Abscisic acid controls sugar accumulation essential to strawberry fruit ripening via the FaRIPK1-FaTCP7-FaSTP13/FaSPT module

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SUMMARY

Strawberry is considered as a model plant for studying the ripening of abscisic acid (ABA)-regulated non-climacteric fruits, a process in which sugar plays a fundamental role, while how ABA regulates sugar accumulation remains unclear. This study provides a direct line of physiological, biochemical, and molecular evidence that ABA signaling regulates sugar accumulation via the FaRIPK1-FaTCP7-FaSTP13/FaSPT signaling pathway. Herein, FaRIPK1, a red-initial protein kinase previously identified in strawberry fruit, not only interacted with the transcription factor FaTCP7 (TEOSINTE BRANCHEN 1, CYCLOIDEA, and PCF) but also phosphorylated the critical Ser89 and Thr93 sites of FaTCP7, which negatively regulated strawberry fruit ripening, as evidenced by the transient overexpression (OE) and virus-induced gene silencing transgenic system. Furthermore, the DAP-seq experiments revealed that FvTCP7 bound the motif “GTGGNNCCNC” in the promoters of two sugar transporter genes, FaSTP13 (sugar transport protein 13) and FaSPT (sugar phosphate/phosphate translocator), inhibiting their transcription activities as determined by the electrophoretic mobility shift assay, yeast one-hybrid, and dual-luciferase reporter assays. The downregulated FaSTP13 and FaSPT transcripts in the FaTCP7-OE fruit resulted in a reduction in soluble sugar content. Consistently, the yeast absorption test revealed that the two transporters had hexose transport activity. Especially, the phosphorylation-inhibited binding of FaTCP7 to the promoters of FaSTP13 and FaSPT could result in the release of their transcriptional activities. In addition, the phosphomimetic form FaTCP7S89D or FaTCP7T93D could rescue the phenotype of FaTCP7-OE fruits. Importantly, exogenous ABA treatment enhanced the FaRIPK1–FaTCP7 interaction. Overall, we found direct evidence that ABA signaling controls sugar accumulation during strawberry fruit ripening via the “FaRIPK1-FaTCP7-FaSTP13/FaSPT” module.

Keywords: strawberry fruit ripening, abscisic acid signaling, FaRIPK1, FaTCP7, sugar transport, FaSTP13, FaSPT.

INTRODUCTION

As a primary biological energy carrier in plants, photosynthetic glucose metabolism-derived triphosphadene (ATP) participates in almost all essential cell metabolism and signaling processes via protein kinase/phosphatase-mediated reversible protein phosphorylation, a cooperation “on-and-off” switch for controlling major forms of post-translational modification (Hunter, 1995; Sun & Shen, 2023). It underscores abscisic acid (ABA) receptor pyrabactin resistance 1/Pyr1-like regulatory components of ABA receptors (PYR1/PYL/RCAR)-mediated classical core signaling by the protein kinase/phosphatase pair “PP2C-SnRK2.6/OST1” (protein phosphatase 2C-sucrose non-fermenting 1 [SNF1]-associated protein kinase 2/OPEN STOMATA1; Fujii et al., 2009; Ma et al., 2009; Melcher et al., 2009; Nishimura et al., 2009; Park et al., 2009; Santiago et al., 2009). Interestingly, besides linking with mitogen-activated protein kinase kinase kinase 18 (MAPKKK18) through its interaction with both SnRK2.6 and ABI1 (PP2C1) and SnRK2.6-MAPKKK18 in the nucleus (Tajdel et al., 2016), this core signaling also associates with several other protein kinases, including Arabidopsis Raf10 (a Raf-like novel MAPKKK; Nguyen et al., 2019) and guard cell hydrogen peroxide-resistant 1 (GHR1); a Raf- and receptor-like protein kinase; Hsu
Overall, protein kinase/phosphatase-mediated reversible protein phosphorylation is a major signaling mechanism underlying ABA action in plant development and adaptation. However, compared to the significant progress in ABA signaling-mediated stressful responses, stomatal movement, seed germination, and seedling growth, signaling in ABA-mediated fruit ripening, an important plant developmental process critical to our food, nutrition, and health, is lagging far behind.

Strawberry (Fragaria ×ananassa) is considered as a model plant for studying ABA-regulated non-climacteric fruit ripening (Jia et al., 2011; Kim et al., 2019; Liao et al., 2020; Symons et al., 2012), a process in which the classical ABA core signaling mechanism “ABA-PYR/PYL/RCAR-PP2C-SnRK2.6” appears conservative and distinct (Bai et al., 2020). For example, AtSnRK2.6 is a positive regulator of Arabidopsis leaf stomatal movement (Belin et al., 2006), whereas FaSnRK2.6 is a negative mediator regulating strawberry fruit ripening (Han et al., 2015); similarly, ClSnRK2.3 is also a negative regulator of watermelon fruit ripening (Wang et al., 2023). Additionally, the activated mitogen-activated protein kinase 3 (FvMAPK3) acts as the downstream target of MAPK kinase 4 (FvMKK4) and FvSnRK2.6 to phosphorylate the TF FvMYB10 (an R2R3 MYB TF) and reduce its transcriptional activity, thereby inhibiting fruit coloration in response to low temperatures (Mao et al., 2022). Furthermore, strawberry FvMAPK6 can interact with, phosphorylate, and activate the ripening-inducing factor (FvRIF), a NAC (NAM, ATAF, and CUC) TF, ultimately promoting strawberry fruit ripening (Li et al., 2023). This classical ABA core signaling is conserved and distinct and mostly links with various MAPK-related protein kinases.

A strawberry leu-rich repeat (LRR) receptor-like kinase (RLK) designated as FaRIPK1 with four conserved domains, including LRRs (from 81 to 276), serine-rich region of AP3B1 (SEEEED, clathrin-adaptor complex, from 344 to 463), ethylene-responsive protein kinase Le-CTR1 (EDR1, from 544 to 776), and serine/threonine-protein kinase (STK, from 837 to 1045) with MAPK activity, was screened from strawberry fruit (Hou et al., 2018) using another putative ABA receptor bait, ABAR (Jia et al., 2011; Wu et al., 2009), which is a multifunctional protein (Sun & Shen, 2023). Furthermore, FaABAR and FaRIPK1 could interact in the nucleus, positively regulating strawberry fruit ripening (Hou et al., 2018). Intriguingly, the FaRIPK1–FaSnRK2.6 interaction and mutual phosphorylation exist (Chen et al., 2022), and Arabidopsis SnRK2.6 interacts with and functions downstream of ABAR in guard cell signaling in response to ABA (Liang et al., 2015). Notably, a recent report found the interaction of strawberry ripening regulation protein/clathrin-related protein (FaRRP1/SCD2) with both FaPYL2 and FaABAR, highlighting the three proteins binding to ABA and positively regulating strawberry fruit ripening (Li & Shen, 2023). These reports collectively imply a link between PYL and ABAR in fruit ripening, a process in which FaRIPK1 is a remarkable kinase. However, the bona fide downstream target of FaRIPK1 is yet to be uncovered.

