The potassium channel FaTPK1 plays a critical role in fruit quality formation in strawberry (Fragaria × ananassa)

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Summary
Potassium (K⁺), an abundant cation in plant cells, is important in fruit development and plant resistance. However, how cellular K⁺ is directed by potassium channels in fruit development and quality formation of strawberry (Fragaria × ananassa) is not yet fully clear. Here, a two-pore K⁺ (TPK) channel gene in strawberry, FaTPK1, was cloned using reverse transcription–PCR. A green fluorescent protein subcellular localization analysis showed that FaTPK1 localized in the vacuole membrane. A transcription analysis indicated that the mRNA expression level of FaTPK1 increased rapidly and was maintained at a high level in ripened fruit, which was coupled with the fruit’s red colour development, suggesting that FaTPK1 is related to fruit quality formation. The down- and up-regulation of the FaTPK1 mRNA expression levels using RNA interference and overexpression, respectively, inhibited and promoted fruit ripening, respectively, as demonstrated by consistent changes in firmness and the contents of soluble sugars, anthocyanin and abscisic acid, as well as the transcript levels of ripening-regulated genes PG1 (polygalacturonase), GAL6 (beta-galactosidase), XYL2 (D-xylulose reductase), SUT1 (sucrose transporter), CHS (chalcone synthase) and CHI (chalcone flavanone isomerase). Additionally, the regulatory changes influenced fruit resistance to Botrytis cinerea. An isothermal calorimetry analysis showed that the Escherichia coli-expressed FaTPK1 recombinant protein could bind K⁺ with a binding constant of 2.1 × 10⁻⁹ M⁻¹ and a dissociation constant of 476 μM. Thus, the strawberry TPK1 is a ubiquitously expressed, tonoplast-localized two-pore potassium channel that plays important roles in fruit ripening and quality formation.

Introduction
Potassium (K⁺), an important and abundant cation in plants, is not only involved in anion neutralization, pH homeostasis, and mediating membrane electrical potential and cell osmotic pressure but also takes part in protein synthesis, cell metabolism and photosynthesis. Thus, a large quantity of K⁺ is essential and inevitable for plant growth and development, processes in which the cellular vacuoles play critical roles (Latz et al., 2007; Nieves-Cordones et al., 2016; Sharma et al., 2013). Although K⁺ also influences fruit yield and quality in many crops, such as grapevine, maize, wheat, soya bean and cotton (Conde et al., 2006; Pettigrew, 2008), how K⁺ is directed by K⁺ channels in the vacuoles of fleshy fruits is yet unknown.

In plants, K⁺ uptake and fluxes are mediated by several families of transporters and channels, including Shaker, two-pore K⁺ (TPK) channels and K⁺ inward rectifier-like channels (Lebaudy et al., 2007; Sharma et al., 2013). In plants, TPK proteins, as vacuolar K⁺ channels with two-pore domains, are localized to the vacuolar membrane and play pivotal roles in maintaining K⁺ homeostasis (Hedrich, 2012; Latz et al., 2007; Lebaudy et al., 2007). In the model plant Arabidopsis, there are six members of the K⁺ channels, five TPK (TPK1–TPK5) and a single K⁺ inward rectifier-like channel, that are located on the vacuolar membrane, except for TPK4, which localizes to the plasma membrane (Voelker et al., 2010). AtTPK1 mediates intracellular K⁺ homeostasis between cytoplasmic and vacuolar compartments, and is a voltage-independent, Ca²⁺/pH-activated K⁺ channel that functions in germination, seedling growth, stomatal closure and intracellular osmosensing through interactions with 14-3-3 proteins in Arabidopsis (Enyedi and Czirjak, 2010; Gobert et al., 2007; Latz et al., 2007; Maathuis, 2011). In contrast, the rice genome only encodes two ubiquitously expressed TPK isoforms, TPKa, which localizes predominantly to the large lytic vacuole, and TPKb, which localizes primarily to the small vacuoles (Isayenkov et al., 2011). TPKb can alter the K⁺ status of small vacuoles and is important for cellular K⁺ homeostasis in response to stress tolerance (Ahmad et al., 2016). In Nicotiana tabacum, NtTPK1 targets the tonoplasts in tobacco cells, exhibiting a strong selectivity for K⁺ over Na⁺, and a higher Ca²⁺ concentration or lower pH markedly increases NtTPK1-mediated K⁺ currents (Hamamoto et al., 2008). The vacuolar TPK channels regulated by Ca²⁺, 14-3-3 proteins and cytosolic pH, have been characterized only in a few plants.

