Genome-wide survey of sucrose non-fermenting 1-related protein kinase 2 in Rosaceae and expression analysis of PbrSnRK2 in response to ABA stress

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Abstract

Background

The members of the sucrose non-fermenting 1-related protein kinase 2 (SnRK2) family are specific serine/threonine protein kinases in plants, which play important roles in stress signal transduction and adaptation. Because of these positive regulatory roles in response to adversity, the genes encoding them are considered potential candidates for breeding of plants for disease resistance and genetic improvement. However, there is far less information about this kinase family and the function of these genes has not been explored in Rosaceae.

Results

A genome-wide survey and analysis of the genes encoding members of the SnRK2 family were performed in pear (*Pyrus bretschneideri*) and seven other Rosaceae species. A total of 71 SnRK2 genes were identified from the eight Rosaceae species and classified into three subgroups based on phylogenetic analysis and structural characteristics. Purifying selection played a crucial role in the evolution of SnRK2 genes, and whole-genome duplication and dispersed duplication were the main forces underlying the characteristics of the SnRK2 gene family in Rosaceae. Transcriptome data and qRT-PCR assays revealed that the distribution of PbrSnRK2s was very extensive including across the roots, leaves, pollen, styles, and flowers, although most of them were mainly expressed in leaves. In addition, under stress conditions, the transcript levels of some of the genes were upregulated in leaves in response to ABA treatments.

Conclusions

This study provides useful information and a theoretical introduction for the study of the evolution, expression, and functions of the SnRK2 gene family in plants.

Background

During growth and agricultural production, plants are frequently affected by a variety of biotic and abiotic stresses such as waterlogging, salinity, and cold and drought stresses. Because of their immovable nature and the inability to choose suitable environmental conditions, plants must change their characteristics to adapt to adverse environments. To cope with these stresses, the plants have established a network of defense-related metabolic mechanisms by regulating the production of beneficial substances or the expressions of related genes over the long course of evolution [1]. As important adversity signal regulators, protein kinases and phosphorylation play an essential roles in the process of recognizing and transmitting stress signals to different parts of cells [2]. Sucrose non-fermenting 1-related protein kinase subfamily 2 (SnRK2) is a plant-specific serine/threonine (Ser/Thr) family of protein kinases that are particularly involved in the adversity stress response and play an

important roles in plant stress signal transduction [3–5]. Recent studies have also shown that SnRK2s are involved in plant growth and development, as well as in responses to various stress signals such as osmotic stress, saline stress and ABA signaling [4, 6]. For example, SnRK2.6 and SnRK2.8 act as regulators of the carbohydrate metabolism and the drought resistance response in *Arabidopsis*, respectively [7, 8], whereas SnRK2.4 and SnRK2.10 play essential roles in root growth and architecture under saline conditions in *Arabidopsis* [9]. Furthermore, in other species, overexpression of SnRK2.8 enhances resistance to various adverse stresses in wheat [10], whereas ZmSAPK8, OsSAPK8, GhSnRK2.6, and MpSnRK2.10 are related to salt tolerance in maize, rice, cotton, and apple, respectively [11–14].

Similar to other SnRK family members, SnRK2 proteins possess a typical structure composed of structurally and functionally conserved domains. The amino acid sequence of SnRK2s can be divided into two regions: the highly conserved N-terminal catalytic domain, which is similar to that of SNF1/AMP kinases, and the relatively differentiated C-terminal regulatory domain, which is short [15]. In addition, earlier research found that the N-terminus of SnRK2 is a highly conserved kinase region, which is closely related to its kinase activity, and contains the highly conserved ATP-binding site (DXGXGNFGVAXL) and Ser/Thr kinase activity domain (KICDFGYSKSXXXHGXPK) [16]. It was also found that the C-terminus was composed of two subdomains (Domains I and II), which contain stretches of acidic patches of either glutamic acid or aspartic acid [16]. Domain I is a region shared by all members of the SnRK2 family that is located 20 amino acids away from the catalytic domain and is the necessary structural basis for independent ABA response to osmotic stress [17]. Domain II exists mainly in the members of the third subfamily and is the necessary structural basis for the response to ABA signals [17].

