A subunit gene of sucrose non-fermenting 1 related kinase, PpSnRK1γ, confers flat fruit abortion in peach by regulating sugar and starch metabolism

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Abstract

**Background:** Fruit abortion is a major limiting factor for fruit production. In flat peach, fruit abortion is present in the whole tree of some accessions during early fruit development. However, the physiological factors and genetic mechanism underlying flat fruit abortion remain largely elusive.

**Results:** In this study, we have revealed that the fertilization process was accomplished and the reduction of sucrose and starch contents might result in flat fruit abortion. By combining association and gene expression analysis, a key candidate gene, \(PpSnRK1\gamma\), was identified. A 1.67-Mb inversion co-segregated with flat fruit shape altered the promoter activity of \(PpSnRK1\gamma\), resulting in much lower expression in aborting flat peach. Ectopic transformation in tomato and transient overexpression in peach fruit have shown that \(PpSnRK1\gamma\) could increase sugar and starch contents. Comparative transcriptome analysis further confirmed that \(PpSnRK1\gamma\) participated in carbohydrate metabolism. Subcellular localization found that \(PpSnRK1\gamma\) was located in nucleus.

**Conclusions:** This study clarified the reason for flat fruit abortion and identified a critical candidate gene, \(PpSnRK1\gamma\), responsible for fruit abortion in peach. The results would provide great help in peach breeding and facilitate gene identification for fruit abortion in other plant species.

Background

To feed the ever-growing number of global human population, food security is becoming an urgent task, including fruit crop yield. Peach (\(Prunus persica\) L.) is the fourth economically important fruit crop in the world (FAO, [http://faostat.fao.org](http://faostat.fao.org)), which is regarded as a model plant in Roseceae family due to its small genome size and relatively short juvenile [1]. Flat peach was first cultivated in China [2] and had been introduced to the world, which had favorite flavor and edible convenient fruit shape [3, 4]. However, the fruits aborted in some flat peach accessions during early fruit development [5-8], which has greatly limited the flat peach breeding and industry.

The flat fruit shape is controlled by a single dominant gene \(S\) (saucer-shaped) [9]. This gene was further mapped to the distal part of chromosome 6 using linkage analysis [5, 6] and association mapping [8, 10]. Dirlewanger et al. (2006) [6] found that some descendants from an F2 population between flat (‘Ferjalou Jalousia’) and round (‘Fantasia’) cultivars exhibited abnormal fruits which aborted soon after fruit set. This recessive character has been named \(Af\) and demonstrated to be linked to the flat fruit shape gene [6]. Similarly, such flat fruit abortion phenomenon was also found by other researchers and the recessive \(Af\) locus was supported by the segregation ratio. The very few mature fruits in the aborting flat peach tree class were also identified, which exhibited abnormal shape of fruit and seed, showing cracking fruit phenotype at the pistillar side and absent of kernel [11]. Therefore, the allele \(S\) linked with \(af\) and \(s\) linked with \(Af\). In this case, \(S/s\) determines flat fruit shape and \(Af/af\) is responsible for flat fruit abortion trait.

Recently, the key gene responsible for flat fruit shape, \(PpOFP1\), has been identified and validated, and overexpression of \(PpOFP1\) did not result in fruit abortion in tomato and \(Arabidopsis\) [8, 12]. These studies
indicated that flat shape and fruit abortion were genetically controlled by different genes, and the adjacent gene underling flat fruit abortion still remained unknown.

Fruit and seed abortion is a major limiting factor for achieving crop yield [13-15]. As sink organs, fruit and seed development is accompanied with the accumulation of storage products, mainly proteins, starch and oils, which are typical features of growth and maturation stages [16]. Sugars play a vital role, though many factors participate in fruit and seed set, such as source-sink interaction, hormonal signaling and other metabolic pathways [17]. Under abiotic stress, sugar limitation is a well-known factor leading to fruit and seed abortion [13, 18, 19]. In tomato, silencing gene LIN5 which encodes an invertase resulted in fruit abortion and reduced fruit size [20]. As well, overexpression a sucrose synthase gene in cotton reduced seed abortion [21]. Therefore, genes associated with sugar metabolism might regulate fruit and seed abortion.

