the floral-dip method (Clough and Bent, 1998). T1 transgenic plants with *Smk2* transgene were selected on BASTA-containing medium, and plants with both *Smk2* and *ZmPDX1.1* transgenes were selected on BASTA- and hygromycin-containing medium. Homozygous *pdx2.1* plants were identified by PCR with primers pdx2.1-F1 and pdx2.1-R1. Transgenic plants containing *Smk2* transgene were verified by PCR with primers SMK2-RTF2 and SMK2-RTR2, and those containing *ZmPDX1.1* transgene were verified by PCR with primers ZmPDX1.1-F1 and GWB2-R1. Information of all primers used in this study is listed in Supplemental Table S1.

### Yeast Complementation

*Saccharomyces cerevisiae* MML21, a knockout of the yeast *SNO1* gene, was obtained from Dr. Jose E. Perez-Ortin (University of Valencia, Spain). For the complementation analysis, the full-length *Smk2* CDS was amplified and ligated into the yeast expression vector pESC-HIS, which places *Smk2* expression under the control of the *GAL10* promoter. Transformation and screening of transformants were performed according to the protocol of Clontech (Takara). The transformants were cultured in synthetic dropout medium without vitamin $B_6$ for 30 h. And then, the starter culture was spotted onto solid medium or grown in liquid medium with optical density ($OD_{600}$) of 0.5 and 0.3, respectively.

### Rescue of *smk2-1* Kernels

The ears of self-pollinated *Smk2-1* heterozygous plants were sprayed daily with 2mM pyridoxine from the first DAP. At 18 DAP, the embryos were isolated from sprayed kernels and cultured on an enriched-vitamin solid medium, composed of Murashige and Skoog salts, 3% Suc, 100 mg/L myoinositol, 500 mg/L MES, 0.9% agar, 0.1 mg/L 1-naphthylacetic acid, 1 mg/L 6-benzylaminopurine, 1 mg/L biotin, 1 mg/L nicotinic acid, 1 mg/L thiamine, and 100 $\mu$M pyridoxine, adjusted to pH 5.7 with NaOH. As a control, *smk2-1* and wild-type embryos were isolated from unsprayed kernels and placed in a medium without pyridoxine. Each embryo cultured on enriched-vitamin solid medium was dripped with enriched-vitamin liquid medium every third day. After 20 d of embryo culture, rescued plants were transferred to soil and watered daily with 2 mM pyridoxine.

### Subcellular Localization of SMK2

Full-length *Smk2* CDS without stop codon was amplified by primers Smk2-F1 and Smk2-R1 from the maize inbred line B73 and cloned into the binary vector pGWB5 (for SMK2-GFP) by the GATEWAY in vitro site-specific recombination methodology (Invitrogen). Full-length *AtADH* CDS without stop codon was amplified by primers ADH-F1 and ADH-R1 and cloned into pH7RWG2 to generate the ADH-RFP construct (Karimi et al., 2002). To transiently express GFP-SMK2, full-length *Smk2* CDS was introduced into pGWB6 vector. The binary constructs were introduced into *Agrobacterium* strain EHA105. Leaves of 4-week-old tobacco were coinfiltrated with the strains carrying ADH-RFP and SMK2-GFP or GFP-SMK2, respectively. Fluorescent signals were detected with an Olympus FV1000-IX81 confocal microscope at 36 h after infiltration.

For western-blotting analysis of the SMK2-GFP and GFP-SMK2 protein, total protein was extracted from leaves at 36 h after infiltration, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis,and analyzed by western blotting with an anti-GFP antibody (Sigma).

### Light Microscopy of Cytological Sections

Wild-type and *smk2-1* kernels were harvested from the same ear of a self-crossed heterozygous plant at 18 DAP. The kernels were cut along the longitudinal axis in three equal parts. The central slice containing the embryo was fixed for 1 d at 4°C in 4% paraformaldehyde. The fixed materials were dehydrated in a graded ethanol series (30%, 50%, 75%, 95%, and 100%), infiltrated, and embedded in paraffin. The samples were sectioned at 8 to 10 $\mu$m with a microtome (Jung Biocut 2035, Leica), stained with Johansen's Safranin O, and observed with a Nikon ECLIPSE 80i microscope.