Herein, we chose FaTCP7 (teosinte branchen 1, cycloidea, PCF; Li, 2015), a plant-specific TF, by yeast library screening using FaRIPK1 as a bait. Subsequently, through the yeast two-hybrid (Y2H), bimolecular fluorescence complementation (BiFC), co-immunoprecipitation (Co-IP), and GST pull-down assays, we further confirmed that FaRIPK1 interacted with FaTCP7 both in vitro and in vivo and that the TCP domain and C-terminal of FaTCP7 were sufficient to bind FaRIPK1. Following that, through a series of molecular analyses, we demonstrated that FaRIPK1 could phosphorylate FaTCP7 and that ABA enhanced the effect. Specifically, FaTCP7 negatively regulated strawberry fruit ripening by binding to the promoter of the sugar transporter genes sugar transport protein 13 (FaSTP13) and sugar phosphate/phosphate translocator (FaSPT), inhibiting their promoter activities, which were controlled by phosphorylation modification. Therefore, we proposed the ABA-FaRIPK1-FaTCP7-FaSTP13/FaSPT signaling module for regulating strawberry fruit ripening. This provides a novel insight into ABA-controlled fruit ripening through sugar, helping us understand the mechanism of ABA signaling network in the regulation of plant reproductive growth.

RESULTS

Protein kinase FaRIPK1 interacts with the TF FaTCP7

The candidate interacting proteins of FaRIPK1 were subjected to yeast library screening to explore how FaRIPK1 promotes strawberry fruit ripening. Given that the full length of FaRIPK1 contains 1152 amino acids, FaRIPK1 was weakly expressed in yeast, leading to poor screening results. Consequently, based on the predicted structural length of FaRIPK1 contains 1152 amino acids, FaRIPK1 was subjected to yeast library screening to explore how FaRIPK1 promotes strawberry fruit ripening. Given that the full length of FaRIPK1 contains 1152 amino acids, FaRIPK1 was weakly expressed in yeast, leading to poor screening results. Consequently, based on the predicted structural domain, we obtained the truncated versions of FaRIPK1, including FaRIPK1-N (N-terminal) and FaRIPK1-CD (catalytic domain), for yeast library screening (Figure 1a). Compared to those of FaRIPK1-N, the FaRIPK1-CD-based screening results were better (Figure 1a,b), revealing that the candidate interacting proteins contained TF FaTCP7, FabHLH3, ethylene-responsive TF RAP2, and F-box protein FLL (Figure 1b). The interaction of FaTCP7 and FabHLH3 with FaRIPK1-CD was then confirmed in the Y2H assay (Figure 1b). This study mainly focused on the FaTCP7–FaRIPK1 interaction.

First, we examined the subcellular localization and discovered that FaTCP7 and FaRIPK1 were both localized in the nucleus in Nicotiana benthamiana leaves and...
Figure 1. Protein kinase FaRIPK1 interacts with transcription factor FaTCP7 in vitro and in vivo.

(a) The schematic illustration of the FaRIPK1 protein. CD, catalytic domain.

(b) FaRIPK1-CD interacts with FaTCP7 as determined by the yeast two-hybrid (Y2H) assay. –LW, synthetic dropout medium without Leu and Trp; –LWHA, synthetic dropout medium without Leu, Trp, His, and Ade.

(c) Luciferase complementation imaging (LCI) assay displaying that FaRIPK1, FaRIPK1-N, or FaRIPK1-CD interact with FaTCP7. The coding sequence (CDS) of FaRIPK1, FaRIPK1-N, or FaRIPK1-CD was cloned into the pCAMBIA1300-NLUC vector, and the CDS of FaTCP7 was introduced into pCAMBIA1300-CCLUC. The constructs were co-transfected into Nicotiana benthamiana leaves. Luminescence signals were captured using a cooled charge-coupled device camera after 48–72 h.

(d) Bimolecular fluorescence complementation (BiFC) assay illustrating that FaRIPK1 interacts with FaTCP7 in the nucleus. The CDS of FaRIPK1 was fused to the N-terminus of yellow fluorescent protein (YFP), and the CDS of FaTCP7 was fused to the C-terminus of yellow fluorescent protein (YFP). The constructs were co-transfected into N. benthamiana leaves. Green fluorescent protein (GFP) signals were detected after 48–72 h using a confocal microscope. Bar = 20 μm.

(e) FaRIPK1-MYC interacts with FaTCP7-GFP in vitro. The ProSP1300-FaRIPK1-MYC and ProSP1300-FaTCP7-GFP constructs were co-transfected into N. benthamiana leaves. The total protein was incubated with MYC beads for 2 h. Proteins were detected with anti-MYC and anti-GFP antibodies.

(f) The full-length and truncated schematic illustrations of FaTCP7 protein.

(g) Glutathione S-transferase (GST) pull-down assay indicates that FaRIPK1 interacts with FaTCP7, FaTCP7-T, or FaTCP7-NT, but not with FaTCP7-N in vitro. Recombinant GST-FaRIPK1 purified from Escherichia coli was incubated with glutathione Sepharose beads for 1 h. Next, the beads were incubated with maltose-binding protein (MBP)-FaTCP7. Proteins were detected with anti-MBP and anti-GST antibodies. The experiments were repeated three times with similar results.
Arabidopsis protoplasts (Figure S1c,d), further supporting the potential association of FaRIPK1 with FaTCP7. Subsequently, we performed the firefly luciferase complementation imaging (LCI) assay and detected an obvious luciferase signal between FaTCP7 and FaRIPK1, FaRIPK1-N, or FaRIPK1-CD (Figure 1c). We used GUS as a negative control. We then performed the BiFC assay to verify the FaRIPK1-FaTCP7 interaction (Figure 1d). We observed a GFP signal in the nucleus when FaRIPK1-YFP-N and FaTCP7-YFP-C were co-expressed in tobacco leaves. In addition, we employed the Co-IP assay to assess protein interaction (Figure 1e). Furthermore, the GST pull-down assay was performed to determine which domain(s) in FaTCP7 could be responsible for its association with FaRIPK1. According to the results, the TCP domain and C-terminal of FaTCP7 were sufficient to bind FaRIPK1 (Figure 1f,g). These findings collectively demonstrate that FaRIPK1 interacts with FaTCP7 both in vitro and in vivo.