Differences in the functions of SnRK2 genes have been widely determined. In contrast to other protein kinases, SnRK2s are essential components and positive regulators of the ABA signaling pathway that are specific to plants. Based on the structural characteristics of the SnRK2 C-terminus and their response to ABA signals, SnRK2s were divided into three subfamilies [14]. Among them, members of Group I are rich in glutamic acid at the C-terminus and barely respond to ABA signals. In contrast, members of Groups II and Group III are rich in aspartic acid; the SnRK2s in Group II do not or weakly respond to ABA signals, whereas SnRK2s in Group III are strongly activated by ABA [4, 18]. Furthermore, the promoter region of SnRK2s usually contains *cis*-acting regulatory elements that are involved in stress responses, such as drought-responsive elements, ABA-responsive elements, and cold-responsive elements [6]. Previous research has shown that the kinase activity of SnRK2 is related to the reversible phosphorylation of key amino acid sites. For example, the kinase activity of NtOSAK is regulated by the phosphorylation of Ser154 and Ser158 within its activated ring in the tobacco [19]. Moreover, there are some differences in the kinase activity of SnRK2s because of their different phosphorylation sites. Regarding SnRK2.6 gene in *Arabidopsis*, the phosphorylation sites Ser7, Ser18, Ser29, and Ser43 are mainly related to the regulation of self-phosphorylation, whereas Ser175 and Thr176 are primarily associated with kinase activity [20].

Because of the critical regulatory functions of SnRK2 genes in plant responses to various adversity stresses and developmental processes, the SnRK2 gene family has been widely studied in the model

plant *Arabidopsis* as well as in non-model plants such as rice, maize, wheat, soybean, apple, and rubber [6, 15, 18, 21–23]. Compared with other species, the members of the SnRK2 gene family have not been extensively examined in Rosaceae. Pear (*Pyrus bretschneideri*) is a member of the Rosaceae family and a commercially important crop that is cultivated in temperate regions worldwide. In addition, the genome of the Chinese white pear (*P. bretschneideri* Rehd. cv. 'Dangshansuli') has been fully sequenced. In recent years, the development of high-throughput sequencing technology has allowed genome sequencing in many organisms for genomic analysis. For example, genome sequences are available for seven other Rosaceae species (apple, strawberry, peach, Chinese plum, black raspberry, cherry, and European pear), which provides an opportunity to further analyze the SnRK2 gene family in Rosaceae. Therefore, the present study aimed to (1) annotate the full-length *SnRK2* genes in Chinese white pear and in additional seven Rosaceae fruit species; (2) explore their responses to adversity stresses, as elicited by salinity, ABA, drought and osmotic stress; and (3) infer their expansion, evolutionary history, and expression patterns, thus providing valuable information for further investigation of SnRK2 gene functions in Rosaceae. These results will be useful for revealing the mechanisms of stress resistance in fruit trees and will lay a foundation for further investigations that will use genetic engineering for molecular breeding.

Results

Identification and classification of *SnRK2* genes in Rosaceae

To investigate the *SnRK2* gene family in Rosaceae, the protein sequences of SnRK2s from *Arabidopsis* and rice were used as queries. Moreover, an Hidden Markov Model (HMM) search using the *SnRK2* gene domain HMM profile (PF00069.1) was used to screen the Rosaceae genome, respectively. A total of 318 candidate SnRK2 genes were screened using these two strategies. Finally, the online program SMART (http://smart.embl-heidelberg.de/) was used to check the Ser/Thr protein kinase catalytic domains, followed by the removal of redundant sequences, incomplete gene sequences, and transcripts of the same gene. Subsequently, 71 nonredundant SnRK2 genes were identified in the Rosaceae genome. Among them, 10 SnRK2 proteins were identified in Chinese white pear (PbrSnRK2s), 14 in apple (MdSnRK2s), 8 in strawberry (FvSnRK2s), 7 in peach (PpeSnRK2s), 7 in Chinese plum (PmSnRK2s), 5 in black raspberry (RocSnRK2s), 7 in cherry (PavSnRK2s), and 13 in European pear (PcSnRK2s) (Fig. 1).

To classify and investigate the evolutionary relationships among SnRK2 genes, a phylogenetic tree was constructed using multiple sequence alignment of SnRK2 protein sequences from the eight Rosaceae species, *Arabidopsis*, and rice. The results of this analysis showed that the SnRK2 gene family was clustered into three well-supported clades (Groups I, II, and III; Fig. 1), which is consistent with the findings of a previous study performed in *Arabidopsis* and rice [15]. Among them, 22 members belonged to Group I, 25 to Group II, and 24 to Group III (Fig. 1).

