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Original Article

Arabidopsis inositol 1,3,4-trisphosphate 5/6 kinase 2 is required for seed coat development

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Inositol 1,3,4-trisphosphate 5/6 kinase (ITPK) phosphorylates inositol 1,3,4-trisphosphate to form inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4,6-tetrakisphosphate which can be finally transferred to inositol hexaphosphate (IP_6) and play important roles during plant growth and development. There are 4 putative ITPK members in *Arabidopsis*. Expression pattern analysis showed that *ITPK2* is constitutively expressed in various tissues. A T-DNA knockout mutant of *ITPK2* was identified and scanning electron microscopy (SEM) analysis showed that the epidermis structure of seed coat was irregularly formed in seeds of *itpk2-1* mutant, resulting in the increased permeability of seed coat to tetrazolium salts. Further analysis by gas chromatography coupled with mass spectrometry of lipid polyester monomers in cell wall confirmed a dramatic decrease in composition of suberin and cutin, which relate to the permeability of seed coat and the formation of which is accompanied with seed coat development. These results indicate that ITPK2 plays an essential role in seed coat development and lipid polyester barrier formation.

Keywords *Arabidopsis thaliana*; inositol 1,3,4-trisphosphate 5/6 kinase (ITPK); seed coat; suberin; cutin

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Introduction

Inositol polyphosphate synthesis is very important for phosphate storage, nutrition utilization, and signal transduction in both animals and plants. In higher plants, studies have shown that inositol-1,2,4,5,6-pentakisphosphate (IP_5) is a cofactor of COI1 (CORONATINE INSENSITIVE 1) and is necessary for perception and transduction of jasmonic acid [1,2]; IP_6 (phytic acid) can chelate mineral cations such as calcium, iron, zinc, and potassium, and is an anti-nutrient factor in animals [3]. In addition, inositol polyphosphates are major storage compound of phosphorus in plant seeds. During seed germination, inositol polyphosphates are metabolized to supply the phosphate and inositol for growing seedlings, and the status of inositol polyphosphates and relevant kinases are important for normal seed development. In soybean, silencing of myo-inositol-1-phosphate synthase gene 1 (*MIPS1*) results in decreased IP_6 content and suppressed seed development (much smaller seeds in which development stops at 2–3 mm size) [4]. Similarly, loss of *Arabidopsis AtMIPS1* results in smaller plants with curly leaves and wrinkled seeds [5].

Synthesis of inositol polyphosphate involves a series of kinases. Inositol 1,3,4-trisphosphate 5/6 kinase (ITPK) is a key enzyme that phosphorylates inositol 1,3,4-trisphosphate to form inositol 1,3,4,5-tetrakisphosphate or inositol 1,3,4,6-tetrakisphosphate. In some cases, ITPK can also phosphorylate inositol 3,4,5,6-tetrakisphosphate to form inositol 1,3,4,5,6-pentakisphosphate [6]. ITPK belongs to a larger family of ATP-grasp fold protein, which can bind ATP in a cleft formed between the β sheets of the central and C-terminal domain [7,8]. In addition to the kinase activity against inositol polyphosphates, ITPKs can also phosphorylate transcription factors, c-Jun and ATF-2 [9], as a serine/threonine protein kinase. There are only a few reports on the ITPKs functions in plants. The OsITL1 (OsITPK1) inversely regulates plant responses to osmotic stress [10]. There are four ITPK members in *Arabidopsis*, and AtITPK1 is a protein kinase that involves in photomorphogenesis possibly *via* interaction with COP9 signalosome (CSN) under red light. The *itpk1* mutant exhibits short hypocotyl under red light, which is similar to that of *csn* mutant [11]. However, physiological functions of the rest of the ITPKs are still unknown.

The *Arabidopsis* seed is composed of an embryo, an endosperm (aleurone), and an external protective seed coat that absorbs water for embryo germination. The seed coat, which differentiates from cells of ovule integuments after fertilization, is the protective surface covering the embryo. The seed coat of immature seeds consists of two layers: outer two-layered integument and inner three-layered

integument [12,13]. At the beginning of seed maturation, the innermost layer synthesizes proanthocyanidin, which stains a brown color of seed coat. The other two inner layers cells are crushed to each other until the cells die. In addition, differentiation of outer integument layers determines the shape of seed surface.

During seed development, the starch-containing amyloplasts firstly accumulate at the center of cells of both outer integuments. Then the cells of outer layer synthesize and secrete a large quantity of mucilage between outer primary wall and protoplast in a ring surrounding the starch granules. Following mucilage synthesis, the starch granules are shaped into a column as a volcano in the center of cell, and the secondary cell wall is produced around the column and along the radial cell wall. The sub-epidermal layer produces a thickened wall as a palisade under the column. At final stage of seed development, the starch granules are degraded, and then the cells of seed coat layers are compressed together and dehydrated to die, resulting in the visible columella and the outer radial walls in mature seed coat as a well-regulated hexagonal volcano [13]. During seed coat development, the hydrophobic lipid polyesters layers, cutin and suberin, are synthesized from common C_{16} and C_{18} cellular fatty acids to form the barriers that protect the embryo from various stresses. The cuticle that covers the outer layer of seed protects embryo from pathogen infection and insects, and controls the gas exchange. The suberin that associates with the inner layer of seed coat forms a barrier to water and solute diffusion through cell walls, and relates to the permeability of seed coat. Formation of cuticle and suberin is accompanied with seed coat development [14,15].

Here, we reported a physiological study of *Arabidopsis ITPK2* which is expressed during seed development. Genetics studies showed that *itpk2-1* mutant has a normal embryo with distorted seed coat. Further studies revealed the content of cutin and suberin dramatically reduced in *itpk2-1* mutant, demonstrating that ITPK2 plays an important role in seed coat development.

Materials and methods

Plant material and growth conditions

All *Arabidopsis* lines used in this study were with Columbia-0 ecotype. Seeds were surface-sterilized with 20% bleach for 15 min and washed four times with sterile water, then sown on plates containing Murashige and Skoog mineral salts and 1% agar, and stratified at 4°C for two days. For phenotypic analysis and growth assays, the plants were grown at 22°C under 16-h-light/8-h-dark condition.

Identification of T-DNA insertions and complementation studies of *ITPK2*

The *itpk2-1* mutant (SAIL_25_H09) was obtained from *Arabidopsis* biological resource center (http://arbc.osu.edu/). The mutant carried a T-DNA insertion in the 7th intron and was confirmed by polymerase chain reaction (PCR) amplification using primers ITPK2-1 (5′-AGCAGCTAGTGCTGATGCAG-3′) and ITPK2-1 (5′-CAAAGACTTGCTCGTAGTCTGG-3′).

The *ITPK2* cDNA clone (U84276) was ordered from ABRC (*Arabidopsis* biological resource center, Columbus, USA). The entire cDNA fragment was digested by *Sfi*I and subcloned into pCAMBIA1301 to generate construct p35S:ITPK2 harboring *ITPK2* in the sense orientation. The construct was transformed into *itpk2-1* and obtained transformants were selected by hygromycin resistance. Transcription levels of *ITPK2* in *itpk2-1* mutant were confirmed by quantitative real-time PCR (qRT-PCR) analysis.