The sucrose non-fermenting 1 related kinases (SnRKs), also known as sucrose non-fermenting 1 (SNF1) in yeast and AMP-activated protein kinase (AMPK) in mammalian, are crucial components in plant growth and development by regulating transcriptional and metabolic processes [22-24]. There are three subfamilies of SnRKs, including SnRK1s, SnRK2s and SnRK3s [25, 26]. The SnRK2 and SnRK3 subfamily are mostly involved in stress and abscisic acid (ABA) signaling [27-31], while SnRK1s regulate carbohydrate metabolism and is crucial for normal development and response to stress [32-34]. The SnRK1 exist as heterotrimeric holoenzyme, comprising a catalytic α subunit, a regulatory γ or βγ subunit, and β subunit as a scaffold linking α and γ subunits [35, 36]. The α subunit genes has been reported in regulating sugar and starch metabolism [37] and loss of function exhibit embryonic lethality in Arabidopsis [38]. The γ subunits, also known as SNF4 in yeast and AMPKγ in animal, are found in complexes with β subunits [22, 39]. In pea (Pisum sativum), SnRK1-antisense seeds have maturation defects and result in seeds abortion [40, 41]. In peach, overexpression of PpSnRK1α in tomato has increased sugar and starch contents, however little is known about the function of γ subunit in sugar metabolism, especially in regulating fruit and seed abortion.

In this study, we investigated the physiological factors for flat fruit abortion and reported a critical candidate gene, PpSnRK1γ, promoting the accumulation of sugars and starch in seed and fruit, which might be responsible for flat fruit abortion in peach. These results further enriched the function of the γ subunit of SnRK1s in carbohydrate metabolism and provided valuable genetic basis for future flat peach breeding.

**Results**

**Phenotypic analysis of flat peach abortion**

The aborting flat peach cultivar was observed in our peach germplasm, showing similar phenotype with individuals obtained by Dirlewanger et al. (2006) and Picañol et al. (2012). To investigate the reason for flat peach abortion, we analyzed the phenotypic characteristics for round (‘JH’), viable flat (‘ZH’), and aborting flat fruits (‘XJ2’). Firstly, we found that the aborted flat peach stopped growing and tend to drop
at 30 DAFB (days after full bloom) (Fig. 1a, b). Then, we supposed whether there was defect in fertilization process in aborting flat fruit, because the style size in flower organ was different from the round and viable flat peach [6]. Pollen germination and growth in pollen tube were studied, which showed that the pollen could germinate and grow into pollen tube normally (Fig. 1c). Due to the relatively larger size of ovary, the signal of germinated pollen in it could not be viewed clearly under microscope. Therefore, the flow cytometer checking ploidy of the fertilized embryo was carried out to determine the fertilization process. This result further showed that the fertilization process was accomplished in ‘XJ2’, because the triploid cells were observed (Fig. 1d). Taken together, these results indicated that the fertilization process was accomplished in the aborting flat peach and there should be other factors contributing to flat fruit abortion.

It was reported that the deficiency of carbohydrate could induce seed abortion [38, 40, 41]. In this study, we found that the seed of the dropped flat peach was withered at around 30 DAFB (Supplementary Figure 1). It has been reported that sugars play a vital role in fruit and seed set [17]. To investigate whether carbohydrate was associated with flat fruit abortion, we detected the sugar and starch content in seed and flesh for round (‘JH’), viable flat (‘ZH’), and aborting flat (‘XJ2’) peach at 15 DAFB, when all these three peach types still under healthy growth status without fruit dropping phenomenon. In this section, much lower contents of starch and sucrose in ‘XJ2’ were observed than those in viable flat and round peach in seed and fruit flesh (Fig. 1e). This observation was also consistent with their genotypes at flat fruit abortion locus, showing af/af for ‘XJ2’, Af/af for ‘ZH’ and Af/Af for ‘JH’. These results showed that the deficiency of sugar and starch in seed might play vital roles in flat fruit abortion, indicating that carbohydrate metabolism participated in fruit abortion process.