Characteristics of the SnRK2 genes in Rosaceae

To study the functions of the SnRK2 proteins, we performed systematic analysis of the physicochemical properties of the SnRK2 proteins in Rosaceae. We found that the SnRK2 protein sequences ranged from

198 to 891 amino acids, and that most of them contained 220 to 402 amino acids. The isoelectric point of 87.3% of the SnRK2 proteins was acidic, which indicates that SnRK2 proteins from Rosaceae are rich in acidic amino acids. Moreover, the molecular weights of these proteins ranged from 30.09 to 85.3 kDa (Table 1). The negative and positive GRAVY scores of proteins reflect their hydrophobicity and hydrophilicity, respectively [24]. The grand average of the hydropathy scores of all SnRK2 proteins was negative in Rosaceae, which indicates that these proteins are hydrophilic. In addition, we found that the aliphatic index ranged from 80.76 to 94.08 for the SnRK2 proteins from Rosaceae, which indicated that all of them are thermally stable (Table 1).

Structural diversity of SnRK2 genes in pear

The analysis of the arrangement of introns and exons can provide unique insights into the evolution and functions of gene families [25]. To better understand the evolutionary model of *PbrSnRK2* genes, exon/intron analyses were performed by aligning genomic sequences with their corresponding coding domains from SnRKs in pear and *Arabidopsis*. The number of exons identified in the members of the SnRK2 gene family ranged from 4 to 10 in pear and *Arabidopsis* (Fig. 2). Most members of the individual groups exhibited a different number of exons/introns and a varying length of the coding sequence in pear, which confirmed the phylogenetic classification of SnRK2 genes. For example, SnRK2 genes in subgroup I in pear contained 9 exons. Most members of subgroup II included 5–6 exons, except *PbrSnRK2.4* containing 9 exons. In addition, subgroup III contained a number of exons (i.e., 7–8), except *PbrSnRK2.10* containing 4 exons (Fig. 2). Thus, the conservation of the number of exons in each subgroup strongly supports the close evolutionary relationship of PbrSnRK2 genes. This may be the result of replication events in the evolution of the gene family, which means that these subgroups originated via different evolutionary paths.

Sequence alignment of PbrSnRK2 genes

To understand the functional characteristics of PbrSnRK2 genes and their conserved domains, multiple sequence alignment of PbrSnRK2 and AtSnRK2 proteins was performed. It was found that all members of the PbrSnRK2 family have two conserved kinase domains in the N-terminal regions: an ATP-binding signature containing a lysine residue as an ATP-binding site and a Ser/Thr protein kinase active site signature (Fig. 3). In addition, the C-terminus included two distinct domains, which were termed Domain I and Domain II. Domain I is necessary in all SnRK2s and is needed for activation by osmotic stress, whereas Domain II only exists in strongly ABA-responsive kinases [26].

Synteny analysis of the SnRK2 genes in Rosaceae

To explore the evolutionary process of SnRK2 genes, a comparative syntenic map was constructed for Rosaceae. The *PbrSnRK2* genes were distributed on 4 out of the 17 pear chromosomes, with 4 SnRK2 genes anchored on chromosome 15 (Fig. 4). Thirteen genes were assigned to 7 of the 17 chromosomes in apple, with 5 genes anchored to chromosome 15. In addition, 7 genes were distributed on 5 of the 8 chromosomes in peach, with 3 genes anchored to chromosome 1. Finally, 8 genes were assigned to 4 of

the 7 chromosomes in strawberry, with 4 genes anchored to chromosome 5 (Fig. 4). Remarkably, similar to these observations, the SnRK2 genes in other Rosaceae species showed random chromosomal distributions (Fig. S1).