DNA gel blot analysis

The BASTA gene was labeled by PCR with primers BASTA-1 (5′-ATGAGCCCAGAACGACGCC-3′) and BASTA-2 (5′-ATATCCGAGCGCCTCGTG-3′) according to the manufacturer's instruction (Roche, Basel, Switzerland). Forty micrograms of *Arabidopsis* genomic DNA were digested with different restriction enzymes (*Eco*RI, *Hin*dIII, and *Xba*I) and separated in 1% agarose gel. After transmembrane cross-linking, hybridization, washing, blocking, and antibody incubation, the signal was detected with disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(59-chloro)tricycle [3.3.1.13,7]decan}-4-yl) phenyl phosphate (CSPD; Roche).

qRT-PCR analysis

qRT-PCR analyses were performed to study the expression pattern of *AtITPKs* in various tissues and to examine the *ITPK2* expression in transgenic lines, or the transcription of *AtITPKs* under ABA treatment or phosphorus deficiency. The RNAs were extracted from various tissues and seedlings grown under ABA treatment or phosphorus deficiency by using TRIzol reagent (Invitrogen, Carlsbad, USA). Obtained RNAs were incubated with DNAase (TaKaRa, Dalian, China) and then reverse-transcribed (Toyobo, Osaka, Japan). The resultant first-strand DNA was used for qRT-PCR analysis by using the SYBR Green qRCR kit (Toyobo) with the RotorGene 3000 system (Corbett Research, Sydney, Australia). The primers used were ITPK1 (5′-TAGGGATGCCAAAGATGCTAATA-3′ and 5′-GTCCCAGAAGAACTCAGTCAACA-3′); ITPK2 (5′-CAAGGTATTTGTGGTGGGTGAT-3′ and 5′-GAGGGTCCAAGTCTGCGTTAT-3′); ITPK3 (5′-ATCGTCGCCGTGTTCGTTAGT-3′ and 5′-AAACGGACCCTGCTCTGAAAGT-3′); ITPK4 (5′-GCCATCTCGGGTAGAGGACTTT-3′ and 5′-AGCAGTTCAGTTCA

ATGGACAAGA-3′); and TIR1 (5′-GCTAGGAACCGTCC CAAC-3′ and 5′-TCCCAATGTATTCAAAAA-3′).

Promoter–GUS fusion study and histochemical analysis of GUS activity

The ~1.9-kb promoter region of *ITPK2* was amplified by PCR with primers ITPK2-5 (5′-GCTCTAGAGCCTAA CAAAAAGGAGCATA-3′, added *Xba*I site underlined) and ITPK2-6 (5′-TCCCCCGGGAGAAGAAGCAAAGGG AGAAT-3′, added *Sma*I site underlined) using *Arabidopsis* genomic DNA as template. The amplified DNA fragment was subcloned into the modified pCAMBIA1300 + pBI101.1 [16] and the resultant construct was transformed into *Arabidopsis* and *Agrobacterium tumefaciens*. Positive transgenic plants were selected by hygromycin resistance screening, and independent lines of T2 and T3 homozygous progeny were used to detect the GUS activity.

SEM analysis

Mature seeds were dried with a silica gel self indicator and fixed on SEM sample holders with a carbon conductive tab. After a gold-palladium coating within a cool sputter coater SCD 050, the samples were observed by a JSM-B360LV scanning electron microscope (JEOL, Tokyo, Japan).

Seed staining and mucilage content determination

Ruthenium red (Sigma, St Louis, USA) was dissolved in water (0.03%, w/v). Seeds were imbedded in ruthenium red solution for 30 min at 25°C, and then observed under a light microscope [17].

To quantify the ammonium-oxalate soluble mucilage content, 50 mg of dry seeds were incubated in 0.2% (w/v) ammonium oxalate solution at 37°C for 8 h (with vigorous shaking for 2 min every hour). After centrifugation, the supernatant was transferred to a new tube, and five volumes of ethanol were added. The tube was placed on ice for 5 min, and then centrifuged at 21,000 *g* for 30 min. The supernatant was then removed and the polysaccharides precipitate was washed with 70% ethanol, air-dried, and weighed [18]. Averages of three replicates and SE were calculated.

Tetrazolium salt uptake

Seeds (~50 mg) of WT and *itpk2-1* mutant were incubated in 500 μl 1% aqueous solution of 2,3,5-triphenyltetra zolium (TTC) at 30°C for 1, 12, 24, or 48 h in dark. After incubation, the sample was observed with a microscope.

To quantify the permeability of seed coat, the seeds were firstly incubated in TTC, washed twice with water, and then centrifuged at 10,000 *g* for 1 min. Seeds were resuspended in 1 ml of 95% ethanol and ground with mortar and pestle to extract formazans. The mixture was collected and the final volume was adjusted to 2 ml with 95% ethanol and then centrifuged at 15,000 *g* for 3 min. The supernatant was collected and the absorbance was measured at 485 nm [19]. Assay was repeated three times.

Analysis of seed lipid polyesters

For each replicate, mature seeds (200 mg) per sample were ground in liquid nitrogen with mortar and pestle before solvent extraction, and then the sample was immersed in 5 ml boiling isopropanol and heated for 10 min at 80°C. After cooling, the sample was shaken for 3 h at 300 rpm and then centrifuged at 800 *g* for 10 min. The insoluble residue was shaken overnight at 300 rpm with 5 ml isopropanol. After centrifuging for 10 min (800 *g*), the insoluble residue was shaken for 8 h with 5 ml chloroform/methanol (2:1, v/v) and then the residue was shaken overnight with 5 ml chloroform/methanol (1:2, v/v). The residue was air dried after centrifugation and ground into small particles, and then dilapidated with the following treatments: methanol (30 min), water (30 min), 2 M NaCl (1 h), water (30 min), methanol (30 min), chloroform/methanol 1:2 (v/v) (1 h to overnight), chloroform/methanol 2:1 (v/v) (1 h to overnight), and methanol (1 h). The material was dried at room temperature and the resultant dry residue was depolymerized using base-catalyzed methanolysis (15%, v/v, methyl acetate and 6% sodium methoxide in methanol) for 2 h (60°C). Methyl heptadecanoate was used as internal standards. The monomers were extracted with two volumes of dichloromethane and one volume of 0.9% (w/v) NaCl. After aqueous washing, the lower organic phase was dried with anhydrous sodium sulfate and evaporated to dryness under a gentle stream of nitrogen gas. The dry monomers were derivatized by sylilation and analyzed by GS-MS [14].

Measurement of cellulose and IP_6 contents

Cellulose content was analyzed by colorimetry. Seeds (0.2 g) were broken to pieces by a tissue grinder (MM301; Retsch, Haan, Germany) and the seed powders were then digested in 100 ml of 60% H_2SO_4 for 30 min. The mixture was filtrated by bushner funnel, and 5 ml of filtered fluid was diluted to 100 ml by water. The diluted solution (2 ml) was transferred to a new test tube, and then 0.5 ml of 2% anthrone and 5 ml H_2SO_4 were added. The absorbance was measured at 620 nm after 12 min.