**Identification and expression analysis of PpSnRK1γ**

In order to elucidate the genetic basis for flat fruit abortion, candidate gene identification was carried out. We have already identified a 1.67-Mb chromosome inversion co-segregated with flat fruit shape and validated it in 336 peach accessions [8] (Fig. 2a). One key gene PpOFP1 has been validated in controlling flat fruit shape [8, 12]. Using transcriptome data generated in our previous study [7], only 7 differentially expressed genes (DEGs) were identified in this 1.67 Mb inversion region, including Prupe.6G303900, Prupe.6G311400, Prupe.6G313000, Prupe.6G314000, Prupe.6G314100, Prupe.6G317200, and Prupe.6G319200. According to their genotypes, 5 of them with FPKM = 0 at some fruit developmental stages were further eliminated. One of the left two genes was annotated as unknown function protein and the other was ribonuclease, which showed no correlations with sugar and starch metabolism.

We have already analyzed the four genes around the two breakpoints of this inversion, and found that the expression levels of Prupe.6G290900 (PpOFP1, flat fruit shape gene) and Prupe.6G323700 were both related to the allelic genotypes of flat and round peach [8]. Given that the close position between flat shape and flat fruit abortion locus, and the recessive trait, Prupe.6G323700 was hypothesized as the candidate gene responsible for flat fruit abortion, which was annotated as γ subunit of SNF1-related protein kinase. In present study, we identified the possible factors for flat fruit abortion, which was the
deficiency of sucrose and starch in seeds, but not the unaccomplished fertilization process. Therefore, gene expression was firstly performed in seeds at 15 DAFB and 30 DAFB. The real-time PCR results showed that Prupe.6G323700 had much higher expression in round than viable flat and aborting flat peach (Fig. 2b). Gene expression pattern was consistent with their genotypes, as fruit abortion trait was recessive [6, 8]. As well, tissue specific analysis showed that this gene was mostly expressed in seed and fruit (Fig. 2c), which further indicated the reliability of this candidate gene. Not only in seeds, gene expression was also performed in fruits. During fruit development, the candidate gene was much less expressed in ‘XJ2’ than in ‘ZH’ and ‘JH’, which suggested that this gene might play roles both in seeds and fruits (Fig. 2d). These results indicated that Prupe.6G323700 might be a critical candidate gene regulating flat fruit abortion. According to its functional annotation, we named it PpSnRK1γ.

Subcellular localization of PpSnRK1γ

To further understand the potential gene function of PpSnRK1γ, subcellular localization assay was carried out. Three vectors, 35S:PpSnRK1γ-GFP, 35S:GFP and 35S:NLS-mKate, were used for transient expression in tobacco (N. benthamiana) leaves. The 35S:NLS-mKate was specifically localized in nucleus. The 35S:PpSnRK1γ-GFP and 35S:GFP were co-transformed with 35S:NLS-mKate respectively. In the present study, the PpSnRK1γ-GFP fusion protein was localized in nucleus (Fig. 3), which was consistent with other research [42]. This result indicated that PpSnRK1γ might play functions by interacting with other related genes.

Phylogenetic analysis of SnRK gene family

To fully understanding the SnRK gene family in peach, genome wide gene identification was carried out. Firstly, we searched and downloaded the 43 SnRK genes in Arabidopsis from National Center for Biotechnology Information (NCBI), including three SnRK1αs, three SnRK1βs, two SnRK1γs, 10 SnRK2s, and 25 SnRK3s. We searched the similar genes in peach genome using local blast [43] and made gene annotation using Mercator [44]. As shown, one SnRK1α, three SnRK1βs, three SnRK1γs, seven SnRK2s, and 17 SnRK3s were identified (Supplementary Figure 2). The candidate gene identified in our study, PpSnRK1γ, was clustered with the γ subunit gene and closely adjacent to ATSNF4 (Fig. 4) which had been proved to be a functional γ subunit [45].