K⁺ regulates in stomata opening in the guard cells (Allen and Sanders, 1995). The tonoplast AtTPK1 regulates stomatal conductance using K⁺ released from vacuoles (Gobert et al., 2007). The accumulation of K⁺ and sugars results in stomatal conductance with increasing water uptake (Shimazaki et al., 2007 and Talbott and Zeiger, 1998). The vacuolar K⁺ channel AtTPK1 plays a main role in ABA-dependent stomatal closure (Gobert et al., 2007).
An osmotic-driven enlargement of the vacuole promotes fleshy fruit development, which involves in cell division, cell expansion and fruit ripening (Ho, 1996). Thus, fruit quality is dependent on the stored compounds in the vacuole, including pigments, sugars, organic acids and other secondary metabolites (Martinoia et al., 2007). Because the TPK channels serve as vacuolar osmosensors (Maathuis, 2011), we postulated that they may play an important role in the regulation of fruit development, ripening and quality formation. To date, although K+ is important in fleshy fruit quality formation, to our knowledge, only the grapevine KAT-type K+ channels (Pratelli et al., 2002) and a role of FaKAT1 (an ABA-induced K+ channel gene) in strawberry fruit ripening have been reported (Song et al., 2017). The AtKAT1 is a downstream component in ABA signalling through an ABA-activated SnRK2.6 protein kinase (Sato et al., 2009). Because of the vital role of the vacuole in fleshy fruit development and quality formation, the vacuole membrane-localized TPK-type K+ channels need to be identified in fleshy fruits.

The fruit of strawberry (Fragaria ananassa) is an ideal model plant in the study of fruit development, especially the ripening of nonclimacteric fruit (Given et al., 1988; Li et al., 2011). To explore the roles of TPKs in fruit ripening and quality formation, a strawberry TPK gene, FaTPK1, was identified by RNA-sequencing and was cloned by reverse transcription-PCR (RT-PCR); its gene functions and protein attributes were then studied using physiological, molecular and biochemical analysis, including HPLC, virus-induced gene silencing (VIGS), intron-containing hairpin RNA (ihpRNA), prokaryotic expression and isothermal calorimetry (iTC). Our results demonstrated that FaTPK1 is a tonoplast-localized, fruit quality-regulating K+ channel.

Results

Changes of exterior morphology and physiological parameters in developmental strawberry fruit

Observed morphological processes of ‘Sweet charlie’ strawberry fruit (Chai et al., 2011; Jia et al., 2011) have been divided into seven developmental stages: small green (SG), big green (BG), de-green (DG), white (Wt), initial red (IR), partially red (PR) and fully red (FR) (Figure 1a). Distinct physiological changes also occur in the
developmental receptacles, with the anthocyanin contents sharply increasing (Figure 1b), and the total soluble sugar contents rapidly and continually increasing after the Wt stage (Figure 1c). The K⁺ contents, on the whole, showed a decreasing and oscillatory trend during the strawberry fruit development (Figure 1d).

**Transcriptome analysis and cloning of FaTPK1**

Based on transcriptome data (Zhao et al., 2017), RNA-seq (RNA-sequencing) technology has been performed using the four-stage (LG, WT, IR and PR) fruit around the onset of the strawberry fruit. At log2 gene expression levels, four TPK-like homologues were detected and divided into three groups, in which the highest expressing contigs (comp72579_c0_seq2 and comp72837_c0_seq1) were coupled with the fruit’s red colouring, and these were used as query in a BLAST algorithm-based search of the NCBI databases and a high homologous sequence (GenBank NO. for a query in a BLAST algorithm-based search of the NCBI databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). They matched the TPK channel 1-like protein (LOC101294428) in Fragaria vesca, and this was named FaTPK1 (Figure 2a).

To clone the FaTPK1 gene, the RNA-seq sequences were used for a query in a BLAST algorithm-based search of the NCBI databases and a high homologous sequence (GenBank NO. XM_004291514) was identified. Based on the cDNA length, including the coding sequence of FaTPK1, the full-length FaTPK1 gene was cloned using RT-PCR. A 1.041-bp sequence was gained and encoded a deduced 346 amino acids. The identity between FaTPK1 and FvTPK1 (GenBank NO. XM_004291514.2) is 99.42% (Figure S1), and the amino acid sequence similarity is the same. A search for both conserved domains using the NCBI website (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi) and transmembrane domains using the website Expasy (http://www.expasy.org/protteomics) revealed that FaTPK1 is a transmembrane protein with two ion_trans_2 conserved domains, which include a GYGD K⁺ selectivity motif (Figure 2b,c).