Content of IP_6 in seeds was also analyzed by colorimetry [20]. Seeds were broken into pieces and digested in the extraction buffer (1.2% HCl/10% Na_2SO_4) for 2 h. After incubation, the extraction solution was centrifuged at 4000 *g* for 30 min and equal volume of 15% TCA was added into the supernatant for 2 h on ice. The reaction solution was centrifuged at 4000 *g* for 30 min and the supernatant was transferred into a new test tube to adjust pH to 6.0–6.5 by using 0.75 M NaOH. Finally, 0.3% 5-sulfosalicylic acid

dehydrate/0.03% $FeCl_3 \cdot 6H_2O$ was added, and after gently mixing, the IP_6 content was determined by measuring the absorbance at 500 nm.

Measurement of α-amylase activity

The α-amylase digests starch to generate reducing sugars such as maltose or dextrin, which can then reduce 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The reduced compound has a brownish red color, which can be quantified by measuring the absorbance at 540 nm. In addition, the α-amylase activity positively correlates with the absorbance. The activity of α-amylase of 7 day's silique was analyzed by using plant-α-amylase activity assay kit (GMS50040.3; Genmed, Shanghai, China).

Statistical analysis

Data were presented as the mean ± SE. P value <0.05 was considered statistically significant.

Results

Expression pattern of ITPK2

To investigate the physiological function of *Arabidopsis* ITPKs, ITPK2, which comprises nine exons and eight introns and encodes a 391-amino acid protein, was selected. qRT-PCR analysis of the expression of *ITPKs* in different tissues showed that *ITPK2* is expressed in various tissues including seedling, root, flower, leaf, stem, and silique; however, its expression is relatively lower when compared with *ITPK3*, which was highly expressed in flower and leaf [**Fig. 1(A)**]. In addition, the expression of *ITPK4* is hardly detected and that of *ITPK1* was high in silique [**Fig. 1(A)**].

Analysis of the independent transgenic lines harboring promoter-reporter (GUS) genes showed that *ITPK2* was highly expressed in cotyledon and seedling root, rosette leaf, silique and seed [**Fig. 1(B)**]. Interestingly, *ITPK2* was expressed in the seed coat and detailed observation showed that *ITPK2* expression was relatively higher at early stage of seed development [**Fig. 1(C)**, two days after fertilization] and cannot be detected at Day 10, and that there was an intermediate level of expression at Days 3 and 4 after fertilization. This particular expression pattern implied that *ITPK2* might be involved in seed coat development.

ITPK2 deficiency results in enhanced seed coat corrodibility by bleach

To study the physiological function of ITPK2, a T-DNA insertion mutant was obtained from the SALK T-DNA collection [SAIL_25_H09, the T-DNA was inserted in the seventh intron of *ITPK2*, **Fig. 2(A)**] and designated as *itpk2-1*. Southern blot analysis confirmed the single insertion of T-DNA and qRT-PCR analysis confirmed the deficient *ITPK2* expression in the homozygous mutant [**Fig. 2(B)**].

Observation of the homozygous *itpk2-1* line indicated that there is no obvious phenotype in the developmental processes. Interestingly, after surface-sterilization with 20% bleach for 15 min, the seed coat of *itpk2-1* mutant was corroded, which was hardly observed in WT seeds [**Fig. 2(C)**]. Considering the oxidation ability of bleach, the seed coat of *itpk2-1* is relatively easy to be oxidized. The 'Karst Cave' on the surface of *itpk2-1* seed coat suggested that the structure or composition of *itpk2-1* seed coat is different from those of WT (not sensitive to oxidant).

Complementation studies by transforming IPTK2 (p35S:ITPK2) into *itpk2-1* mutant rescued the *ITPK2* expression [**Fig. 2(B)**] and recovered the oxidized seed coat of *itpk2-1* mutant by bleach [**Fig. 2(D)**], confirming the role of *ITPK2* in seed coat development.

ITPK2 deficiency results in the distorted seed coat and crumpled columellas

To gain further insights into the structure of seed coat, SEM analysis was performed and results showed that the morphology of epidermal cells of mature seed coat in *itpk2-1* mutant was severely distorted, compared with the uniform cell shape of WT seeds which are hexagonal, with a columella at the center surrounded with thick radial cell wall [**Fig. 3(A)**]. In the mature seeds of *itpk2-1* mutant, the radial cell wall of seed coat was snatchier [**Fig. 3(A)**], suggesting that collapse of cell wall resulted in an indistinguishable border between neighboring cells and appearance of cell fusion.

As the uniform epidermal cell of mature seeds of *itpk2-1* mutants and distorted seed coat of WT were also observed by SEM analysis, the percentage of distorted seed coat was thus calculated. The results showed that ~78% mutant seeds were with distorted surface of seed coat, while only ~28.6% in WT seeds (**Table 1**), confirming that ITPK2 deficiency resulted in distorted seed coat of mature seeds.

Previous studies showed that ITPK2 is an enzyme which is related to the synthesis of IP_6, a cofactor of TIR1. However, analysis of seed coat in *itpk3* (*ITPK3* is the closest homologous gene of *ITPK2*), *ip2k* (IP_6-deficient mutant) [21,22], *tir1* (deficiency of auxin receptor TIR1) and *axr2-1* mutants (auxin signaling mutant that is insensitive to auxin) showed that *tir1* and *axr2-1* mutants had high percentage of distorted seed coat, similar to that of *itpk2-1*. In addition, other IP_6-related mutants, such as *itpk3* and *ip2k*, did not show any difference compared with WT seeds (**Table 1**). Further analysis of the IP_6 content in mature seeds showed that the content was decreased by ~40% in *ip2k* mutant, but only ~12% in *itpk2-1* mutant (**Table 2**), which was similar to that in a previous report [22]. These results indicated that ITPK2 is not a key enzyme of IP_6