A chromosome inversion alters promoter activity

To further demonstrate that PpSnRK1γ expression levels were reduced by the 1.67Mb chromosome inversion, different parts of its promoter were analyzed as shown P1, P2, P3 in Fig. 2a. These promoter fragments and their re-combinations were fused with luciferase reporter separately. Transient expressions were carried out using tobacco leaves through Agrobacterium tumefaciens mediated methods. As expected, the luciferase reporter gene driven by P2+P1 fragment, which was the promoter sequence from round peach, was highly expressed than the other promoter fragments, especially higher than P3RC+P1, which was the promoter of flat peach (Fig. 5a,b), indicating that this 1.67-Mb inversion changed and reduced the promoter activity of PpSnRK1γ. Furthermore, this result was verified by gene expression in
'ZPT15' (flat fruit shape) and its bud mutant 'ZPT15-Mut' (round fruit shape) peach fruits, which showed much higher expression in 'ZPT15-Mut' (Supplementary Figure 3). All these results illustrated that the 1.67Mb inversion affected the promoter activity of *PpSnRK1γ*, resulting in its much lower expression in viable and aborting flat peach than the round one.

**Overexpression of** *PpSnRK1γ* **in tomato increased sugar and starch contents**

Due to the technical limitation of stable transformation in peach, we could not confirm the function of *PpSnRK1γ* by generating transgenic peach lines. As an alternative approach, we expressed *PpSnRK1γ* in the Micro-Tom tomato genotype. Ten transgenic lines with different levels of *PpSnRK1γ* expression were generated (Fig. 6a). Three of them with much higher expression levels, OE1, OE3 and OE9, were selected for sugar and starch contents determination. The results showed that the sugar and starch contents in these three OE lines were significantly higher than those in wild type (WT) (Fig. 6b) (*P* < 0.05), indicating that *PpSnRK1γ* had function in sugar and starch biosynthesis.

**Carbohydrate metabolism related genes were enriched**

During fruit development, differentially expressed genes (DEGs) were identified using transcriptome data (SRA: SRP116734) generated in our previous study [7]. In present study, we only considered the last two developmental stage from 15 DAFB to 55 DAFB. Totally, 1350 DEGs were identified and 43 of which were related to carbohydrate metabolism. 19 of 43 were highly expressed in ‘ZH’ (flat peach) and 24 in ‘HY’ (round peach) (Fig. 7a). Considering all the 31 SnRK genes identified in peach genome, only *PpSnRK1γ* was differentially expressed, which further suggested its role in carbohydrate metabolism during fruit development.

**Transient expression and comparative transcriptome analysis in peach**

Although stable transformation in peach was limited, transient over-expression in peach fruit was executable. To validate the gene function, the 35S:*PpSnRK1γ* was transiently expressed in ‘Hakuho’ peach cultivar at 50 DAFB using 35S:GFP as control. After transformation, samples were collected for sugar and starch contents determination, and also for comparative transcriptome analysis. The results showed that the sugar and starch contents were significantly increased in transient over-expression peach fruit (Fig. 7b), which further validated the gene function in carbohydrate metabolism. In addition, comparative transcriptome analysis between transient and the control samples found that 411 genes were differentially expressed and some of which were enriched in polysaccharide, glucan, carbohydrate metabolic process (Fig. 7c), including 176 up-regulated and 235 down-regulated genes (Supplementary Table 1). Comparative transcriptome analysis and GO enrichment facilitated to propose a model for fruit abortion (Fig. 8). Up-regulation of *PpSnRK1γ* affected carbohydrate metabolism by down-regulating *T6P*, *VIN* and *INV* genes, which further increased sugar and starch content in seed and fruit, resulting in fruit set. On the opposite, the flat fruit would abort (Fig. 8).