To further confirm transcripts of the gene in developing fruit, SqRT–PCR was used to investigate the six stages fruit. A similar expression trend for this gene was detected around onset of strawberry fruit ripening (Figure 2d), suggesting that the ripening of strawberry fruit was required for a certain stable expression level.

**Expression pattern and localization analysis of FaTPK1**

To explore whether FaTPK1 is involved in strawberry fruit ripening, the expression pattern of FaTPK1 over the seven developmental fruit stages was determined using transcriptome data and SqRT–PCR. During fruit enlargement and de-greening, FaTPK1 transcripts decreased slowly from the SG to DG stages, and reached the lowest level in the Wt stage. Then, with the red fruit colour development, FaTPK1 transcripts increased rapidly and reached their highest level in the FR stage (Figure 2d).

An enhanced green fluorescence protein (EGFP)–FaTPK1 fusion protein in onion epidermis cells revealed that the transiently expressed fusion protein was targeted to the tonoplasts (Figure 3), indicating that FaTPK1 was localized on the vacuole membrane.

**Functional analysis of FaTPK1 in fruit**

To investigate the role of FaTPK1 in strawberry fruit development, we generated VIGS fruit (Jia et al., 2011) ihpRNA fruit (Hoffmann et al., 2006), and overexpression (OE) fruits. *Agrobacterium tumefaciens* strain GV3101 cultures containing pTRV1 and pTRV2-FaTPK1 in a 1:1 ratio was infiltrated into DG fruits, and the control fruits were infiltrated with TRV empty vector alone.

Seven days after infiltration, the surface of the control fruits turned fully red, while, in contrast, the inoculated sector on the surface of the RNA interference (RNAi) fruits remained white (Figure 4a, TRV), which was accompanied by a decrease in FaTPK1 transcripts (Figure 4b,c, TRV).

An ihpRNA construct of FaTPK1 using a 650-bp FaTPK1 gene was cloned into pFGC5941 vector. The RNAi transient expression of FaTPK1 was made by injecting the *Agrobacterium tumefaciens* strain GV3101 carrying FaTPK1-RNAi into the DG fruits. The injection of empty pFGC5941 vector was adopted as control. The down-regulation of FaTPK1 expression (Figure 4b,c, hairpin) inhibited the FaTPK1-RNAi fruit’s red colour development (Figure 4a, hairpin).

A FaTPK1-OE construct was created by cloning the coding sequence of FaTPK1 into pCAMBIA1304 vector at the KpnI and EcoRI restriction sites. Expression of the target gene is controlled through a CaMV 35S promoter. After injection of the FaTPK1-OE construct and the control vector of empty pCAMBIA1304 into the DG fruits, respectively. The mRNA expression levels of FaTPK1 and the phenotypes of strawberry are shown in Figure 4; the up-regulation of FaTPK1 expression (Figure 4b,c, OE) accelerated the fruit’s red colour development (Figure 4a, OE) compared with the control (Figure 4a, control). Thus, FaTPK1 plays a role in fruit ripening.

**Altered FaTPK1 expression affects the ripening-related physiological processes**

To further understand the role of FaTPK1 in strawberry fruit ripening, we analysed the physiological conditions of fruit firmness, soluble sugar (glucose, fructose, and sucrose) contents and anthocyanin contents in transgenic fruits in which FaTPK1 was down-regulated and up-regulated compared with the control fruits. Transcripts of genes regulated to these physiological processes: polygalacturonase (PG1), beta-galactosidase (GAL6) and D-xylulose reductase (XYL2) for fruit firmness; sucrose transporter (SUT1) for sugars; and chalcone synthase (CHS) and chalcone flavanone isomerase (CHI) for anthocyanins (Jia et al., 2011, 2013; Tian et al., 2012) were also assessed. The fruit firmness increased in RNAi fruits but declined in OE fruits (Figure 5a), whereas the anthocyanin (Figure 5b) and sugar (Figure 5c) contents were down-regulated in RNAi fruit and up-regulated in OE fruit compared with the control fruits. The K⁺ contents were determined using the inductively coupled plasma mass spectrometry method. The tonoplast FaTPK1 is an outward potassium channel and controls K⁺ release from vacuoles; during strawberry fruit ripening, K⁺ was maintained at a high level. In the RNAi, control and OE fruits, there was a declining trend in the K⁺ content (Figure 5d) with sugar accumulation (Figure 5c).

The SqRT–PCR (Figure 6a) and qPCR (Figure 6b) analysis showed that the mRNA expression levels of all of the tested genes were down-regulated in RNAi fruits and up-regulated in OE fruits (Figure 6). The changes in transcripts of firmness (-PG1, XYL2 and GAL6), sugar- (SUT1) and pigment-related (CHS and CHI) genes in these transgenic fruits were accordant to a role of FaTPK1 in ripening.