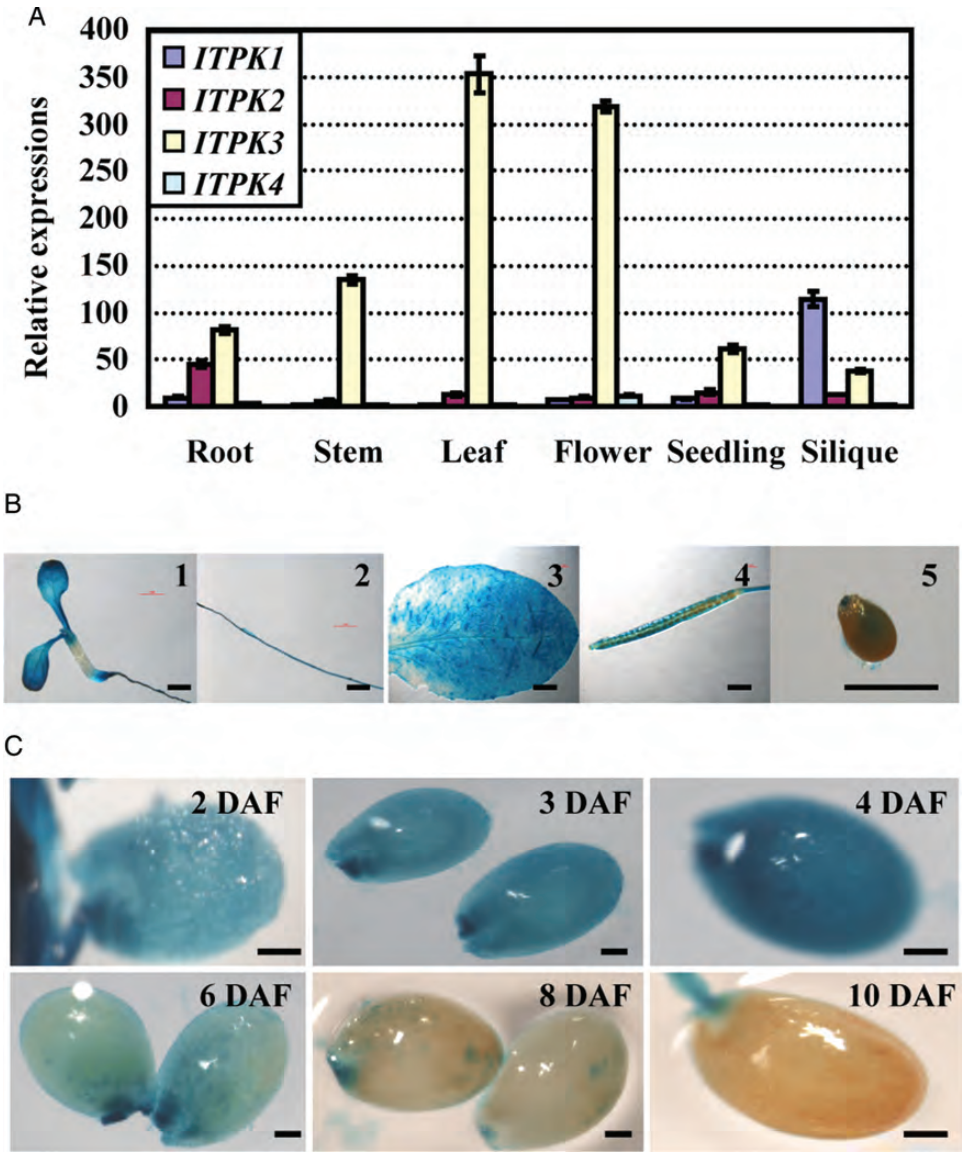


Figure 1 Expression pattern of *Arabidopsis ITPK2* (A) qRT-PCR analysis of *Arabidopsis ITPKs* expression in various tissues. The *ACTIN7* gene was used as a positive internal control and the transcript level of *ITPK4* in silique was set as 1.0. $n = 3$. (B) Promoter−GUS fusion studies revealed the expression of *ITPK2* in seedling (1), root (2), rosette leaf (3), silique (4), and seed (5) at 7 days after fertilization. Bar = 1 mm. (C) Promoter−GUS fusion studies revealed the expression of *ITPK2* during seed development at different days after fertilization (DAF). Bar = 0.1 mm.

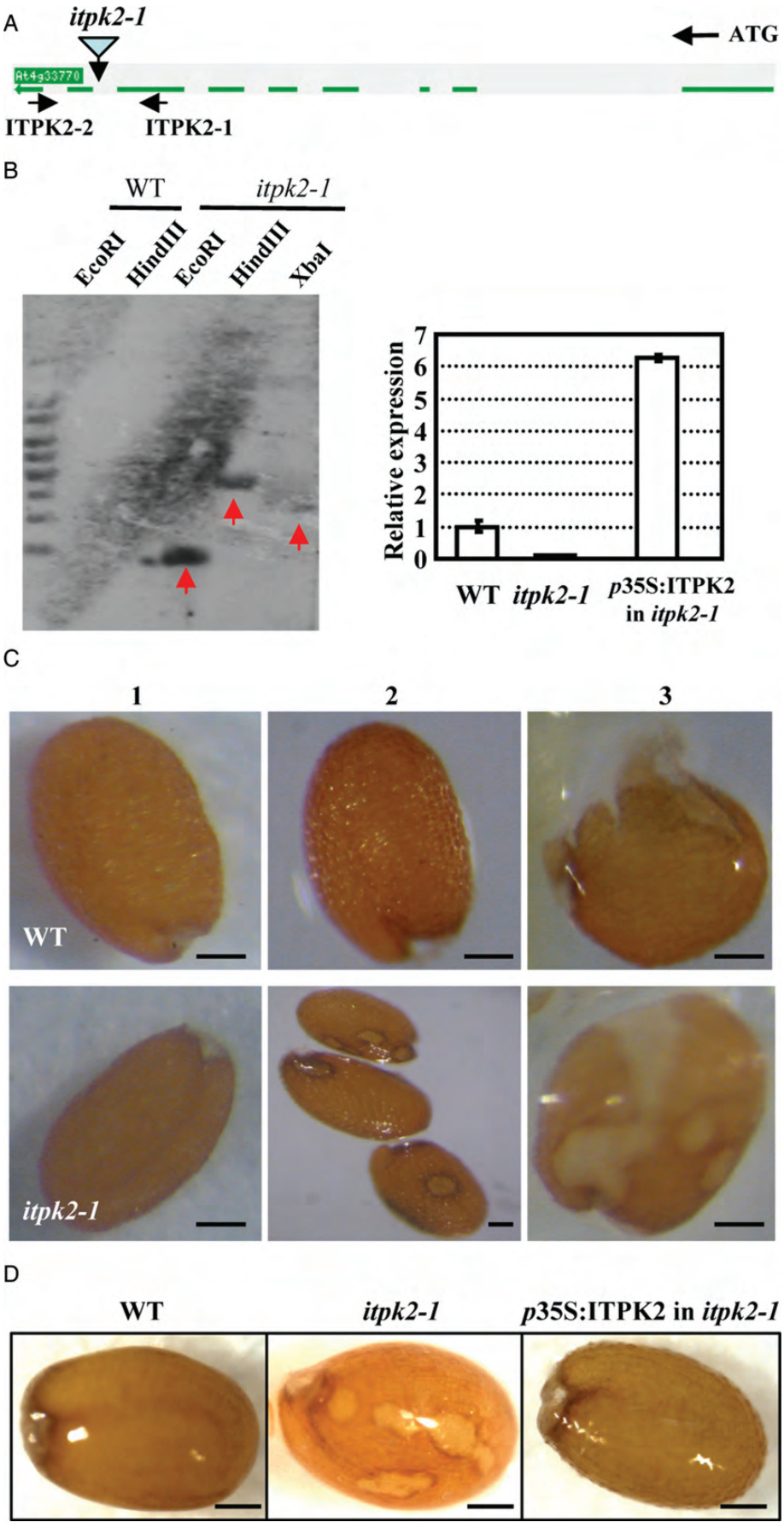
synthesis in *Arabidopsis* seeds and IP_6 deficiency is not the main reason for the defective seed coat development, and additionally, auxin may regulate the seed coat development.