**Discussion**
Fruit abortion has great influence on fruit production and food security. In peach, fruit abortion exists severely in the whole tree of some flat peach individuals. The aborting flat peach progenies were observed in a F2 population generated from flat ('Ferjalou Jalousia') and round ('Fantasia') cultivars, which could be explained either by a single dominant gene (S/s) or by two tightly linked gene (S/s and Af/af) [6]. It was reported that quite a few fruits could be abnormally mature with no seeds and cracking fruit phenotypes in these aborting flat peach progenies [11]. In addition, overexpression the flat shape gene, *PpOP1*, did not result in fruit abortion in tomato [8] and *Arabidopsis* [12]. These results indicated that *PpOP1* was the flat shape gene and the flat fruit abortion trait should be controlled by the adjacent *Af* gene locus.

To find out the casual gene responsible for fruit abortion, physiological factors were characterized. For most flowering plants, pollination and fertilization is a pre-requisite for fruit and seed set. In this study, we found that the pollination and fertilization processes were accomplished in aborting flat peach cultivar 'XJ2', which indicated that other processes should be involved in peach fruit abortion (Fig. 1c,d). Seed and fruit set are established soon after fertilization and transit from ovule to seed and ovary to fruit. During this process, cell division, expansion and coordinated development of seed and fruit take place immediately, including the accumulation of sugars and starch [16, 18, 46]. It has been reported that fruit and seed abortion could be induced by photoassimilate limitation in grain [47] and fruit crops [48], such as sugars and starch [13, 49]. Under optimal conditions, fruit and seed set are accomplished with sufficient nutrients, including sucrose, glucose and starch [17]. In this study, we determined the contents of sugars and starch in seeds of aborting flat, viable flat and round peach at 15 DAFB to clarify the possible reason for flat fruit abortion. The results showed that the sucrose and starch contents were significantly reduced in aborting flat fruit, which suggested that carbohydrate metabolism might be involved in fruit abortion process (Fig. 1e). This finding was consistent with the studies on fruit and seed abortion in grain and fruit crops, and could enhance the understanding of fruit abortion in other fruit crops.

The SnRK1 subfamily comprise three subunits which play function as heterotrimeric holoenzyme, including a catalytic α subunit, a regulatory γ or βγ subunit, and β subunit as a scaffold linking α and γ subunits [35, 36]. It has been reported that the α subunit has function in regulating sugar and starch metabolism [37]. For instance, SnRK1-antisense seeds have maturation defects and result in seeds abortion by interaction of carbohydrate and hormone metabolism in pea (*Pisum sativum*) [40, 41]. As well, overexpression of *PpSnRK1a* in tomato has increased sugar and starch contents, and regulated fruit maturity and salt stress in peach [33, 34]. For β subunit, it has also been reported be involved in sugar signaling [36]. For γ subunit, it can interact with α subunit and SnRK2s [45, 50]. Although *KINβγ* was reported in controlling pollen development [42], hybridization between ‘JH’ (male) and ‘XJ2’ (female) was also performed in this study, which still did not alter the fruit abortion fate (data not shown). This result indicated that *PpSnRK1γ* should play roles in other aspects but not pollen development.

Little is known about the function of γ subunit in sugar metabolism, especially in regulating fruit and seed abortion. In this study, gene function of *PpSnRK1γ*, was validated using stable ectopic
transformation in tomato and transient overexpression in peach, which both increased the sugar and starch contents (Fig. 6b,7b). However, in this study, we further found that $PpSnRK1\gamma$ could not interact with $PpSnRK1\alpha$ (data not shown), which indicated that the $\beta$ subunit might be necessary in linking $PpSnRK1\alpha$ and $PpSnRK1\gamma$[35, 36]. After transient overexpression of $PpSnRK1\gamma$ in peach fruit, 411 genes were differentially expressed and some of which were enriched in polysaccharide, glucan, carbohydrate metabolic process (Fig. 7c).