Analysis of the expansion of SnRK2 genes in Rosaceae

Several gene duplication patterns drive the evolution of protein-coding gene families, which include whole-genome duplication (WGD) or segmental duplication, tandem and segmental duplications, and rearrangements at the gene and chromosomal levels [27]. The origins of duplicated genes were explored in the SnRK2 gene family in eight Rosaceae genomes using the MCScanX package. Each member of the SnRK2 gene family was allocated to one of five different categories: WGD or segmental, singleton, proximal, tandem, or dispersed. Five types of duplication events contributed to the expansion of the SnRK2 gene family in Rosaceae: 50% WGD, 19.7% dispersed, 15.2% transposed, 9% proximal, and 6% tandem (Fig. 5). Among them, WGD events occurred in each of the Rosaceae species; in particular, 60% of the SnRK2 genes in Chinese white pear, 86% in apple, 57.1% in peach, and 50% in strawberry were duplicated and retained from WGD events compared with only 40% in black raspberry, 28.6% in Chinese plum, 28.6% in cherry, and 7.7% in European pear (Table S2). In addition, the proportions of dispersed SnRK2 gene duplication events in black raspberry (40%), European pear (30%), peach (28.6%), strawberry (28.6%), cherry (28.6%), and apple (7%) were assessed (Table S2). Therefore, gene losses, genome rearrangements, and RNA- and DNA-based transposed duplications may have occurred in these species. These results indicate that WGD and dispersed duplication play key roles in the expansion of the SnRK2 gene family in Rosaceae.

Ks value and Ka/Ks ratio reveal dates and driving forces of evolution

Purifying selection (negative selection) is the process via which disadvantageous mutations are removed, whereas Darwinian selection (positive selection) accumulates new advantageous mutations and spreads them throughout the population [27]. To identify the selection process that drove the evolution of the SnRK2 gene family, the Ka value and Ka/Ks ratio of its paralogs were examined in the eight Rosaceae species based on coding sequences. We found that all values were <1 in the studied Rosaceae species (Fig. 6A), implying that this family underwent a purifying selection pressure during its evolution in Rosaceae and that its evolution was very conservative.

The Ks value is extensively used to evaluate the evolutionary dates of WGD or segmental duplication events. To explore the evolutionary dates of the duplication events among the SnRK2 gene family members, Ks values were analyzed in the Rosaceae species. The results showed that the Ks values for the SnRK2 gene pairs ranged from 0.107 to 4.0487 in Rosaceae; moreover, the WGD gene pairs *PbrSnRK2.1– PbrSnRK2.2* (Ks, ~0.1028), *PbrSnRK2.7– PbrSnRK2.8* (Ks, ~0.1363), *PCP022180.1–PCP002062.1* (Ks, ~0.182), *MD01G1035000– MD15G1321000* (Ks, ~0.1644), *MD02G1166500–MD15G1279000* (Ks, ~0.1757), *MD04G1054400– MD06G1046300* (Ks, ~0.1635), *MD08G1187200–MD15G1373000* (Ks, ~0.1138), and *MD08G1236500– MD15G1428500* (Ks, ~0.107) indicated that some SnRK2 genes underwent WGD events more recently (30–45 MYA). Furthermore, other duplicated gene

pairs (such as *PbrSnRK2.5* and *PbrSnRK2.6*) possessed higher Ks values (2.26–4.0487), indicating that they might have stemmed from a more ancient duplication event (Fig. 6B).

Expressions of *PbrSnRK2* genes in different tissues

To understand the expression patterns and functional properties of SnRK2 genes in different tissues, we constructed a heat map at the transcriptional level using MeV to depict the overall expression patterns of SnRK2 genes in pear. The results showed that the SnRK2 genes were expressed in most organizations of pear, i.e., three genes (*PbrSnRK2.2*/*2.5*/*2.7*) were mainly expressed in fruits, two (*PbrSnRK2.3*/*2.8*) in leaves, and one (*PbrSnRK2.4*) in roots (Fig. S2). In addition, to validate the reliability of SnRK2 gene expression patterns based on the transcriptome data from pear, these SnRK2 genes were analyzed by qRT-PCR using gene-specific primers (Table S1). The results showed that SnRK2 genes were expressed in all tissues of pear, although there were differences in the absolute fold changes in the expression patterns between the two methods. For example, most genes were expressed in leaves and flowers, which indicated that SnRK2 genes mainly play important roles in these structures and respond to abiotic stress; in addition, two genes (*PbrSnRK2.1/2.2*) were mainly expressed in pollen and may play roles in regulating pollen germination (Fig. 7).