SEM analysis indicated that the cell wall structure of seed coat is altered in *itpk2-1* mutant. We then analyzed the seed coat by I_2/KI-stained sections of silique at 5, 7 and 9 DPA (days post-anthesis). At 7 DPA, the starch granules are piled up to form a column in the center of the surface cells in WT, while the columellas are not observed in *itpk2-1* [**Fig. 3(B)**] and the starch granules seemed being degraded. These results suggested that defective expression of *ITPK2* mutant affects the degradation of starch granules and the frame of radial cell wall in seed coat, which led to the distorted seed coat of *itpk2-1* mutant.

Seed coat of *itpk2-1* mutant presents high permeability to TTC and has less mucilage

When contacting water, the seeds normally release the mucilage which is encircled in the central columella [23] and swells to form a gelatinous coating over the seed. Previous studies showed that released mucilage can be visualized by staining with ruthenium red [23,24]. Indeed, histological staining [**Fig. 4(A)**, left panel] and quantification [**Fig. 4(A)**, right panel] of mucilage indicated that the mucilage synthesis was decreased in *itpk2-1* mutant.

The regular seed coat forms a protective barrier to isolate embryo. Tetrazolium salts have been used to reflect the permeability of seed coat [25,26]. After incubation with TTC for 24 h, seeds of *itpk2-1* mutant showed perceptible red


A
itpk2-1
ATG
At4g33770
ITPK2-2
ITPK2-1
B
WT
itpk2-1
EcoRI
HindIII
EcoRI
HindIII
XbaI
Relative expression
7
6
5
4
3
2
1
0
WT
itpk2-1
p35S:ITPK2 in itpk2-1
C
1
2
3
WT
itpk2-1
D
WT
itpk2-1
p35S:ITPK2 in itpk2-1

staining, while those of WT seed and *tir1* mutant remained impermeable [**Fig. 4(B)**]. Further measurement showed a 10-fold increase of formazans in *itpk2-1* mutant seeds after incubation with TTC for 24 h [**Fig. 4(C)**], implying the reduced barrier of seed and protection of embryo.

Alcohol is an anti-germination reagent [27] and is toxic for plant growth. With the high permeation to stress, seeds of *itpk2-1* mutant were not able to germinate after being washed with 95% alcohol for 15 min, while WT seeds showed normal germination [**Fig. 4(D)**], which further suggested that seed coat of *itpk2-1* mutant was more permeable to water than the WT seed coat.

Reduced contents of cutin and suberin in seed coat of *itpk2-1* mutant

Based on the early degradation of starch granules of seed coat, activity of α-amylase, a main enzyme for starch degradation in seed [28], was analyzed. Results showed that the α-amylase activity in *itpk2-1* mutant was higher than that in WT seed [**Fig. 5(A)**], which might explain the less starch granules observed in silique of *itpk2-1* mutant.

Seed coat is the protective outer cover surrounding plant embryo. The two cell-layered integument of seed coat combines with cuticle, suberin and mucilage to form this barrier. In mature seeds, main constituent of these two layers is dead cell with thick cell wall. Analysis of cellulose content, which is a base part of cell wall [29], revealed no difference between WT seed and *itpk2-1* mutant [**Fig. 5(B)**], suggesting that the basic cell framework of seed coat in *itpk2-1* mutant is normal and does not lead to the high permeability to TTC.

Suberized cell walls and cuticles are highly hydrophobic barriers in seed coat [15]. To determine whether the cell wall-associated polyesters in the seed coat were affected, the composition of lipid polyester monomers arising from mature seeds of *itpk2-1* mutant and WT seed was analyzed by GC-MS. The results showed that the content of ω-hydroxy fatty acids including 24-Hydroxytetracosanoic acid and 18-hydroxyoctadecenoic acid was obviously reduced in *itpk2-1* mutant. In addition, the content of 1,ω-dicarboxylic acids (1,22-docosane dioic acid) and ferulate were also reduced [**Fig. 5(C)**]. On count of the C16 and C18 monomers that are major components of cutin in *Arabidopsis*, the cuticle in seed coat was observed by transmission electron microscopy [26,30]. In WT seeds, the electron-dense outer layer covered the seed coat, while this layer was not obvious in the seed coat of *itpk2-1* mutant [**Fig. 5(D)**]. Considering that C22 and C24 monomers combined with ferulate are major components of suberin [14], further examination of seed hilum region indicated that the accumulation of suberin was reduced in *itpk2-1* mutant [**Fig. 5(E)**]. These data implied that *ITPK2* deficiency resulted in altered polyester monomer composition and organization in seed coat.

ABA or phosphorus deficiency induces *ITPK2* expression

According to the data in GENEVESTIGOR (https://www.genevestigator.com/gv/), *ITPK2* was induced under phosphorus deficiency. qRT-PCR analysis confirmed that the *ITPK2* expression was significantly induced under phosphorus deficiency or ABA treatment [**Fig. 6(A)**]. Being consistent, *itpk2-1* mutant was more sensitive to ABA [**Fig. 6(B)**], implying that ITPK2 is involved in response to ABA response or phosphorus deficiency.

Discussion

There are four candidate ITPKs in *Arabidopsis* genome. ITPK1 was proved to be a protein kinase that is involved in photomorphogenesis [11]. ITPK4 was confirmed as an outlier to the AtITPKs. Functions of ITPK2 and ITPK3 are still unknown. Analysis showed that ITPK2 and ITPK3 share 54.4% sequence identity and 69.6% similarity by using Ins(1,4,6)P_3, Ins(3,4,6)P_3, and Ins(1,3,4)P_3 as substrates. In addition, ITPK2 and ITPK3 display unique Ins(3,4,5,6)P_4 1-kinase activity [6].

The null mutant of *ITPK2* did not result in significantly reduced IP_6 content when compared with that of *ip2k* mutant (IP_6-deficient mutant) (**Table 2**), which is consistent with previous studies [21]. *Arabidopsis ITPK4* is much more homologous to *EnITPK* and *HsITPK*, and phylogenetic analysis indicated that the function of *Arabidopsis* ITPK2 and ITPK3 is different from ITPK4. Previous studies showed that ITPK2 and ITPK3 use Ins(1,3,4)P_3, Ins(1,4,6)P_3, Ins(3,4,6)P_3, and Ins(3,4,5,6)P_4 as substrates, while ITPK4 uses Ins(1,3,4)P_3, Ins(1,4,6)P_3, Ins(3,4,6)P_3, and Ins(1,3,4,6)P_4 as substrates [6]. Considering the different kinase activity of ITPK4 and ITPK2 or ITPK3, it was unsurprising that the IP_6 content was distinct between these

Figure 2 Identification and phenotypic analysis of *ITPK2* knockout mutant (A) Schematic representation of the *ITPK2* gene. Position of T-DNA insertion, which is located in seventh intron, is highlighted. The primers ITPK2-1 and ITPK2-2 were used to confirm the T-DNA insertion. (B) Southern blot analysis confirmed the single copy of T-DNA in *itpk2-1* mutant (left panel). Arrows indicate the band after digested by different restriction enzymes. qRT-PCR analysis revealed the deficient *ITPK2* expression in the rosette leaves (20 DAG) of *itpk2-1* and recovered expression of *ITPK2* in *itpk2-1* mutant by transforming p35S:ITPK2 (right panel). The *ACTIN7* gene was used as a positive internal control and the transcript level of *ITPK2* in WT was set as 1.0. $n = 3$. (C) Seed coat of *itpk2-1* was corroded after surface-sterilized with 20% bleach for 15 min. (1) Dry seed; (2) seed after surface sterilization; and (3) immediately germinated seed. Bar = 0.1 mm. (D) The phenotype of *itpk2-1* was rescued by complemented expression of *ITPK2*. Bar = 0.1 mm.