FaRIPK1 phosphorylates FaTCP7 that negatively regulates strawberry fruit ripening

According to previous research, FaRIPK1 exhibits kinase activity in vitro and promotes strawberry fruit ripening (Hou et al., 2018). Given that FaRIPK1 interacted with FaTCP7, we subsequently investigated whether it phosphorylated FaTCP7. The in vitro phosphorylation assay revealed that FaRIPK1 could phosphorylate FaTCP7, FaTCP7-T, FaTCP7-NT, and FaTCP7-C, but not FaTCP7-N (Figure 2a). Since the TCP domain is a conserved functional domain, we subsequently focused on determining which TCP site was phosphorylated. The four Ser/threonine residues in the TCP domain (Ser89, Ser89, Thr93, and Ser104) were mutated to alanine (Ala). Notably, FaTCP7-T was no longer phosphorylated by FaRIPK1 when Thr93 was mutated to Ala, and the FaTCP7-T phosphorylation level was weakened after Ser89 was mutated to Ala (Figure 2b). These findings imply that Ser89 and Thr93 are the critical sites phosphorylated by FaRIPK1. Furthermore, since Ser89 and Thr93 are close to each other, we hypothesized that their phosphorylation has a mutual influence.

The TCP TFs are plant specific. The diploid strawberry contains 19 TCPs, and the phylogenetic tree shows that FvTCP7, together with FvTCP1, FvTCP2, FvTCP8, FvTCP11, FvTCP12, FvTCP16, FvTCP17, FvTCP18, and FvTCP19 belong to class I, while the other members belong to class II (Figure S2a). Moreover, the TCP domain analysis revealed that the TCP domain of FvTCP7 lacks four amino acids (Figure S2b). In Arabidopsis, FvTCP7 is homologous to AtTCP7 and AtTCP21. According to research, AtTCP7 promotes the endogenous replication process, thereby affecting leaf development (Zhang et al., 2019). It also interacts with the circadian clock components to influence photoperiodic plant flowering (Li et al., 2021). On the other hand, AtTCP21 is involved in maintaining the homeostasis of the circadian clock (Wu et al., 2016). However, the function of FvTCP7 in the strawberry fruit remains unknown.

Based on our previous transcription data in developmental fruit (Wang et al., 2017), we discovered that FvTCP7 was dramatically downregulated after the large green stage (Figure 3a), implying that FvTCP7 might negatively regulate fruit ripening. To prove this notion, we injected Agrobacterium GV3101 strains harboring proSuper: FaTCP7-FLAG or proSuper: FaRIPK1-FLAG into 20 white fruits attached to the strawberry plants to overexpress FaTCP7 or FaRIPK1 based on the transient fruit transgenic system commonly employed (Chai et al., 2011; Huang et al., 2019; Li et al., 2013; Medina-Puche et al., 2014; Zhang et al., 2023). Phenotypes were assessed on the sixth-day post-injection. Coloration was markedly inhibited in the FaTCP7-OE fruit, showing white color in the section of transfection on sixth day (Figure 3b), whereas the FaRIPK1-OE fruits were fully ripe, showing deep red compared with the control (Figure 3b), revealing overexpressing FaTCP7 delayed and overexpressing FaRIPK1 hastened fruit ripening. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses confirmed that the FaTCP7 or FaRIPK1 mRNA expression level was significantly higher in the OE fruits than in the control fruits (Figure 3d). Moreover, TRV-mediated virus-induced gene silencing (VIGS) was utilized to verify the function of FaTCP7. We silenced the FaTCP7 or FaRIPK1 gene in white fruits, using a mixture of Agrobacterium cultures containing pTRV1 and pTRV2 carrying a 300 bp of FaTCP7 (FaTCP7-pTRV2) or FaRIPK1 (FaRIPK1-pTRV2), with control fruit being infiltrated with the empty TRV vectors (Control-pTRV2). In contrast, fruits turned fully red faster in the section of transfection in the FaTCP7-pTRV2, while fruits kept white in the FaRIPK1-pTRV2 (Figure 3c), showing that silencing FaTCP7 promoted and silencing FaRIPK1 inhibited fruit ripening. The transcript level of FaTCP7 or FaRIPK1 was significantly lower in the VIGS fruits than in the control fruits (Figure 3d). These findings suggest that FaTCP7 controls fruit ripening by acting as a negative regulator.

FaTCP7 binds to the promoters of sugar transporter genes FaSTP13 and FaSPT to inhibit their expression levels

We sought the downstream gene of FaTCP7 to explore how FaTCP7 regulated fruit ripening. Herein, the purified recombinant FvTCP7 with the same protein sequence as FaTCP7 was subjected to the DAP-seq assay using 3-month-old diploid seedlings. The DAP-seq data were used to analyze the DNA binding characteristics of FvTCP7. The MACS software analysis revealed that FvTCP7 had 5979 binding peaks on the genome. The binding peaks of the two technology repeats had 4434 peaks with
good repeatability (Figure 4a). Further statistical analyses on the distribution of binding peaks in gene functional regions showed that 3148 and 1192 binding peaks were distributed in the distal intergenic and promoter regions, respectively, and that some were located in intron or exon regions (Figure 4b), all of which were primarily distributed within 2 kb upstream and downstream of transcription start sites (Figure 4c). The sequence of the interval where the binding peaks were located was extracted using the MEME software, and the common motifs among the binding peaks were scanned to draw the motif map. We found that GTGGNNCCCNC was the main FvTCP7 binding motif (Figure 4d). Furthermore, the enriched binding peaks were distributed on all seven chromosomes (Figure 4e). According to the KEGG enrichment analysis of genes corresponding to the binding peaks located in the promoter region, some genes related to signals such as hormone signal transduction, the circadian clock, and RNA degradation were significantly enriched (Figure 4f).

Subsequently, we screened and analyzed the genes corresponding to the bindings in the promoter region and discovered some genes potentially associated with fruit ripening. Sugar transporter-related genes (sugar transporter protein 13 [STP13], sugar phosphate/phosphate translocator [SPT], SUC3, and sugars will eventually be exported transporters [SWEET]) and hormone signal transduction-related genes (ERF054/020 [ethylene-responsive TF], auxin-responsive protein IAA11-like isoform X2, gibberellin receptor GID1C, protein kinase constitutive triple response 1 [CTR1], and protein phosphatase PP2C46) were all in the list.

Then, the binding of FaTCP7 to the promoter region of candidate genes was examined using the yeast one-hybrid (Y1H) experiment, and the relationship between TF FaTCP7 and the sugar transporter genes FaSTP13 and FaSPT was studied further. First, we predicted the possible binding sites of FaTCP7 in FaSTP13 and FaSPT promoter sequences using the website PlantRegMap. Biotin probes were then synthesized with the predicted binding motif as the center. The electrophoretic mobility shift assay (EMSA) was performed using the purified FaTCP7 protein. According to the results, although FaTCP7 could bind to the promoter probes of FaSTP13 and FaSPT, the binding was weakened after adding the competing probes (Figure 5a). Furthermore, FaTCP7 bound directly to the promoter regions of FaSTP13 and FaSPT (which required the full length of FaTCP7) in the Y1H assay (Figure 5b). The dual-luciferase reporter assay was then performed to assess the effect of FaTCP7 on the promoter activity of FaSTP13 and FaSPT. The promoter sequences of FaSTP13 or FaSPT were integrated into the pGreenII-0800 vector to create the reporter for evaluating the effect of FaTCP7 on FaSTP13 and FaSPT promoters (Figure 5c). The empty vector and proSuper-FaTCP7-GFP were the effectors (Figure 5c). Compared to proSuper-Empty, co-injection of proSuper-FaTCP7 reduced the LUC/REN ratio; hence, FaTCP7 inhibited the
promoter activity of FaSTP13 and FaSPT (Figure 5d). These findings indicate that FaTCP7 may bind to FaSTP13 and FaSPT promoters, inhibiting their promoter activities.