Expression profiles of *PbrSnRK2* genes under ABA treatment

Many studies have shown that SnRK2s play key roles in response to multiple abiotic stresses such as salinity, dehydration, and hyperosmotic stress [14]. Moreover, the members of the SnRK2 gene family are involved in the regulation of phytohormone pathway responses, particularly ABA signal transduction [4]. To explore the dynamic transcriptional changes in *PbrSnRK2* genes in response to ABA treatment, the expression levels of these genes in leaves of pear were evaluated by qRT-PCR under ABA (50 μM) treatment at four time points: 0, 3, 6, and 9 h. The results showed that in response to exogenous ABA application, the SnRK2 genes exhibited different expression patterns. For example, eight genes (*PbrSnRK2.1/2.2/2.3/2.4/2.6/2.7/2.8/2.9*) were activated by ABA, whereas the expressions of two genes (*PbrSnRK2.5/10*) remained unchanged at each time point (Fig. 8). Among them, the expression levels of *PbrSnRK2.2/2.3/2.6/2.7/2.9* were significantly upregulated at 9 h after ABA treatment. *PbrSnRK2.1* had a waving trend with ABA treatment, whereas *PbrSnRK2.8* was downregulated over time after ABA treatment.

Discussion

SnRK2 is a specific family of Ser/Thr protein kinases in plants that play important roles in the transduction of various signaling pathways, particularly in response to abiotic stress [4, 5]. With the completion of whole-genome sequencing in an increasing number of organisms, it has become possible to comprehensively analyze and study the functions of gene families from the genomic perspective. The members of the SnRK2 gene family have been identified and analyzed in many plant species [26, 28]; however, a comprehensive systematic investigation of this family remains limited in Rosaceae. Here, 71 SnRK2 genes were identified in Rosaceae and the same number of SnRK2 genes was identified in pear,

compared with 8 in *Arabidopsis*. Moreover, these SnRK2 genes were classified into three subfamilies, subgroups I, II, and III, which is consistent with the classification of other SnRK2 genes [15, 16]. In addition, apple (14) and black raspberry (5) exhibited the most and least found members of the SnRK2 gene family in Rosaceae, respectively. Recent genome-wide evidence revealed that pear and apple have undergone not only a recent WGD but also an ancient WGD event; however, strawberry, peach, Chinese plum, black raspberry, and cherry did not undergo this event [27]. Therefore, this WGD event likely led to the different numbers of SnRK2 genes detected in the investigated Rosaceae species.

To investigate the evolutionary process and history of SnRK2 genes in Rosaceae and to explore further their functions in response to various abiotic stresses, gene duplication events, gene syntenic relationships, and Ka/Ks values of SnRK2 genes were investigated in Rosaceae. Dispersed, tandem, proximal, transposed, and genome-wide duplications differentially contribute to the expansion of specific gene families in plant genomes [29]. The results of the synteny analysis performed here indicated that the SnRK2 genes in Chinese white pear, peach, apple, and strawberry were derived primarily from WGD events. Dispersed duplications were the major drivers of SnRK2 gene family expansion in European pear. Furthermore, the expansion of the SnRK2 gene family underwent five types of duplication events, with WGD events accounting for 50% of the SnRK2 genes in Rosaceae. Among these events, four duplicated gene pairs, i.e., *PbrSnRK2.1– PbrSnRK2.2*, *PbrSnRK2.7– PbrSnRK2.8*, *MD08G1236500– MD15G1428500*, and *MD08G1187200– MD15G1373000*, had lower Ks values (0.12, 0.13, 0.11, and 0.11, respectively), suggesting that they resulted from a recent duplication event. However, the Ks values of other duplicated gene pairs, i.e., *MD08G1187200– MD15G1321000* and *PbrSnRK2.5– PbrSnRK2.6*, and *pm02088– pm004465*, were > 3, which indicated that they resulted from a more ancient duplication event. This is similar to previous observation, i.e., > 90% of the increase in functional genes in the *Arabidopsis* lineage is due to expansion resulting from genome duplication events [30]. Therefore, these results indicated that WGD events play a key role in the expansion of the SnRK2 gene family and that SnRK2 genes function in response to various biological and abiotic stresses.

Purifying selection (negative selection) is the process via which deleterious mutations are eliminated. In contrast, Darwinian selection (positive selection) accumulates new advantageous mutations and spreads them across the population [31]. The Ka/Ks ratio was further calculated for exploring the selection process that drove the evolution of the SnRK2 gene family in Rosaceae. In this study, the Ka/Ks ratios of all paralogous genes were < 0.5. Previous studies have shown that the Ka/Ks ratio represents selection magnitude and direction measure: 1 indicates neutral evolution, < 1 indicates negative selection, and > 1 indicates positive selection [32]. Therefore, these results indicated that negative selection played a key role in the evolution of these SnRK2 genes.