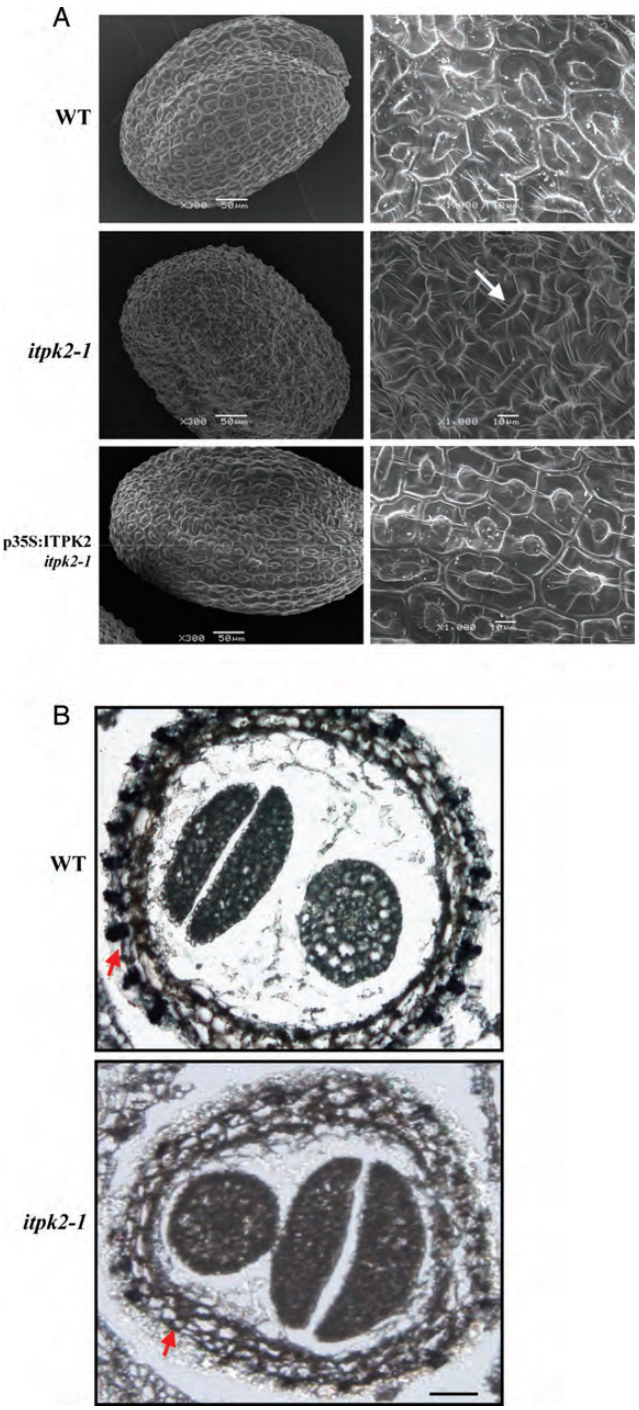


Figure 3 Seed coat of *itpk2-1* mutant is distorted and defective of the regular starch granules (A) Observation of the seed surface by SEM. Compared with the uniform cell shapes of WT seed, the seed coat with collapse and distorted radial cell wall was observed in *itpk2-1* mutant, which could be rescued by complemented expression of *ITPK2*. White arrow indicated the radial cell wall between two cells. Bars = 50 μm (left panel) or10 μm (right panel). (B) I2/KI-stained sections of seeds 7 days after fertilization. Compared with those of WT seed, the starch granules were not piled up to form a columella in *itpk2-1* mutant. Red arrows indicated the starch granules. Bars = 0.1 mm.

Table 1 Statistics of the distorted seed coat by SEM analysis

Group	Distorted	Hexagonal	Percentage
WT	48	120	28.6
itpk2-1 mutant	148	42	77.9
itpk3 mutant	62	120	34.1
ip2k mutant	69	102	40.4
tir1 mutant	144	43	77
axr2-1 mutant	156	28	84.8
p35S:ITPK2 in *itpk2-1* mutant	42	121	25.7

two clade genes. These results also suggested that Ins(3,4,5,6)P_4-1 kinase activity is important for the function of ITPK2 or ITPK3. As *ITPK3* was not expressed in seed coat and not induced by ABA or phosphorus deficiency [**Fig. 6(A)**], which suggested that ITPK3 plays a different role in *Arabidopsis* development.

IP_6 is a cofactor of auxin receptor TIR1 and may play a role in the signal transduction of auxin [31]. Indeed, the seed coat of the *tir1* mutant is also distorted. However, the IP_6 content in seeds of *itpk2-1* mutant is not reduced significantly and *ip2k* mutant (whose null mutant was confirmed with ~50% reduction of IP_6 content) did not show the altered shape of seed coat [22], and the seed permeability was same as that of WT seed, indicating that the distorted seed coat of *itpk2-1* mutant was not relate to TIR1 and its cofactor IP_6, and the phenotype of *itpk2-1* mutant was not caused by disturbed signal transduction of auxin.

The distorted seed coat of *tir1* and *axr2-1* mutants suggested that auxin plays an essential role in seed coat development of *Arabidopsis*. Auxin induces the activity of α-amylase in pea cotyledon [32] and the degradation of starch granules in seed coat is important for seed coat structure arrangement [28]. Therefore, the disarrangement of the seed coat surface structure in *tir1* and *axr2-1* mutants might be resulted from the repressed activity of α-amylase due to the defective auxin signal transduction.

Starch granules are degraded earlier in seeds of *itpk2-1* mutant and the synthesis of mucilage surrounding the column is deficient to fill the space between the outer primary wall and column, resulting in the distorted and irregular rank cell of seed coat. In some cellulose synthase mutant such as *cesa9* mutant, the radial cell wall of seed coat is thinner due to the reduced cellulose synthase [18]. In *itpk2-1* mutant, there is no difference of cellulose content of seeds [**Fig. 5(B)**], suggesting that the distorted seed coat with thin cell border is not caused by abnormal cellulose synthase.

ITPK2 expression was induced by ABA and *itpk2-1* mutant was more sensitive to ABA, suggesting that ITPK2

Table 2 Measurement of IP_6 content in mature seeds by colorimetry

Group	WT	*itpk2-1* mutant	*itpk3* mutant	*ip2k* mutant
IP_6 content (mg/g seed)	12.76 ± 1.02	11.21 ± 0.96	12.133 ± 0.79	7.368 ± 0.82

Data are presented as mean ± SD from two independent assays.