Subsequently, we examined gene expression in the fruits by manipulating FaTCP7 expression. We discovered that FaTCP7 overexpression downregulated the transcription levels of FaSTP13 and FaSPT while silencing FaTCP7 upregulated the transcription levels (Figure 6a). Meanwhile, manipulating FaRIPK1 expression presented an opposite trend (Figure 6a). The hexose transporter FaSTP13 is homologous with the Arabidopsis hexose transporter AtSTP13 (Lemonnier et al., 2014; Schofield et al., 2009; Yamada et al., 2016). On the other hand, the transporter FaSPT has no reported homologous proteins. According to TMHMM prediction, FaSTP13 and FaSPT had 12 and 10 transmembrane domains, respectively (data not shown). Herein, we transformed the vectors into a hexose transporter-deficient yeast strain EBY.VW4000 to verify the function of FaSTP13 and FaSPT. After gradient dilution, the spots were placed on SD/C0 Ura medium containing different concentrations of glucose (Glc), fructose (Fru), sorbitol (Sor), and galactose (Gal). The deficient strains transformed with FaSTP13 grew on the deficient medium containing glucose, fructose, sorbitol, or galactose, whereas the deficient strains transformed with FaSPT grew on the deficient medium.

Figure 3. FaTCP7 negatively regulates strawberry fruit ripening.
(a) Changes in the FaTCP7 transcripts during strawberry fruit ripening in the transcriptome data. IR, initial red; LG, large green; PR, partial red; SG, small green; WT, white. Data are presented means ± SE (n = 3).
(b) Transient overexpression of FaTCP7 inhibits strawberry fruit ripening. The ProSP1300-FLAG (Control-OE), ProSP1300-FaTCP7-FLAG (FaTCP7-OE), or ProSP1300-FaRIPK1-FLAG (FaRIPK1-OE) construct was transfected into white fruits and was recorded through photography. Twenty white fruits were collected for each reaction. The section of the dotted line represents the range of transfection. Bar = 1 cm.
(c) Virus-induced gene silencing-induced silencing of FaTCP7 promotes strawberry fruit ripening. The TRV2 (Control-RNAi) carrying a fragment of FaTCP7 (FaTCP7-RNAi) or FaRIPK1 (FaRIPK1-RNAi) construct was transfected into white fruits and was recorded through photography. Twenty white fruits were collected for each reaction. The section of the dotted line represents the range of transfection. Bar = 1 cm.
(d) The transcript level of FaTCP7 or FaRIPK1 in transient transgenic fruits of (b, c). Data are shown as means ± SE (n = 3). *Represents significant difference compared with the control as determined by one-way ANOVA followed by Tukey’s test, **P < 0.001, ns, no significance.

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containing fructose, sorbitol, or galactose (Figure 6b). These results indicate that both FaSTP13 and FaSPT were able to transport hexose in yeast. We then assessed the soluble sugar levels in the transgenic fruits and found that the transient FaTCP7 overexpression decreased and silencing FaTCP7 increased the soluble sugar content, including the fructose, glucose, and sucrose levels (Figure 6c). In contrast, the soluble sugar content was increased and decreased in FaRIPK1-OE and FaRIPK1-pTRV2, respectively (Figure 6c). In brief, FaTCP7 could negatively regulate soluble sugar content by repressing the transcription levels of FaSTP13 and FaSPT.

Figure 4. DAP-seq assay shows the binding sites of FvTCP7.
(a) The binding peaks between two technical repeats are consistent. MACS software was employed to determine the binding peaks and IDR software was utilized to merge binding peaks between two technical repeats. Results showed that there were 4434 overlapping binding peaks between the two technical repeats.
(b) Annotation statistics of binding peaks in gene functional regions. The binding peaks were annotated with HOMER, and the functional regions were separated into promoter, exon, intron, downstream, distal intergenic, 5' UTR, and 3' UTR regions.
(c) The distribution of the enriched binding peaks near the transcription starting site (TSS).
(d) FvTCP7 binds to the motif GTGGNCCCNC. Sequences of binding peaks were extracted using the MEME software. The common motifs were scanned and exhibited using position-correlation weight matrix.
(e) The enriched binding peaks are distributed across all seven chromosomes.
(f) KEGG analysis of the promoter region-related genes. Analysis of the corresponding genes whose promoter contains binding peaks revealed that the genes involved in hormone signal transduction, circadian rhythm, and RNA degradation were enriched. Term: pathway name; bubble color: \(-\log(P\text{ Value})\), the larger the value, the darker the color; bubble size: input number, the number of target genes in this pathway; and gene ratio: input number/background number.
Phosphorylation inhibits the transcriptional activity of FaTCP7

Since post-translational modification processes such as phosphorylation may affect protein localization, interaction, or activity, we subsequently examined the influence of FaTCP7 phosphorylation. Consistent with FaTCP7, phosphomimetic forms FaTCP7S89D, FaTCP7T93D, and FaTCP7S89D T93D were all localized in the nucleus.

Figure 6. FaTCP7 may negatively regulate soluble sugar content through repressing the expression of FaSPT13 and FaSPT.

(a) Transient overexpression of FaTCP7 inhibits virus-induced gene silencing (VIGS)-induced silencing of FaTCP7 induces FaSPT13 and FaSPT expression. RNA was extracted from the fruits and reverse transcribed for further RT-qPCR. Statistical results showing that FaTCP7 overexpression significantly decreased the transcript level of FaSPT13 and FaSPT, while knock down of FaTCP7 increased the transcript level. Data were shown as means ± SE (n = 3). *Represents significant difference compared with the control as determined by Student’s t-test, ***P < 0.001.