### RNA Extraction, RT-PCR, and qRT-PCR

Total RNA was isolated by using the Qiagen Plant RNeasy kit (Qiagen) according to the manufacturer's instructions and then treated with DNase I (New England BioLabs) to eliminate genomic DNA contamination. Complete removal of DNA was verified by performing PCR analysis on RNA samples before converting to cDNA. Reverse transcription reactions were performed using 1 $\mu$g of total RNA with random hexamers by SuperScript II Reverse Transcriptase (Invitrogen). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad). Relative quantification was carried out using the $2^{-\Delta\Delta CT}$ method. *ZmActin* was used as an internal control for RNA normalization. The expression level of *Smk2* in silk was set to 1. The RT-PCR was performed in three technical replicates. The *Smk2* cDNA was amplified with primers SMK2-CF1 and SMK2-CR1 from RNAs extracted from W22 leaves.

### DNA Extraction, Southern Analysis, and Construction, and Screening of Genomic Library

Genomic DNA extraction and Southern hybridization analysis were performed as described previously (Tan et al., 2011). To reduce the high active *Mu* copy number, *smk2-1* mutant was backcrossed twice with W22 and then self-pollinated to create an F2 segregating family. As homozygous *smk2-1* mutants are embryo lethal, only heterozygotes and wild-type seeds can germinate. The genotype of each F2 plant was determined by scoring the selfed ear for segregating (S) or nonsegregating (N) the *smk2-1* mutants. DNAs of the F2 plants were hybridized with different *Mu*-specific probes (Tan et al., 2011). Construction and screening of genomic libraries were carried out according to the method described previously (Tan et al., 1997).

### In Situ Hybridization

In situ hybridization was performed as previously described (Shen et al., 2013). The DIG-labeled sense and antisense probes were synthesized by T7 and SP6 polymerases, respectively. The probe used to detect *Smk2* transcripts corresponds to a 210-bp fragment from +29 to +238 bp of the *Smk2* cDNA.

### Vitamin $B_6$ Analysis by HPLC

Vitamin $B_6$ was extracted from maize embryos and endosperms and Arabidopsis rosette leaf tissue from *pdx2.1/Smk2*-OX, *pdx2.1/Smk2+ZmPDX1.1*-OX in Col-0 ecotype. To determine vitamin $B_6$ content, three independent maize

kernel samples were obtained from three different cobs, and three independent Arabidopsis lines were sampled using six leaves per line. Vitamin $B_6$ was extracted by 5% (w/v) trichloroacetic acid in darkness (González et al., 2007). HPLC analysis of $B_6$ vitamer levels was performed as described previously (Valls et al., 2001). For alkaline phosphatase treatment, the embryo and endosperm were ground into dry powder under liquid nitrogen. One-half milliliter of 0.05 M phosphate buffer (pH 7.5) was added to 0.625 g of dry powder and followed by 100 U of alkaline phosphatase. The mixture was incubated at 37°C for 1 h. Then 5% (w/v) trichloroacetic acid was added and the sample was mixed and analyzed. Standards PMP, PLP, pyridoxal hydrochloride, pyridoxine hydrochloride, and pyridoxamine dihydrochloride were purchased from Sigma. PNP is not available commercially.

## Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers SMK2 (AY109859), SNO1 (NP_013813), AtPDX2 (AT5G60540), YaaE (AKL87126), AtADH (AT1G77120), ZmPDX1.1 (NM_001147185), and ZmActin (NM_001155179).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Scheme of de novo and salvage vitamin $B_6$ biosynthetic pathways.

**Supplemental Figure S2.** The development of transfer cell layer at 18 DAP.

**Supplemental Figure S3.** The expression of *Smk2* transcript in embryo and endosperm of wild type and *smk2-1* mutant.

**Supplemental Figure S4.** Representative $B_6$ vitamer profiles of maize embryo and endosperm.

**Supplemental Figure S5.** Characterization of Arabidopsis *pdx2.1* mutants.

**Supplemental Figure S6.** Characterization of transgenic plants.

**Supplemental Figure S7.** Alignment of ZmPDX1s and AtPDX1s.

**Supplemental Table S1.** Primer information.

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