**Altered FaTPK1 expression affects fruit pathogen resistance**

Ripened fruit is susceptible to pathogen infections, which limits the fruit storage periods and preservation. To explore the role of FaTPK1 in pathogen resistance, *B. cinerea* was used to infect strawberry fruits. Five days after inoculation, the degree of infection was
divided into four grades: level one (no infection), level two (20% infection), level three (60% infection) and level four (100% infection). As shown in Figure 7, fruits infected with 10^6 CFU/mL spores exhibited B. cinerea lesions at 5 days after inoculation, while the FaTPK1-RNAi fruits appeared only lightly infected. By contrast, the control fruits had moderate infection levels and

**Figure 2** Two-pore potassium (TPK) channel gene transcriptome data of strawberry, the characterization of the FaTPK1 gene sequence and its semi-quantitative detection. (a) Four TPK-like homologues were detected and divided into three groups based on log2 gene expression levels and the four fruit stages, DG, WT, IR and PR, that occur near the onset of strawberry fruit ripening. The sequence alignment in NCBI found that the first group had two complementary hits, comp72679_c0_seq2 and comp72837_c0_seq1, that aligned with the Fragaria vesca TPK channel 1-like (LOC101294428) sequence, and was named FaTPK1; the second group, comp58158_c0_seq2 aligns with F. vesca TPK channel 1-like (LOC101294725); the last group, comp104834_c0_seq1 aligns with F. vesca TPK channel 3-like (LOC101307905). (b) The conserved-domain regions were determined for the FaTPK1 protein using a 364-amino acid polypeptide BLAST algorithm-based search of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). (c) FaTPK1 protein has two ion_trans_2 domains, each pore containing a GYGD K⁺ selectivity motif. (d) Relative gene expression of FaTPK1 in six developmental processes of strawberry fruits.

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the FaTPK1-OE fruits were severely infected at 5 days after inoculation. The disease indices of the FaTPK1-RNAi, control and FaTPK1-OE fruits were 51.67, 76.67 and 96.67, respectively (Table 1). Thus, FaTPK1-RNAi fruits were significantly resistant to B. cinerea compared to the control, and in contrast, FaTPK1-OE fruits were sensitive to the pathogens, indicating that FaTPK1 is involved in pathogen resistance.

Analysis of the FaTPK1 protein using ITC

To further explore the binding capacity of the FaTPK1 protein with K\(^+\), the coding sequence of FaTPK1 was amplified and expressed in Escherichia coli cells, and 1.2 mg/mL of a ~65-kD recombinant fusion protein was purified using Beaver Beads\textsuperscript{TM} GHS (Glutathione S-transferase) tag (Figure 8a). The protein was identified using a one-step western kit with horseradish peroxidase (Figure 8b). Then, an ITC analysis was performed using 20 \(\mu\)M of FaTPK1 recombinant protein and 100 mM of KCl in an ITC200 calorimeter, in which the protein samples were titrated with a 2-\(\mu\)L injection of KCl per 150 s. The KCl–buffer–FaTPK1 recombinant protein titration system showed that the binding of KCl to FaTPK1 followed a saturation kinetics curve with a binding constant of \(2.1 \times 10^{-3} \text{ M}^{-1}\) and a dissociation constant of 476 \(\mu\)M (Figure 8c). The control results gained from the (Tris–HCl) buffered protein did not follow a saturation curve (data not shown). Thus, the FaTPK1 protein could bind K\(^+\).

Down-regulation and overexpression of the FaTPK1 gene alter the ABA content

To investigate a relationship of FaTPK1 with ABA during ripening, the ABA content in the FaTPK1-RNAi fruits decreased compared with the control fruits, while it increased in the OE fruits. The average ABA contents were 76.816 ± 3.546 ng/g, 108.877 ± 2.752 ng/g and 95.689 ± 2.378 ng/g in RNAi, OE and control fruits, respectively. Thus, the ABA content was significantly down-regulated in infiltrated RNAi fruits but significantly up-regulated in the OE fruits compared to the control fruits (Figure 9a). ABA can inhibit FaTPK1 transcription in vitro, and when exogenous ABA was sprayed on intact strawberry fruit, the FaTPK1 expression decreased (Figure 9b, c). Thus, ABA could inhibit FaTPK1 transcription.
Discussion

The developmental processes of fleshy fruits have three distinct, overlapping phases: cell division (early stage), cell expansion (middle stage) and ripening (late stage). Most of the cell's volume is occupied by a large central vacuole, and its osmotic-driven enlargement determines the fruit’s size and development (Ho, 1996). An H+-coupled accumulation of soluble sugars localizes predominantly in the vacuole throughout tomato fruit growth, and the osmotic strength of the vacuole is highly controlled by sucrose import during fruit development (Beauvoit et al., 2014).