(b) FaSPT13 and FaSPT possess hexose transport activity in yeast. The coding sequence (CDS) of FaSPT13 or FaSPT was cloned into pDR195 vector. The constructs in (c) were co-transfected into Nicotiana benthamiana leaves. The enzyme activities were detected after 48-72 h. The results indicate that LUC/REN value declined following co-transfection with proSP1300-FaTCP7. Data were shown as means ± SE (n = 3). *Represents significant difference compared with the control as determined by Student’s t-test, ***P < 0.001.
Moreover, phosphorylation did not affect the interaction with FaRIPK1 in the Y2H and LCI assays (Figure S3b,c). Subsequently, we examined whether phosphorylation affected the transcriptional activity of FaTCP7. The EMSA results revealed that the phospho-deficient form FaTCP7S89A did not affect binding to the probe, while phosphomimetic forms FaTCP7S89D and FaTCP7S89E weakened the binding to the probe. Moreover, FaTCP7T93A...
FaTCP7T93D, and FaTCP7T93E did not bind to the probe (Figure 7a,b). Consistent with the EMSA results, compared to FaTCP7, FaTCP7S89D and FaTCP7S89E bound to the FaSTP13 or FaSPT promoters weakly, while the Y1H assay revealed that FaTCP7T93A, FaTCP7T93D, and FaTCP7T93E were no longer bound to the promoters (Figure 7c). The preceding assays showed that FaTCP7 inhibited the promoter activity of FaSTP13 and FaSPT (Figure 5). The dual-luciferase reporter assay was then employed to further

Figure 7. Phosphorylation of Thr93 blocks the binding ability of FaTCP7 to the promoter of FaSTP13 or FaSPT.
(a) Phosphomimetic or non-phosphorylated mutated form of FaTCP binds to the promoter of FaSTP13 or FaSPT differently as detected by the electrophoretic mobility shift assay (EMSA). Recombinant proteins purified from Escherichia coli and synthesized probes were employed to conduct the EMSA. S89A did not alter the binding. FaTCP7S89D and FaTCP7S89E showed weak binding to the probe, but FaTCP7T93A, FaTCP7T93D, and FaTCP7T93E did not bind to the probe.
(b) CBB picture corresponding to the proteins in (a).
(c) Y1H assay showing that Thr93 of FaTCP7 is required for the binding to the promoter of FaSTP13 or FaSPT. The 300 bp promoter sequence of FaSTP13 or FaSPT was cloned into pAbAi vector. The coding sequence (CDS) of FaTCP7, FaTCP7S89A, FaTCP7S89D, FaTCP7S89E, FaTCP7T93A, FaTCP7T93D, or FaTCP7T93E was cloned into pADT7 vector. Similar to the results shown in (a), FaTCP7S89D and FaTCP7S89E showed weak binding to the promoter, and FaTCP7T93A, FaTCP7T93D, FaTCP7T93E, FaTCP7S89A T93A, FaTCP7S89D T93D, or FaTCP7S89E T93E did not bind to the promoter.
(d) Phosphorylation of Ser89 attenuates the transcriptional inhibition of FaSTP13 by FaTCP7. When co-transfected with proSP1300-FaTCP7S89A, LUC/REN value was not significantly different compared with the sample co-transfected with proSP1300-FaTCP7. LUC/REN value increased following co-transfection with proSP1300-FaTCP7S89D or proSP1300-FaTCP7S89E.
(e) Phosphorylation of Thr93 attenuates the transcriptional inhibition of FaSTP13 by FaTCP7. LUC/REN value increased following co-infiltration with proSP1300-FaTCP7S89A, proSP1300-FaTCP7S89D, or proSP1300-FaTCP7S89E compared with proSP1300-FaTCP7. Data in (d, e) are shown as means ± SE (n = 3). *Represents significant difference determined by one-way ANOVA followed by Tukey’s test, *P < 0.05 and ***P < 0.001.
detect the influence of phosphorylation on the transcriptional activity of FaTCP7. The results showed that S89D and S89E compromised the decrease in transcriptional activity of FaTCP7 to some extent (Figure 7d). Furthermore, T93A, T93D, or T93E significantly attenuated the transcriptional inhibition of FaSTP13 by FaTCP7 (Figure 7e). Overall, FaRIPK1 phosphorylates FaTCP7 and represses its transcriptional activity, eventually relieving the inhibition of the sugar transporter genes FaSTP13 and FaSPT.

Next, we transiently overexpressed the phosphomimetic form FaTCP7S89D or FaTCP7T93D to check the effect of phosphorylation of FaTCP7 on fruit ripening. As shown in the results, the inhibition of FaTCP7 on fruit ripening was compromised in FaTCP7S89D-GFP or FaTCP7T93D-GFP fruits, the coloration of which was similar to the control (Figure 8a). In terms of the transcription levels of FaSTP13 and FaSPT, and the soluble sugar content, FaTCP7S89D or FaTCP7T93D rescued inhibitory effect of FaTCP7 (Figure 8b, c).

Figure 8. The phosphomimetic form FaTCP7S89D or FaTCP7T93D rescues the phenotype of FaTCP7-OE fruits. (a) Transient overexpressing the phosphomimetic form FaTCP7S89D or FaTCP7T93D shows phenotype similar to the control. The ProSP1300-GFP (EV-GFP), ProSP1300-FaTCP7-GFP, ProSP1300-FaTCP7S89D-GFP, ProSP1300-FaTCP7T93D-GFP, or ProSP1300-FaRIPK1-GFP construct was transfected into white fruits and was recorded through photography. Twenty white fruits were collected for each reaction. The section of the dotted line represents the range of transfection. Bar = 1 cm. (b) The transcript level of FaTCP7, FaRIPK1, FaSTP13, or FaSPT in transient transgenic fruits of (a). The results showed that the expression level of FaSTP13 or FaSPT was rescued in transgenic FaTCP7S89D-GFP and FaTCP7T93D-GFP. (c) The soluble sugar content was increased in transgenic FaTCP7S89D-GFP and FaTCP7T93D-GFP compared with FaTCP7-GFP. Data in (b, c) were shown as means ± SE (n = 3). *Represents significant difference determined by one-way ANOVA followed by Tukey’s test, *P < 0.05, **P < 0.01, and ***P < 0.001.
receptor complex PYR1/PYLs-PP2Cs or putative receptor ABAR. The downstream kinases FaSnRK2.6 interacted with FaRIPK1, phosphorylating each other. Additionally, ABA enhanced the FaRIPK1–FaTCP7 interaction. As a result, FaRIPK1 phosphorylated FaTCP7 and inhibited the binding of FaTCP7 to the promoter of the sugar transporter genes FaSTP13 and FaSPT, eventually relieving the transcriptional inhibition of FaTCP7 on FaSTP13 and FaSPT genes, promoting sugar accumulation and fruit ripening.

**DISCUSSION**

The discovery of FaTCP7 in strawberry fruit fills a gap in how ABA signaling regulates the ripening by sugar

Although ABA can enhance the transportation of photoasimilates into the economical organs of both grain and fruit, the mechanisms involved are mostly unknown (Murcia et al., 2016). Notably, two recent reports have provided a line of cues to aid our understanding of plant development and adaptation as well as fruit ripening through the multiple functions of the H subunit of magnesium chelatase/putative ABA receptor genomics uncoupled 5 (CHLH/ABAR/GUN5), which is integrated into chloroplast-derived signaling (Sun & Shen, 2023) and linked to FaPYL2 signaling by FaRRP1/SCD2-mediated vesicle transport (Li & Shen, 2023), a process associated with the SnRK2.6-RIPK1 interaction of two ABA core signaling components (Chen et al., 2022). Our FaBAR-based discovery of FaTCP7-targeting sugar transporters in strawberry fruit fills the gap in understanding ABA regulation of the ripening by sugar to some extent.