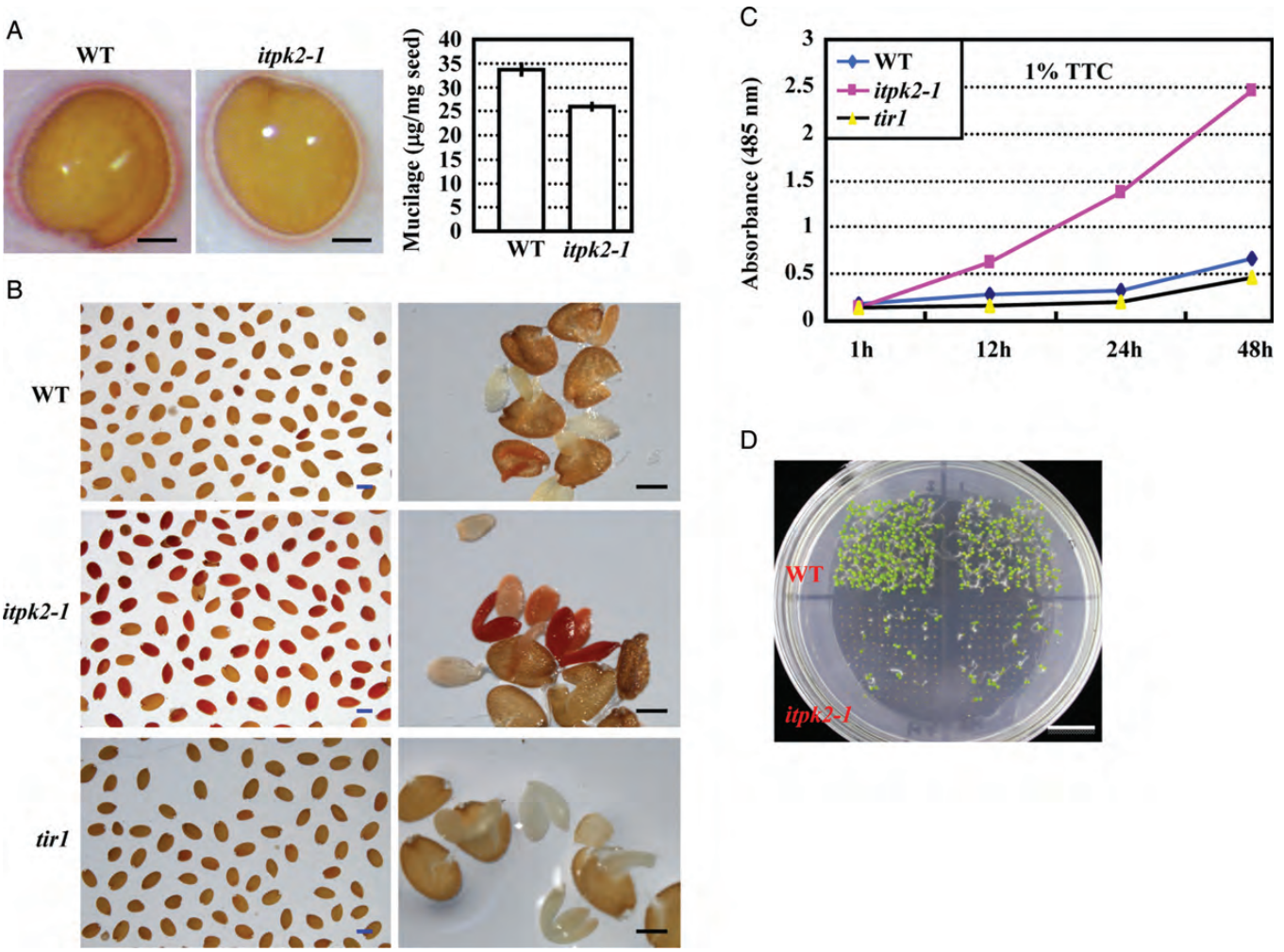


Figure 4 ***ITPK2*** **deficiency results in the reduced mucilage content and high permeability of seed coat to TTC** (A) Observation by ruthenium red-staining (light microscopy, leaf panel) and measurement revealed the reduced mucilage weight in *itpk2-1* (right panel). $n = 3$. (B) Observation of WT seed, *tir1* and *itpk2-1* mutant seeds by a microscope after incubation with tetrazolium salt for 24 h. The embryo of *itpk2-1* was stained red while that of WT and *tir1* was still normal. Bar = 0.2 mm. (C) Measurement of the formazans by absorbance at 485 nm revealed the altered permeability of *itpk2-1* seed coat. The TTC will be reduced to red-colored formazans by NADH-dependent reductase in embryo, which was measured after incubation for 1, 12, 24, or 48 h. Experiments are repeated two times. (D) *itpk2-1* mutant seeds failed to germinate after washed by 95% ethanol for 15 min. WT and *itpk2-1* mutant seeds were grown on Murashige and Skoog (MS) medium for 7 days.

plays an important role in ABA signaling. ABA is accumulated during seed maturation [33] and promotes the seed dehydration, which is the last step of seed coat maturation followed by the starch granules degradation and mucilage synthesis. In *itpk2-1* mutant, enhanced starch granules degradation and less mucilage synthesis demonstrate that the promotion of seed dehydration by ABA is possibly advanced. Reduced ABA in *itpk2-1* mutant at later stage in seed development may activate seed dehydration, which influence the final formation of surface cells in outer layers. The phosphorus is involved in many metabolic processes and provides energy for vital movement. As *ITPK2* expression is specially induced by phosphorus deficiency, it was suggested that ITPK2 may involve in phosphorus responses by mobilizing phosphorus storage *in vivo*. However, further studies are needed to prove this hypothesis.