Tonoplast TPK1 mediates K+-selective currents between the cytoplasm and vacuole. TPK1 activity is regulated by cytoplasmic Ca2+ and the cytoplasmic pH. TPK1 is also involved in the translocation of intracellular K+ and the redistribution of K+ among the different plant tissues (Gobert et al., 2007). The variation in the K+ distribution of different genotypes may result from differences in TPK1 expression. Gobert et al. (2007) also found that K+ release is delayed in the presence of ABA when the TPK1 gene is knocked out, which can inhibit plant growth because of a slower stomatal closure rate. This suggests that TPK1 could be a possible pathway for vacuolar K+ release during stomatal closure in the presence of ABA.

The rapid accumulation of apoplast-unloaded sucrose and subsequent sucrose synthase-mediated sucrolysis resulted in an increase in the contents of glucose, fructose and especially sucrose, which promoted strawberry fruit ripening (Jia et al., 2011; Li et al., 2012a; Tian et al., 2012; Zhao et al., 2017). Here, we showed that the soluble sugar contents rapidly and continually increased during fruit ripening (Figure 1). The contents of the soluble sugars were down-regulated in FaTPK1-RNAi fruits but up-regulated in the FaTPK1-OE fruits (Figure 5), suggesting that a link may be present between FaTPK1 and soluble sugar accumulation, especially sucrose accumulation.

The vacuole-mediated K+ uptake and efflux are essential and inevitable for plant growth and development, as well as stress responses, because they control the cell water potential and turgor during osmotic regulation (Latz et al., 2007; Nieves-Cordones et al., 2016; Osakabe et al., 2013; Sharma et al., 2013). In Vicia faba leaf guard cells, stomatal opening is correlated with more K+ uptake and less sucrose accumulation, and maximal apertures are coupled with a drastic decrease in the K+ content, while sucrose becomes the dominant solute (Talbott and Zeiger, 1996, 1998). In the present study, we showed that K+ is the most abundant element in strawberry fruit (Table S1), and its contents, on the whole, showed a declining and oscillating trend during fruit development (Figure 1). It was up-regulated in FaTPK1-RNAi fruits but down-regulated in FaTPK1-OE fruits (Figure 5), demonstrating that fruit ripening is coupled with a rapidly accumulating sucrose content and a decrease in the K+ contents. Thus, FaTPK1 can regulate the K+ contents, which modulates sucrose metabolism and affects fruit firmness, anthocyanin content and soluble sugar accumulation, as well as the biotic stress responses and the...
transcriptional levels of the ripening-regulated genes GAL6, XYL2, PG1, CHS, CHI and SUT1 (Figure 6). Previous study demonstrated that K+ is released rapidly from the vacuole (MacRorie, 2006) after the application of ABA, which may play a role in the ripening of nonclimatic fruit (Jia et al., 2011). Thus, ABA can affect the activity of FaTPK1. In our study, the ABA contents were down-regulated in FaTPK1-RNAi fruits and up-regulated in FaTPK1-OE fruits compared to control (Figure 8a). This suggested that silencing FaTPK1 could inhibit fruit ripening but overexpressing it could promote fruit ripening. Thus, our results indicate that FaTPK1-mediated K+ oscillation plays a role in soluble sugar accumulation and, as a result, regulates fruit ripening and quality.

It is known that soluble sugar main stored in vacuoles of ripened fleshy fruit. The strawberry K+ channel FaKAT1 plays a role in sugar absorption through tonoplast membrane. Given that ripened strawberry fruit mainly accumulates sucrose (Jia et al., 2011) and sucrose is a signal molecule in strawberry fruit ripening (Jia et al., 2013). Thus, the mechanism of coordinated regulation of FaKAT1, FaTPK1 and sucrose in sugar accumulation is interesting work in the future. FaTPK1, a K+ channel on tonoplasts, has the function of controlling K+ effluxes from vacuoles. Thus, the efflux of K+ may promote the accumulation of sucrose in strawberry ripening.