It has been reported that CHLH, as a multi-function protein, is involved in chlorophyll (Chl) biosynthesis, retrograde signaling, sugar metabolism, ABA response, and fruit ripening (Sun & Shen, 2023). According to the research, CHLH interacts with the anti-sigma factor SigE, which positively regulates sugar catabolism in a light-dependent manner in *Synechocystis* sp. PCC 6803 (Mochizuki et al., 2001; Osanai et al., 2009). Similarly, strawberry CHLH/ABAR interacts with FaSigE to regulate sugar accumulation and fruit ripening (Zhang et al., 2017). However, the mechanism underlying CHLH/ABAR-mediated signaling in association with sugar is unclear. Therefore, using FaABAR as bait, we screened the cDNA library of strawberry
fruit to positively identify FaRIPK1 and establish its role in the strawberry fruit ripening process (Hou et al., 2018). Subsequently, FaRIPK1 was used as bait to isolate FaTCP7, a member of Class I FaTCPs (Figure 1), through two successive Y2H experiments. Herein, we demonstrated that FaRIPK1 controls strawberry fruit ripening through the phosphorylation-mediated post-translational modification of FaTCP7 (Figure 2), which directly regulates sugar accumulation by the sugar transporter genes FaSTP13 and FaSPT (Figures 7 and 8). In this regard, the “ABA-FaABAR-FaRIPK1-FaTCP7-FaSTP13/FaSPT” signaling module first establishes a direct link between ABA signaling and sugar accumulation.

The interaction among FaABAR, FaRIPK1, and FaTCP7 in the nucleus lays a basis for the signaling cascade as a feedback regulation of sugar homeostasis, contributing to strawberry fruit ripening.

Most fruit species, including strawberries and grapes, transport photosynthates (sugar) into fruit, mainly in the form of sucrose. Sugar levels and compositions, mainly including sucrose, glucose, and fructose, influence fruit development and ripening to a great extent. Consistent with the rapid accumulation of sugar and ABA, the strawberry fruit ripening process is coupled with a color transition (green to red). Specifically, the green strawberry fruit accumulates glucose and fructose rather than sucrose, whereas the red-colored fruit mainly accumulates sucrose, which may function as a signal to accelerate fruit ripening by stimulating ABA biosynthesis. Therefore, downregulating FaCHLH/ABAR may reduce the levels of sucrose, glucose, and fructose while inducing ABA accumulation (Li & Shen, 2023). It has been established that MAPKKK18 regulates the classical core ABA signaling “PYLs-PP2Cs-SnRK2s” to interact with both SnRK2.6 and ABI1 in the nucleus (Tajdel et al., 2016). Furthermore, we discovered that FaRIPK1, as a MAPKK protein kinase, is integrated into the ABA signaling network through its reciprocal phosphorylation with FaSnRK2.6 (Chen et al., 2022; Hou et al., 2018). Herein, we also found that FaRIPK1 could phosphorylate TF FaTCP7 (Figure 2). Additionally, among the 19 TCPs in strawberries, FaRIPK1 interacted with FaTCP11 and FaTCP12 (Figure S4), implying that FaTCP7, 11, and 12 may act redundantly in fruit ripening.

Our findings also revealed that FaTCP7 was bound to the promoter of two hexose carrier genes, FaSTP13 and FaSPT, acting as a negative regulator for gene expression and fruit ripening (Figures 5 and 6). Sugar and ABA reciprocal promotion concertedly accelerates strawberry fruit ripening to some extent (Jia et al., 2011, 2013, 2016). Unlike previous reports that posited the involvement of TCPs primarily in physiological processes such as leaf and flower development, circadian clock regulation, thermomorphogenesis, and photomorphogenesis (Nicolas & Cubas, 2016), the present study revealed a new TCP function of FaTCP7. Specifically, the phosphorylation of the TCP domain at the Ser89 and Thr93 sites inhibits the transcription activity of FaTCP7 (Figures 2 and 7), demonstrating unique mechanisms of action. For example, OST1 phosphorylates Arabidopsis ICE1, resulting in a stronger transcriptional activity, which activates cold-signaling-related gene expression downstream (Ding et al., 2015). Similarly, MaMPK6-3 phosphorylated MabZIP21 in Musa acuminata to enhance its transcriptional activity and promote banana ripening (Wu et al., 2022). In this regard, the role of FaTCP7 in targeting sugar transporter genes offers novel insights into the underlying mechanism of the TCP family in plants.
Based on the amino acid characteristics of their TCP domains, TCP TFs are classified into Class I and Class II. Compared to Class II TCP domains, the Class I TCP domains lack four amino acids (Li, 2015). In Arabidopsis thaliana, TCPs contain 24 members, among which the Class I member, AtTCP14/15, regulates plant growth, trichoid branching, stratum cornum formation, and high-temperature-induced elongation of the petiole and hypocotyl (Camoimano et al., 2020; Daviere et al., 2014; Ferrero et al., 2019). On the other hand, Class II member AtTCP4 integrates auxin and BR signals to promote hypocotyl elongation (Challa et al., 2016), while AtTCP5, 13, and 17 activate AP1 (APETALA1) to promote flowering (Li et al., 2019). The TCP protein tendril (TEN) in cucumbers also regulate tendril development (Wang et al., 2015). Furthermore, the TCP TF lanceolate (LA) in tomatoes plays a role in leaf maturation (Shleizer-Burko et al., 2011). Interestingly, FvTCP9, a Class II member, regulates both ABA and anthocyanin synthesis in the strawberry fruit (Xie et al., 2020). Collectively, these two classes could concertedly regulate the ABA- and sugar-induced strawberry fruit ripening.

ABA signaling integration in strawberry fruit growth and ripening

In Arabidopsis (Chen et al., 2021) and strawberry (Bai et al., 2020; Li et al., 2022; Perotti et al., 2023), ABA performs versatile functions in plant development and adaptation, as well as fruit ripening co-regulated by both the classical and new signaling pathways, including “ABA-PYR/PYLs/RCARs-PP2Cs-SnRK2” and “ABA-ABAR-RIPK1.” Based on previous research and our findings herein, we believe that the two signaling integrations contribute rapidly to establishing the balance and facilitating transitions among plant growth, adaptation, and fruit ripening by fine-tuning sugar transport and metabolism.

Both the interaction of FaRIPK1 with FaSnRK2.6 and FaRRP1/SCD2-mediated ABA trafficking and signaling have now integrated the classical and new signaling cross-talk in strawberry plant adaptation and fruit ripening (Chen et al., 2022; Li & Shen, 2023; Sun & Shen, 2023). The discovery of “FaRIPK-FaTCP7-FaSTP13/FaSPT” signaling in this study establishes a direct link to sugar accumulation, aiding our understanding of the regulation of sugar synthesis, transport, accumulation, and metabolism, either directly or indirectly by the multifunctional protein CHLH/GUN5/ABAR, which is critical for Chl synthesis, retrograde signaling, and ABA signaling.