Although the expression levels of $PpSnRK1\gamma$ were much higher at early fruit developmental stages than those at late fruit developmental stages (Fig. 2d), the sugar contents were much higher at late fruit developmental stages (Supplementary Fig. 4). These results indicated that $PpSnRK1\gamma$ might be critical for early fruit and seed development, and could confer sugar biosynthesis in mature peach fruit together with some other related genes. It has been reported that flat peach had much favorite flavor than round peach [3, 4] and there was one soluble solid content (SSC) QTL around the fruit shape locus [51, 52]. By evaluating the SSC for 12 flat and 28 round peaches, we confirmed that the SSC in flat peach was much higher than that in round peach (Supplementary Fig. 5a). Hence, to make sure whether $PpSnRK1\gamma$ could confer much favorite flavor in flat peach fruit, its expression was analyzed at fruit maturation stage, which found that the flat peach cultivars had much higher expression levels than the round ones (Supplementary Fig. 5b). In addition, we used another two cultivars to perform gene expression during fruit development stage, which showed much higher expression in flat peach (‘ZPT10’) than round peach (‘HY’) at fruit maturation stage (Supplementary Fig. 6). Much higher expression of $PpSnRK1\gamma$ at fruit maturation stage might explain why the flat peach has much better flavor than round one. However, the opposite result of $PpSnRK1\gamma$ activation in flat peaches at fruit ripening stage still need to be well explored in the future.

Conclusions

In this study, the physiological factors and genetic basis for flat peach abortion were investigated. we have revealed that the fertilization process was accomplished and the reduction of sucrose and starch contents might result in flat fruit abortion. A key candidate gene, $PpSnRK1\gamma$, was identified by integrating gene expression analysis, comparative transcriptome analysis and gene transformation. A 1.67-Mb inversion co-segregated with flat fruit shape altered the promoter activity of $PpSnRK1\gamma$, resulting in much lower expression in aborting flat peach. Ectopic transformation in tomato and transient overexpression in peach fruit have shown that $PpSnRK1\gamma$ could increase sugar and starch contents. Comparative transcriptome analysis further confirmed that $PpSnRK1\gamma$ participated in carbohydrate metabolism. This study clarified the reason for flat fruit abortion and identified a critical candidate gene, $PpSnRK1\gamma$, might be responsible for flat fruit abortion in peach. The results would provide great help in peach breeding and facilitate gene identification for fruit abortion in other plant species.

Methods

Plant materials and sample collection
Three peach cultivars were used in this study, including one round peach ‘Zhong Nong Jin Hui’ (‘JH’), one viable flat peach ‘Zao Huang Pan Tao’ (‘ZH’) and one aborting flat peach ‘Xinjiang Pan Tao 2#’ (‘XJ2’) for which the fruit aborted at around 30 DAFB. ‘JH’ and ‘ZH’ have similar developmental period. We collected fruit samples at 0, 7, 15, 30, 40, 55, 65 DAFB for sugar and starch content measurements, check diameter measurement and gene expression analyses. Six fruits per stage were measured for the check diameter. Fruit flesh and seeds were separated at 15 DAFB, when developmental seeds were visible and could be stripped out. All cultivars used in this study were grown in the field of National Horticulture Germplasm Resources Center at Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences (34°42′N, 113°42′E).

**Pollen tube elongation**

Flowers of ‘JH’, ‘ZH’ and ‘XJ2’ were emasculated and pollinated with pollen collected from ‘Zhong Tao Hong Yu’ (‘HY’). At 12 hours after pollination, the pistils were collected and incubated in a solution of acetic acid : ethanol at a 1:9 ratio [53] for overnight. The pistils were then passed through an ethanol series (90%, 70% and 50%) and water (three times) at 3-mins intervals separately before they were immersed in 1M NaOH for overnight and stained with 0.1% (w/v) aniline blue dissolved by 0.1 M K$_3$PO$_4$ in dark for than 1 h. Stained pollen tubes in the pistils were observed using an epi-fluorescence microscope fitted with a camera (DP71; Olympus).

**Ploidy detection using flow cytometry**

Nuclei were isolated from young leaves and 15-day-old seeds of ‘XJ2’, and run through a flow-cytometer (PARTEC, Germany) after they were stained with DAPI solution (PARTEC, HR-B) following a protocol reported by Guo et al [54]. Three replicates were carried out in this part.