**Experimental procedures**

**Plant materials**

In this study, strawberry ‘Sweet charlie’ (Fragaria × ananassa) fruits were used. Strawberry plants were cultivated in greenhouse at 23–
28 °C, with 60%–70% relative humidity. Fruits were classified into seven developmental stages: SG, BG, DG, Wt, IR, PR and FR, which were collected at 7, 14, 18, 21, 23, 25 and 28 days after anthesis, respectively. Twenty fruits of uniform size were sampled at every stage (one replication). Fresh fruit (nonseed fruit tissue) was used to measure the physiological indices.

RNA-seq and data analysis

Four stages (BG, Wt, IR and PR) of fruits (n = 3) were sampled for RNA isolation and cDNA synthesis. RNA was extracted from each receptacle using E.Z.N.A. Total RNA Kit (Omega Bio-tek, Norcross, GA), then the RNA was used for cDNA library synthesis after RNase-free DNase digestion based on a RNA library prep kit (New England BioLabs, Ipswich, MA). RNA-seq was performed on the Illumina HiSeq2000 platform (Illumina, San Diego, CA) by Beijing Ori-Gene Science and Technology Corp., LTD, and analysis of the RNA-seq data was carried out according the report (Benjamini and Yekutieli, 2001; Langmead and Salzberg, 2012; Mortazavi et al., 2008; Wang et al., 2010; Zhao et al., 2017).

Cloning of FaTPK1 gene

To clone the FaTPK1 gene, the specific primers (forward, 5'-ATGGATAGGAAATGGATGC-3'; reverse, 5'-TTATGATTGAGCAAGTGTAGAT-3') were designed. The FaTPK1 cloning was performed on according to the following PCR conditions: 98 °C for 30 s, 98 °C for 10 s (35 cycles), 55 °C for 30 s, 72 °C for 40 s and with a final extension of 72 °C for 2 min in 50 μL Q5 PCR mixture (0.5 μL Q5 high-fidelity DNA Polymerases, 5 μL 5× Q5 buffer, 1 μL 10 mM dNTPs, 2.5 μL forward specific primer, 2.5 μL reverse specific primer, 2 μL cDNA template, 31.5 μL ddH2O). The PCR products were ligated into T1-simple cloning vector and subsequently transformed into Escherichia coli competent cell (TransGen Biotech, Beijing, China). Positive colonies were selected and sequenced by Huada China (Beijing, China).

Construction of recombinant plasmids

Using VIGS (Liu et al., 2002), FaTPK1 was silenced in strawberry fruit. A 426-bp cDNA fragment of FaTPK1 was amplified using primers (sense, 5'-GGGATCCATGGATAGGAAATGGATGC-3'; antisense, 5'-GGGGTACCAATAGTCTGCTGCTTGGCTCA-3') by PCR, and the DNA fragments were cloned into KpnI–EcoRI-cut virus vector pTRV2. Agrobacterium-mediated TRV infection was made as described by Fu et al. (2005).

To generate ihpRNAi of FaTPK1, a 650-bp fragment of the FaTPK1 cDNA was PCR amplified using the primers (sense, 5'-CGGGATCCATGGATAGGAAATGGATGC-3'; antisense, 5'-CGGGATCCATGGATAGGAAATGGATGC-3') by PCR, and the DNA fragments were cloned into pFGC5941 vector (Mubin et al., 2011).
at the NcoI and BamHI restriction sites, and the recombinant plasmid was named pFGC650. A 350-bp fragment, which complementary to the 3′ end of the 650-bp fragment, was amplified by PCR using specific primers (sense, 5′-CGGGATCCGCTGTTTATTTCTGTATTGT-3′ and antisense, 5′-GCTCTAGATAAAATGCATCCACTAGGTCCAAT-3′) from strawberry fruit cDNA. The 350-bp fragment was cloned into pFGC650 at BamHI and XbaI restriction sites. By this means, a hairpin structure was produced in the recombinant plasmid.

To generate the FaTPK1 OE construct, the full-length of FaTPK1 was obtained by PCR from cDNA using specific primers (sense, 5′-GGGGATCCATGGATAGGAATGGGATGCG-3′; antisense, 5′-GAATTC TTATGATTGAGAAGTGTTAGAT-3′) from strawberry fruit cDNA. The 350-bp fragment was cloned into the pCAMBIA1304 vector at the KpnI and EcoRI restriction sites.

Transfection of strawberry by agroinfiltration
Agrobacterium tumefaciens strain GV3101, containing pTRV2-FaTPK1, pGCS5941-FaTPK1 and pCAMBIA1304-FaTPK1, was cultured in Luria–Bertani liquid medium at 28 °C containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) and 20 µM acetosyringone with appropriate antibiotics. Vectors pTRV2, pGCS5941 and pCAMBIA1304 were the controls. The Agrobacterium cells were cultured to an optical density at 600 nm (OD600) of 0.8 and Agrobacterium infection as described by Chai et al. (2011) and Jia et al. (2013). Strawberry fruits were treated by
local and whole fruit injection with 200 μL and 1 mL of the Agrobacterium suspension, respectively.