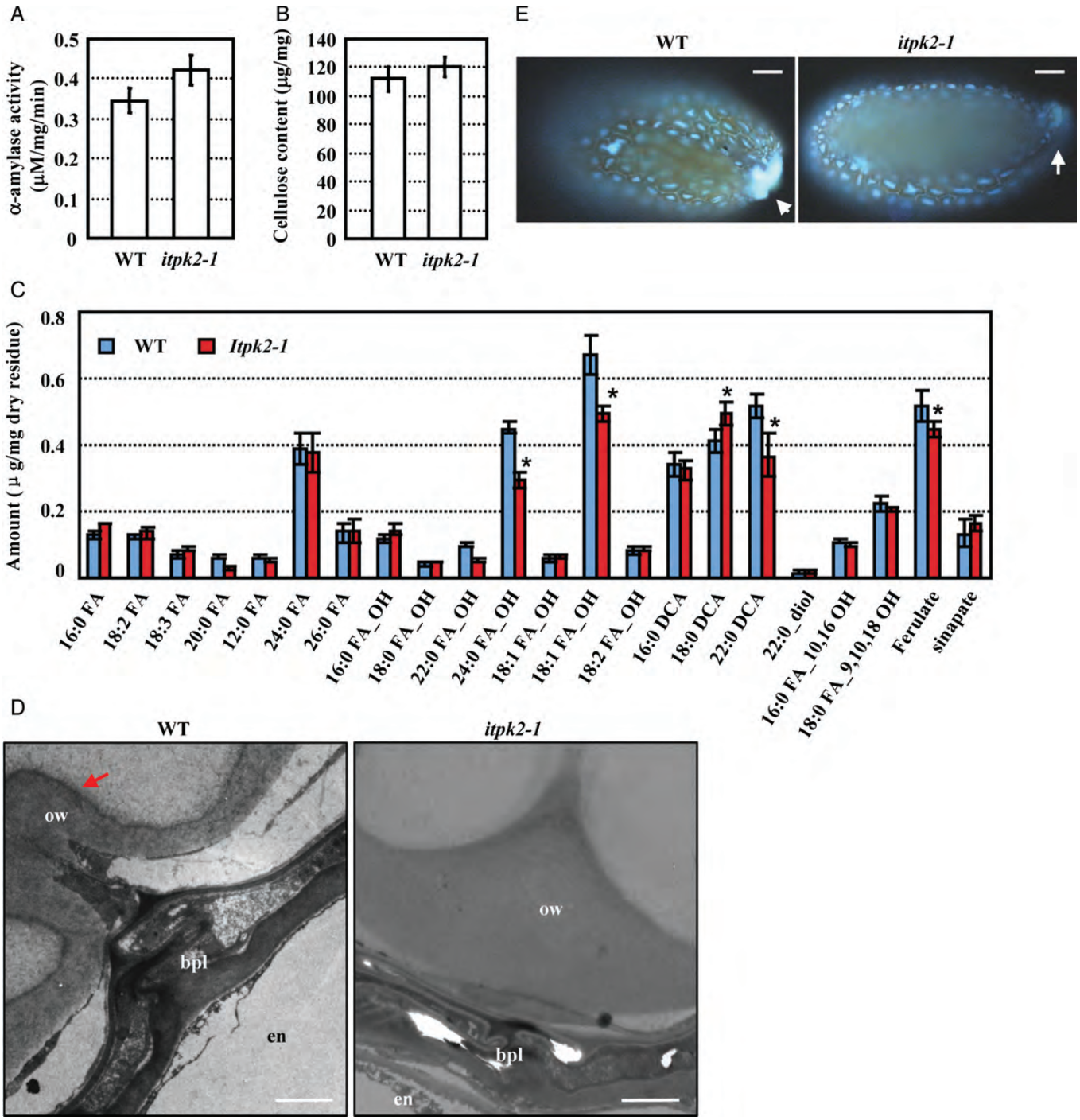


Figure 5 Analysis of the α-amylase activity and observation of seed coat by transmission electron microscope (TEM). (A) The activity of α-amylase is elevated in *itpk2-1* mutant siliques. $n = 3$. (B) Analysis of the cellulose content by colorimetry revealed the unaltered cellulose content. $n = 3$. (C) Polyester and aliphatic monomers composition of WT and *itpk2-1* mutant seed coat by GC/MS analysis from three independent assays. FA, unsubstituted fatty acids; FA-OH, ω-hydroxy fatty acids; DCAs, α, ω-hicarboxylic acids; diol, α-ω diol. (E) Observation of the cuticle in the outer layer of seed coat by TEM. Arrow indicated the cuticle. bpl, brown pigment layer; en, endosperm; ow, outer cell wall. Bar = 2 μm. (F) Analysis of the suberin auto-fluorescence in seeds by illumination under ultra vialet light (365 nm) revealed the reduced suberin accumulation around the hilum of *itpk2-1* seeds. Bar = 0.1 mm.

Cutin and suberin are the polymer matrices for cell wall barriers, which control the movement of gases, water and solutes and convey pathogen resistance, thus play crucial roles in protecting plants from biotic and abiotic stresses [15]. In addition to distorted seed coat, the composition of these lipid polyester monomers was altered under *ITPK2* deficiency, resulting in the high permeability to TTC and hypersensitivity to alcohol [**Fig. 4(B–D)**]. Therefore, the embryo of *itpk2-1* mutant lost the outer protection layer and was sensitive to environmental alteration.

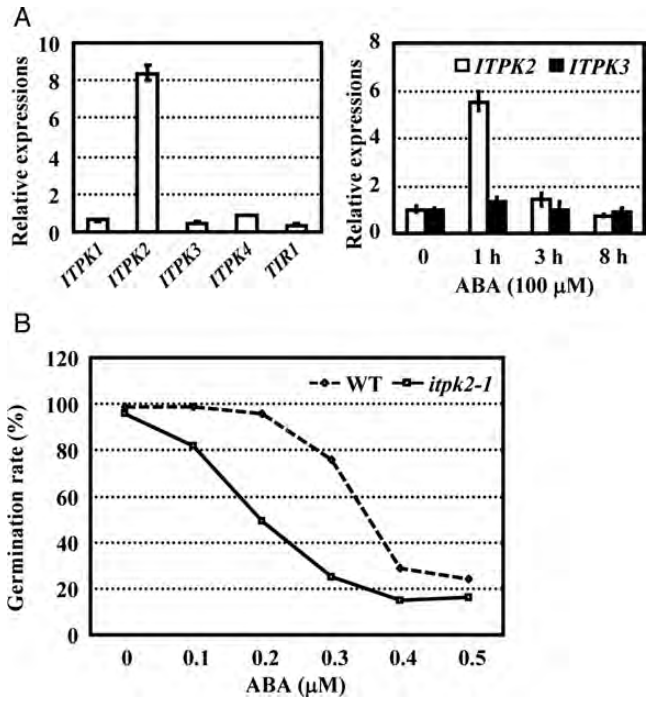


Figure 6 ***ITPK2*** **expression is induced by phosphorus deficiency or ABA** (A) qRT-PCR analysis of *ITPKs* expression revealed the induced expression of *ITPK2* by phosphorus deficiency (left panel) and ABA (right panel). The *ACTIN7* gene was used as a positive internal control and the transcript level of corresponding *ITPKs* with phosphorus or in the absence of ABA was set as 1.0. $n = 3$. (B) *itpk2-1* mutant was hypersensitive to ABA ($n = 200$).

Recently, some oxidoreductase and ω-hydroxy acid dehydrogenase encoding genes which were involved in the biosynthesis of cutin and suberin in *Arabidopsis* seed coat have been identified [15]. In addition, cellulose synthase (CESA9) and UDP-glucose:sterol glucosyltransferase are also involved in cutin formation and suberin biosynthesis [17,18]. These studies confirmed that C16 or C18 ω-hydroxy fatty acids and dicarboxylic acids polyester monomers are the main component of cutin, and C22 or C24 ω-hydroxy fatty acids and dicarboxylic acids polyester monomers are the dominant components of suberin in *Arabidopsis* seed and the content of ω-hydroxy fatty acids and dicarboxylic acids was reduced in the deficiency mutants of these genes. Decrease of dicarboxylic acids suggested that ITPK2 was involved in the lipid polyester biosynthesis and natural barrier formation. However, it is still unknown how ITPK2 affects the formation of structural lipids and there is no evidence that ITPK2 has relationships with genes mentioned above. In view of the reduced starch granules and mucilage, we hypothesized that the whole seed coat development is affected in *itpk2-1* mutant and that ITPK2 is necessary for providing energy to promote the synthesis of lipid polyester such as cutin and suberin.

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