**RNA extraction and gene expression analysis**

The total RNA was extracted from the three cultivars during fruit development using an RNA extraction kit (Waryong, China). First-strand cDNA was synthesized from 0.5 μg of total RNA in a 10-μL reaction volume by ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). Quantitative PCR (qPCR) was conducted on LightCycler 480 (Roche) using SYBR mix (Roche) with the following procedure: 95°C for 5 min, followed by 45 cycles at 95°C for 10 s, 58°C for 10 s and 72°C for 20 s. The relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method [55]. Primers used in this experiment are listed in **Supplementary Table 2**.

**Subcellular localization**

The coding sequence of $PpSnRK1\gamma$ without stop codon was amplified from ‘JH’ using PCR primers listed in **Supplementary Table 2** and in-frame fused to the N-terminal of the GFP in the plant binary expression vector $pBWA(V)HS$-$Glosgfp$ to generate the $35S:PpSnRK1\gamma$-$GFP$ construct. One step cloning kit (Novoprotein, China) was used to form recombination vector. The $35S$-$GFP$ construct in the original vector
was used as a control and 35S:NLS-mKate was used as a nucleus localization marker. *Agrobacterium tumefaciens* strain GV3101 was transformed with these three vectors separately and cultivated in LB medium containing 50 μg/mL kanamycin under 28°C. After cultivation, the Agrobacterium cells were re-suspended with infiltration buffer containing 10 mM MgCl₂, 10 mM MES, and 200 μM acetosyringone to OD600 of 0.6–1.0, and placed at room temperature for 2 h. The cells containing 35S:PpSnRK1γ-GFP and 35S:GFP were mixed with the same volume of cells containing 35S:NLS-mKate, and injected into leaf tissues of tobacco (*N. benthamiana*) using a 1-mL syringe without needle. After infiltration, the tobacco plants were firstly placed in dark at room temperature for 12 h and then moved to conditions of 16 h light and 8 h dark for 48 h. The GFP and mKate (monomeric version of Katushka) fluorescence were observed using a confocal laser scanning microscope (TCS SP5, Leica, Germany).

**Promoter activity analysis**

Based on the chromosome position of the 1.67-Mb inversion, promoter segments with different length were cloned using PCR. As shown in Fig 2c, promoters of P1, P2, P3 and their recombination were used for luciferase activity analysis. The promoter regions of P2+P1, P3RC+P1 and P1, which could stand for the main part of promoter regions of two different alleles in flat and round peach were used for promoter activity analysis using *pGreen-II-0800-LUC* vector. The corresponding vectors were constructed using one step clone kit (NovoProtein, China) and named as *pGreen+P1*, *pGreen+P2+P1* and *pGreen+P3RC+P1*. The fragment of P2+P1 was the promoter region of *PpSnRK1γ* in round peach, while P3RC+P1 was promoter in flat peach. P1 was truncated promoter (Fig. 2a). The transient expression assay was conducted following the protocol described above, using the empty vector *pGreen* as a negative control. Luciferase activities were evaluated using Tanon-5200Multi machine (Tanon, China). Primers used in this experiment are listed in Supplementary Table 2.

**Sugar and starch contents quantification**

For sugar extraction, 100 mg ground fresh sample and 10 mL 80% ethanol were added into a 15-mL tube and incubated in 80°C water bath for 30 min, followed by centrifuging at 12000 x g for 15 min. The supernatant was transferred into a new tube for sugar analysis and the left item was used for starch quantification. For sucrose, fructose, glucose and starch quantification, the measurement kits were used (Bioengineering, China) according to the corresponding protocols.