**qRT-PCR and SYBR real-time PCR**

In each treatment group, strawberry fruits (n = 6) growing at the same rate were acquired using the random sampling method. For analysis of FaTPK1 transcripts by qRT-PCR in RNAi and OE fruits, six fruits were randomly selected for mixed grinding. First-strand cDNA was used as the template for 28 cycles of PCR amplification of FaTPK1 using 20 μL PCR mixture. The reactions contained 0.2 μL LA Taq (5 U/μL), 2 μL 10× LA PCR buffer II (Mg2+ plus), 3.2 μL dNTP Mixture (2.5 mM), 1 μL forward specific primer (10 μM; Sangon, Shanghai, China), 1 μL reverse specific primer (10 μM; Sangon), 1 μL cDNA template and 11.6 μL ddH2O. The experiment above was repeated three times. A 418-bp FaTPK1 was amplified by primers (sense, 5′-TGCTTCACGGGTCATAG-3′; and antisense, 5′-AGTCTGCTCCTGGCTCA-3′). These conditions were selected for the comparison of the relative accumulation of FaTPK1 and Actin. After silence and overexpression of FaTPK1 in strawberry, the ripening-regulated genes GAL6, XYL2, PG1, CHS, TPK1, CHI and SUT1 were investigated by qRT-PCR, primers as shown in Table S2. The primers used for SYBR real-time PCR are showed in Table S3 and made according to the description by Chai et al. (2011). The experiment was performed with three replications.

**Expression and purification of the FaTPK1 recombinant protein**

The expression and purification of the recombinant FaTPK1 protein was performed using a prokaryotic expression system in E. coli. The coding sequence of FaTPK1 was amplified by PCR from a synthesized cDNA using primers forward, 5′-CCCGAGTTTCATGATGAGTAATGATGTCG-3′ (EcoR I site) and reverse, 5′-TAAGAATGCCGCCCGCATATTGTAGGACGACAGTGTTA-3′ (Ndi I site) and cloned into the expression vector PGEX-4T1 in frame with the N-terminal GST fusion tag, which was transformed into E. coli to enable the selection of transformants on LB plates containing 100 μg/mL ampicillin. Ten transformants were selected to confirm the correct fusion frame by sequencing. The purified recombinant plasmids were transformed into E. coli BL21 to select the ampicillin-resistant E. coli transformants. The FaTPK1-GST fusion protein was expressed at 16 °C in LB broth with 1 μM IPTG for 12 h. The purification of the FaTPK1-GST fusion protein was carried using BeaverBeads™ GHS (Beaver, China) according to manufacturer’s protocols. The eluted fusion protein was stored at −80 °C until use.

**Subcellular localization of FaTPK1 in onion inner epidermal cells**

The FaTPK1 was cloned into the pEZS-NL vector (Li et al., 2012a, b) using primers forward, 5′-G GAATTC ATGGATAGGA ATGGGATGCGC-3′ (EcoR I site) and reverse, 5′-GGGTCTCCATGATGAGTAATGATGTCG-3′ (EcoR I site) to obtain an EGF transient plasmid. The plasmids were transformed into onion inner epidermal cells by particle bombardment using biolistic delivery of gold particles (Bio-Rad, PDS1000/He Biolistic Gene Gun, Hercules, CA) to get the subcellular localization information of the expressed proteins. The effective bombardment parameters were 1100 Psi of helium pressure, 84.66 kPa of vacuum pressure and 6 cm of target-shelf distance, and the amount of gold powder for is 0.7 mg. The onion epidermis was incubated for 4 h on MS plates (sucrose 30 g/L, sorbitol 0.15 M, mannitol 0.15 M, agar 8 g/L, pH 5.8).

After bombardment, the onion epidermis cells were incubated for 24 h on MS plates (sucrose 30 g/L, agar 8 g/L, pH 5.8). After incubation at 25 °C for 24 h, the fluorescence of EGFP was observed using a confocal laser scanning microscope (Zeiss LSM 510 META, Germany) with excitation at 488 nm. Confocal observations were made using a Plan-Apochromat X40 dry objective. The experiment was performed with three replications.