The FaRIPK1-FaTCP7 interaction was enhanced by ABA treatment (Figure 9a). Thus, we hypothesize that FaTCP7 inhibits FaSTP13/FaSPT expression and sugar accumulation during strawberry green-fruit stages with low ABA levels, while “ABA-ABAR-RIPK1” signaling inhibits FaTCP7 activity after the white stages with rapid ABA accumulation, releasing the FaTCP7 retard on FaSTP13/FaSPT expression, thereby promoting sugar accumulation and fruit ripening, a process possibly facilitated by “ABA-PYL2-AB1-SnRK2.6” signaling to activate FaRIPK1 activity. This coordinated regulation contributes to fine-tuning the balance and transition between fruit growth and ripening by sugar accumulation and metabolism. In conclusion, this study elucidates the mechanism underlying FaRIPK1-mediated strawberry fruit ripening and identifies FaTCP7 as a FaRIPK1-phosphorylated target. Furthermore, we found that FaTCP7 negatively regulates sugar accumulation through the sugar transporter genes. These findings offer novel insights into ABA-modulated signaling during fruit ripening, highlighting the new role that Class I FaTCPs play in sugar homeostasis and the FaRIPK1 integrator.

MATERIALS AND METHODS

Plant materials and growth conditions

Octoploid strawberries (Fragaria × ananassa Duch. cv Beni-hoppe) for transient transgene were grown in a greenhouse under natural sunlight conditions. Diploid strawberries (Fragaria vesca. cv Rugen) for DAP-seq were cultivated in the light incubator under 16 h:8 h (light:dark) at 22 °C for 3 months.

Yeast cDNA library screening

The coding sequence (CDS) of FaRIPK1-CD was inserted into the pGBK7 vector. BD-FaRIPK1 was transformed into yeast gold strain to measure the protein expression. The yeast spot with good protein expression was subjected to mating with cDNA library obtained from strawberry white strawberry and red fruit. After 22-24 h, the culture solution was plated on SD/–Ade—His—Leu—Trp medium and incubated for 3-5 days at 30 °C. The yeast that was grown on 4D medium was transferred to a new 4D plate, and PCR amplification was carried out using designed AD-F and AD-R primers. The PCR products were sent to a company for sequencing. Protein sequence alignment was performed using NCBI blast.

Yeast two-hybrid assay

The CDS of FaRIPK1, FaRIPK1-N, or FaRIPK1-CD was cloned into pGBK7, whereas the CDS of FaTCP7 was cloned into pGADT7 vector. The plasmids were co-transfected into yeast. The transformed yeast cells were plated on SD/–Leu—Trp medium for 3 days at 30 °C. Subsequently, the yeast was transferred to SD/–Ade—His—Leu—Trp medium and incubated for 3 days. The primers used for Y2H are presented in Table S1.

Firefly LCI assay

The assay was conducted following a protocol described in a previous study (Chen et al., 2008). The CDS of FaRIPK1, FaRIPK1-N, or FaRIPK1-CD was cloned into pCAMBIA1300-NULC, and the CDS of FaTCP7 was introduced into pCAMBIA1300-CULUC. The constructs were co-transfected into N. benthamiana leaves. Luminescence signals were recorded using a cold CCD camera after 48-72 h. The primers used for LCI are listed in Table S1.

BiFC assay

The assay was performed following a protocol described in a previous study (Waad et al., 2008). The CDS of FaRIPK1 was cloned
into pSPYNE173, and the CDS of FaTCP7 was cloned into pSPYCE. The constructs were co-transfected into N. benthamiana leaves. GFP signals were detected after 48–72 h using confocal microscopy. The primers used for BiFC are listed in Table S1.

**Co-IP assay**

The ProSP1300-FaRIPK1-MYC and ProSP1300-FaTCP7-GFP constructs were co-transfected into N. benthamiana leaves. After 2 days, the leaves were collected and used to extract total protein using protein lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, and 0.5% NP-40). The total protein was incubated for 1 h. The results were detected by immunoblotting using anti-MBP and anti-GFP antibodies.

**Protein purification**

The CDS of corresponding genes was cloned into pGEX4T-1 (GST tag) or pMAL-c5X (MBP tag). Proteins were expressed in BL21 Escherichia coli with 0.2 mM IPTG for 14 h at 20°C. The pellet was resuspended in PBS buffer (10 mM NaH₂PO₄, 2 mM KH₂PO₄, 150 mM NaCl, 2.7 mM KCl, pH 7.4, and 1 mM PMSF) for GST and MBP-tagged proteins. The cell suspension was sonicated for 10 min and then centrifuged. The supernatant was incubated with glutathione Sepharose 4B (GST-tagged proteins) or amylose resin (MBP-tagged proteins) for 2 h at 4°C. Finally, GST-tagged proteins were eluted with GSH buffer (50 mM Tris–HCl, pH 8.0, and 10 mM GSH), and MBP-tagged proteins were eluted with 10 mM maltose in PBS buffer. The purified proteins were mixed using Quick Start Bradford Dye Reagent (Bio-Rad, Hercules, CA, USA), and the absorbance of each sample was measured at 595 nm. A standard curve was created using BSA. In addition, 2 μg purified proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue (CBB) to examine the purity. The primers used for the assay are listed in Table S1.

**GST pull-down assay**

Twenty-microgram recombinant GST-FaRIPK1 in a reaction was incubated with 20 μl glutathione Sepharose 4B in pull-down buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM β-mercaptoethanol, 3 mM EDTA, pH 8.0, and 1% NP-40) for 1 h. This was incubated with 3 μg MBP-FaTCP7, FaTCP7-T, FaTCP7-NT, or FaTCP7-C for 1 h. The results were detected by immunoblotting using anti-MBP and anti-GST antibodies.

**In vitro phosphorylation assay**

The in vitro phosphorylation assay was performed for the recombinant proteins GST-FaRIPK1, MBP-FaTCP7, MBP-FaTCP7-Δ339A, MBP-FaTCP7-Δ388A, MBP-FaTCP7-Δ739A, and MBP-FaTCP7-Δ1014A. Incubation with a kinase buffer (2 mM Tris–HCl, pH 7.5, 1 μM CaCl₂, 0.2 mM DTT, 0.5 mM MgCl₂, and 0.1 mM MnCl₂) with 50 μM cold ATP and 1 μCi γ-32P-ATP for 30 min at 30°C. The reaction was terminated by the addition of 4 μl 5× SDS loading buffer followed by denaturation by heating at 95°C for 5 min and the proteins were separated by 10% SDS-PAGE. The gel was stained, decolorized, wrapped tightly with plastic wrap, and then placed in a phosphor screen. Finally, radioactivity was detected using the Typhoon 9410 imager.