**RNA-seq and data analysis**

RNA was extracted from peach fruit in transient overexpression assay. Three biological replicates were conducted and the total RNA were mixed together to construct one library for experimental and control samples respectively. Total RNA of 20 μg was sent to ANBOROAD (Beijing, China) company for library construction and sequencing. The Illumina HiSeq™ X Ten platform in paired-end mode was used for sequencing. The raw reads were generated and the low quality reads were filtered to obtain clean reads using FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The clean reads were mapped to reference peach genome (release version 2.0_a2.1) [56] using tophat and the FPKM (fragments per
kilobase of exon per million reads mapped) and differentially expressed genes (DEGs) were calculated using cufflink [57].

**Phylogenetic and gene structure analysis**

The SnRK family genes in Arabidopsis were searched and downloaded from the National Center for Biotechnology Information (NCBI), including 3 SnRK1αs, 3 SnRK1βs, 2 SnRK1γs, 10 SnRK2s, and 25 SnRK3s. The amino acid sequences of these SnRK genes were downloaded. These 40 genes were used as queries in identifying SnRK family genes in peach genome. The local blast was performed using blast (Version 2.2.26) [43] with the following parameters: blastall -p blastp -m 8 -d -o. The blast results were further managed by keeping E-value equal to 0.0 or identity greater than 70%. The phylogenetic tree was constructed with Mega7 [58] using neighbor-joining method with bootstrap of 1000 and displayed with ITOL (https://itol.embl.de/). The gene structure was displayed using GSDS (http://gsds.cbi.pku.edu.cn/index.php).

**Gene transformation in tomato**

The full-length coding region of *PpSnRK1γ* was amplified from fruit cDNA sample of ‘JH’ by PCR using a high fidelity DNA polymerase (KOD-201, TOYOBO, Japan). The products were cloned into the *pBI121* vector downstream of the cauliflower mosaic virus (CaMV) 35S promoter using a one-step construction kit (C112, Vazyme, China). The constructs were then transformed into Micro-Tom tomato using *Agrobacterium tumefaciens* GV3101 following protocol described in Sun et al [59]. After transformation, transgenic lines were obtained and gene expression levels were analyzed. The three higher expression lines (OE1, OE3, OE9) and wild type (WT) were further used to determine the sugar and starch content in fruit at maturation stage. Primers used in this experiment are listed in Supplementary Table 2.

**Transient overexpression in peach fruit**

To further understand the function of *PpSnRK1γ*, transient expression was carried out in peach fruits. The over-expression vector was the same as it used in tomato transformation. The peach cultivar used for transient expression was ‘Hakuho’ (HK), due to its white flesh and hesitation in browning during exposing in air. The peach fruits were collected at 50 DAFB and cut into 1cm thick cubes and then infiltrated by submerging into *Agrobacterium tumefaciens* suspension under a vacuum of -70kPa for 30 min as described by Liu et al [60]. Then the fruit sections were cultivated on MS medium for two days. After that the sections were collected and stored under -80°C for gene expression and sugar/starch contents analysis. Three replicates were performed in this section.

**Abbreviations**

SnRK1, sucrose non-fermenting 1 related kinase.

DAFB, days after full bloom.
OE, overexpression.
DEGs, differentially expressed genes.
SSC, soluble solid content.
JH, Zhongnong Jin Hui.
ZH, Zaohuang Pan Tao.
XJ2, Xinjiang Pan Tao 2#.
ZPT15, Zhongpantao 15#.
ZPT15-Mut, Zhongpantao 15# mutant.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Not applicable.

Availability of data and materials
Raw data generated in this study were deposited in the NCBI Short Read Archive (SRA) under the accession PRJNA633964. The data under accession PRJNA401307 generated in our previous study was also used. All other relevant data contained within the paper are available in the paper and Supplementary Files.

Competing interests
The authors declare that they have no conflict of interest to this work.

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Authors’ contributions

L.W. and W.G. designed and managed the project. J.G., G.Z., W.F., C.C. and X.W. collected materials. J.G., Y.L., J.W. and L.G. prepared and purified DNA and RNA samples. J.G. performed data analyses. J.G. wrote the paper. L.W., K.C., C.D. and J-L.Y. revised the paper. All authors read and approved the final manuscript.

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