**ITC assay**

The eluted fusion protein concentration was used an Amicon Ultra-4 centrifugal 30-kDa filter (Millipore, Darmstadt, Germany). The FaTPK1 was adjusted to 20 μM in ITC buffer (50 mM Tris-HCl, pH 8.0). The protein samples were titrated using a 2-μL injection with 100 μM of KCl per 150 s in Microcal ITC200 (Malvern, Worcestershire, UK) at 25 °C. The titration of Tris–HCl buffer into the FaTPK1 protein was used as the control. The experiment was repeated three times.

**Determination of mineral element contents by inductively coupled plasma mass spectrometry method**

To determine the mineral element contents of strawberry in the seven developmental stages, six RNAi, OE and control fruits (control fruits for RNAi and OE were mixed) were randomly selected for mixed grinding, respectively. For RNAi fruits, we selected fruits that had delayed maturity by 50% and selected OE fruits that promoted maturity by 200%. Samples (0.5 g, n = 3) were digested in 5 mL of HNO3 and 4 mL of H2O2 at 16 °C using a microwave digestion instrument (MARS-240/50). After cooling samples to room temperature, they were dried by electrothermal heating and their volumes increased to 50 mL. The solvents were used as controls. The mineral element contents of strawberry fruits were determined by inductively coupled plasma mass spectrometry (ICAP6300 Radial, Thermo Fisher, Waltham, MA). Three replications were performed.

**Determination of the soluble sugar and ABA content**

The soluble sugar content was determined using reverse phase HPLC (Agilent Technologies 1200 Series, RID1 A detector). The supernatant was fractionated in 5 mL of 16% HCl for 10 min, carefully collect the supernatant into a 10-ml triangular flask. The residues were mixed with 2.5 mL of methanol (1% HCl) and then centrifuged as described above. The volume was then increased to 10 mL with distilled water, and 1 mL extract was filtered with a 0.22 micron membrane; the filtrate was used for the determination of the anthocyanin content.

The experiment was repeated three times.

**Determination of the anthocyanin content**

Ripening strawberry fruits (n = 10) of consistent maturity were selected and treated by liquid nitrogen grinding. Then, 2.5 mL of methanol (1% HCl) was added to 0.5 g of powder, extracted overnight in the darkness at 4 °C, after centrifugation at 10,000 g for 10 min, carefully collect the supernatant into a 10-mL triangular flask. The residues were mixed with 2.5 mL of methanol (1% HCl) and then centrifuged as described above. The volume was then increased to 10 mL with distilled water, and 1 mL extract was filtered with a 0.22 micron membrane; the filtrate was used for the determination of the anthocyanin content.
The anthocyanin content was measured by reverse phase HPLC using a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm, Agilent) with a linear gradient from solution A (acetonitrile), 0 to 20% for 13 min, 20% to 40% for 20 min and 0% for 25 min, to solution B (10% formic acid) at a flow rate of 1 mL per min. The detection wavelength was 520 nm, the column temperature was 25 °C, and the injection volume was 20 μL. The standard sample used was pelargonidin-3-O-glucoiside. The entire process was repeated three times.

Effect of ABA on strawberry fruit ripening in vivo

WT stage fruits were selected and immersed into 100 μM of ABA (a treatment includes 20 fruits per replication), and ddH₂O was used as the control. The developing fruits still attached to plants were treated four times on alternating days beginning at 14 days after anthesis. Whole strawberry fruits treated with 100 μM ABA or ddH₂O were used for sampling. Seven days after the first treatment, the expression level of FaTPK1 was investigated by qRT–PCR and qPCR.

Fruits infection with B. cinerea

Culture of fungi (B. cinerea) on PDA plating medium with the temperature at 25 °C and the relative humidity reached to 95%. The spores were eluted from the culture medium after 8 days. After removing mycelia, spores were counted and added to the inoculation solution at the concentration of 10⁶ CFU/mL. The FaTPK1-RNAi strawberry fruits (n = 15) and FaTPK1-OE fruits (n = 15) were incubated in an inoculation solution containing 10⁶ CFU/mL spores for 5 min. Then, the strawberry fruits were put into incubator at 25 °C, and the incubators were covered with plastic film to guarantee a relative humidity of 95%–100%. The control fruits (n = 15) were treated with sterile water. After inoculation for 5 days, we count the number of spreading lesions on each fruit to evaluate fruit infection caused by B. Cinerea (Li et al., 2013).

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Supporting information
Additional Supporting Information may be found online in the supporting information tab for this article:
Figure S1 Nucleotide blast of FaTPK1 gene and FvTPK1 gene.
Table S1 Element contents in ripening fruits (FR) and 7-stage fruits (average).
Table S2 The primers used for SqRT-PCR.
Table S3 The primers used for qPCR.