**Transient transgene and qRT-PCR**

Based on the study by Huang et al. (2019), Agrobacterium strain GV3101 carrying the ProSP1300-FaRIPK1-FLAG, ProSP1300-FaTCP7-FLAG, ProSP1300-FaRIPK1-GFP, or ProSP1300-FaTCP7-GFP plasmid was transfected into white strawberry fruits (n = 20 per replicate). For VIGS, the pTRV1 and pTRV2 (tobacco rattle virus) vectors were used as described before (Hou et al., 2018). A 300 bp FaTCP7 or FaRIPK1 cDNA fragment was cloned into pTRV2 using primers in Table S1. Agrobacterium tumefaciens strain GV3101 containing pTRV1, pTRV2, pTRV2-FaTCP7, or pTRV2-FaRIPK1 in a 1:1 ratio was transfected into strawberry fruit. Each fruit received a 200-μl injection using a hypodermic syringe. After 6 days, the phenotypes were recorded through photography. Fruits stripped of achenes were collected and subjected to qRT-PCR. Total RNAs were extracted with the HiPure Plant RNA Mini Kit (Magen, Guangzhou, China; R4151). Four-microgram RNAs were used to synthesize first-strand cDNA using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA; K1882). qRT-PCR was performed on Bio-Rad CFX96 system using TransStart Green qPCR SuperMix. ACTIN was used as the internal control. The primers used for qRT-PCR are listed in Table S1. White strawberry fruits (n = 20, one replication) were transfected with approximately 200 μl of Agrobacterium suspension per fruit using a hypodermic syringe.

**DAP-seq assay**

The 3-month-old “Rugen” seedlings were sent to Bluescience Company for DAP-seq. The following procedures were as follows: genomic DNA was extracted and fragmented to construct DNA library. Protein FvTCP7 was extracted using cell-free protein expression. Next, the library was incubated with FvTCP7 and the binding DNA fragments were eluted for further high-through sequencing analysis.

**Electrophoresis mobility shift assay**

The EMSA was performed using LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific; 20148) following standard protocols with minor modifications. The biotin probes were synthesized based on the predicted binding motif. Recombinant MBP-FaTCP7 purified from Escherichia coli was subjected to EMSA. Subsequently, the protein was incubated with either biotin probe or cold probe for 20 min at 27°C. Following native PAGE gel separation and UV cross-linking, the results were visualized using the Tanon imaging system.

**Yeast one-hybrid**

The 300 bp promoter sequence of FaSTP13 or FaSPT was cloned into pAbAi. The constructs were linearized and transformed into Y1H GOLD to screen the appropriate concentrate of aureobasidin A (AbA). Moreover, the CDS of FaTCP7 was cloned into pADT7 vector. AD-FaTCP7 was integrated into yeast transformed with pBait-AbAi and was plated on SD/C0 plate. The CDS of corresponding genes was cloned into pSPYCE. The constructs were co-transformed into N. benthamiana leaves. After 2 days, the leaves were collected and used to extract total protein using protein lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, and 0.5% NP-40). The total protein was incubated with MYC beads for 2 h. The proteins were detected with anti-MBP and anti-GFP antibodies.

**Dual-LUC reporter assay**

The promoter sequence of FaSTP13 or FaSPT was inserted into the pGreenII-0800 vector to form a reporter. The CDS of FaTCP7 or mutated form was cloned into proSP1300-GFP vector to create the effector together with the empty vector. Different combinations were transfected into the tobacco leaves. Subsequently, the assay was performed following the manufacturer’s instructions on the kit Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA; E1910). Briefly, the sample was lysed, and the supernatant was centrifuged for fluorescence detection. Twenty-microliter
lysate was added to a white opaque culture plate, and three to five repeats were set for each group. Next, 100 μl firefly luciferase reaction buffer was added to measure the LUC enzyme activity. Moreover, the 100 μl Renilla luciferase reaction buffer was added to detect the REN enzyme activity. The ratio of two enzyme activity was calculated.

Measurement of the soluble sugar content

The fruits were powdered and 1 g sample in 50 ml tube was dissolved with 10 ml 80% ethanol and boiled for 30 min for two times. The solution was centrifuged and the supernatant was transferred into a new 50 ml tube and boiled to dry state. Next, the pellet was dissolved in ultra-pure water to 50 ml. The solution was filtered by LC-18 SPE and measured using high performance liquid chromatography. d(+)-glucose, d(-)-fructose, and sucrose were used as standards.

Statistical analysis

The statistical significance of differences was evaluated by Student’s t-test and one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test. Asterisks indicate significant differences (*P < 0.05; **P < 0.01; ***P < 0.001).

ACCESSION NUMBERS

Genes in this article can be found in The Genome Database for Rosaceae (GDR) under the following accession numbers: FvTCP7 (FvH4_4g06720), FvSTP13 (FvH4_4g15150), FvSPT (FvH4_3g16500), FvTCP1 (FvH4_3g31480), FvTCP2 (FvH4_3g31470), FvTCP3 (FvH4_3g05220), FvTCP4 (FvH4_3g08160), FvTCP5 (FvH4_3g18740), FvTCP6 (FvH4_428170), FvTCP8 (FvH4_5g01340), FvTCP9 (FvH4_5g12710), FvTCP10 (FvH4_5g13710), FvTCP11 (FvH4_5g15150), FvTCP12 (FvH4_4g31520), FvTCP13 (FvH4_4g27300), FvTCP14 (FvH4_6g45410), FvTCP15 (FvH4_6g46730), FvTCP16 (FvH4_6g16170), FvTCP17 (FvH4_6g53830), FvTCP18 (FvH4_7g12810), and FvTCP19 (FvH4_7g28770).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. FaRIPK1 and FaTCP7 are localized in the nucleus.

Figure S2. The phylogenetic tree of FvTCPs family and the alignment of TCP domain.

Figure S3. Phosphorylation did not affect the subcellular localization of FaTCP7 and the interaction between FaTCP7 and FaRIPK1.

Figure S4. FaTCP11 and FaTCP12 interact with FaRIPK1 in yeast.

Table S1. Primers used in the present study.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/CYVIN9W.

DATA AVAILABILITY STATEMENT

All the required materials and data supporting this article are included within the manuscript and the supporting information.

REFERENCES

Hsu, P.K., Takahashi, Y., Merilo, E., Costa, A., Zhang, L., Kernig, K. et al. (2021) Raf-like kinases and receptor-like (pseudo) kinase GHR1 are...
Direct evidence of ABA signaling in sugar accumulation


Sun, M. & Shen, Y. (2023) Integrating the multiple functions of CHIL1 into chloroplast-derived signaling fundamental to plant development and adaptation as well as fruit ripening. Plant Science, 338, 111892.


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Wu, C.J., Shan, W., Liu, X.C., Zhu, L.S., Wei, W., Yang, Y.Y. et al. (2022) Phosphorylation of transcription factor bZIP21 by MAP kinase MPK6-3 enhances banana fruit ripening. Plant Physiology, 188, 1665–1685.


Xie, Y.G., Ma, Y.Y., Bi, P.P., Wei, W., Liu, J., Hu, Y. et al. (2020) Transcription factor FvTCP9 promotes strawberry fruit ripening by regulating the biosynthesis of abscisic acid and anthocyanins. Plant Physiology and Biochemistry, 146, 374–383.