Gene expression patterns can provide important clues for describing gene function. Therefore, information pertaining to the specific expressions of *PbrSnRK2* genes was analyzed in various tissues using public RNA-Seq datasets and qRT-PCR. The results revealed that *PbrSnRK2* genes exhibited diverse spatiotemporal expression patterns in different tissues of pear. For example, *PbrSnRK2.3*, *PbrSnRK2.5*, and *PbrSnRK2.8* were preferentially expressed in leaves, implying that the proteins encoded by them

possibly participate in leaf development and stomatal regulation in response to abiotic stress. Conversely, *PbrSnRK2.1*, *PbrSnRK2.2*, *PbrSnRK2.4*, *PbrSnRK2.7*, *PbrSnRK2.9*, and *PbrSnRK2.10* exhibited strong expression in germ cells such as flowers, pollen, and styles, thereby implying that their encoded proteins potentially participate in the reproductive growth of plants and adaptation to various adverse environments. Nevertheless, further studies are necessary to verify whether PbrSnRK2s play a key role in leaf development and the reproductive growth of plants in response to various abiotic stresses. Previous studies have shown that SnRK2s exhibit widespread expressions in various tissues of *Arabidopsis* as well as are involved in seed germination and growth [33], regulation of primary metabolism [7] and stomatal aperture [34], and responses to drought stress signaling. For example, *AtSnRK2.2* is expressed in both seeding and cell suspension [35], and *AtSnRK2.8* is mainly expressed in roots [36], whereas *AtSnRK2.6* and *AtSnRK2.7* are primarily expressed in stems and roots, respectively [7]. Furthermore, the expression patterns of SnRK2 genes have been reported in other species such as *MpSnRK2.4*, which is mainly expressed in fruit and may participate in fruit development in apple [23]; *HbSnRK2.2*, which is high expressed in leaf and flower tissues [15]; and *StSnRK2.4*, which is mainly expressed in shoot tissues and may be involved in responses to stress signaling [37].

Many studies have shown that SnRK2 genes play a key role in response to various environmental stresses such as salinity, low temperature, and drought [26]. Moreover, SnRK2 genes play important roles in the regulation of phytohormones, particularly of ABA signal transduction [28]. In this study, we explored the expression levels of *PbrSnRK2* genes in pear by treating the leaves with ABA. The results expanded our understanding of the functions of PbrSnRK2s in response to phytohormones and showed that all PbrSnRK2s but PbrSnRK2.5 and PbrSnRK2.10 were induced at different time points after ABA treatment. For example, PbrSnRK2.1, PbrSnRK2.3, PbrSnRK2.4, PbrSnRK2.7, and PbrSnRK2.9 were upregulated at 3 h after ABA treatment; among them, the expression levels of PbrSnRK2.3, PbrSnRK2.7, and PbrSnRK2.9 were significantly induced at 9 h after ABA treatment. In contrast, the expression levels of PbrSnRK2.8 were downregulated at 3 h after ABA treatment. These results indicated that the response of most SnRK2 genes to the various abiotic stresses is achieved via the ABA signaling pathway, which is especially involved in the regulation of salt stress and drought stress tolerance in pear. However, additional investigation is needed regarding the functional characterization of PbrSnRK2s. Furthermore, SnRK2 genes reportedly respond to various abiotic stresses in other species. Many of the SnRK2 genes (such as *SAPK8*, *SAPK9*, and *SAPK10*) were differentially regulated by ABA in different organs of rice [18]. The expression levels of *TaSnRK2.7* and *TaSnRK2.8* genes from wheat were induced in response to ABA treatment [4]. *MpSnRK2.1*, *MpSnRK2.8*, *MpSnRK2.9*, and *MpSnRK2.10* exhibited strong responses to ABA application in apple [23]. In *Arabidopsis*, *AtSnRK2.2*, *AtSnRK2.3*, and *AtSnRK2.6* are typically activated by ABA treatment, which can phosphorylate the ABA-responsive elements [20]. In rubber tree, *HbSnRK2.7* was predominant and exhibited strong responses to ABA treatment, thus playing a key role in the ABA signaling pathways [15].

Conclusion

In this study, 71 SnRK2 genes were identified and characterized in eight Rosaceae species; among them, 10 SnRK2 proteins were identified in Chinese white pear. The structural characteristics of the encoded proteins and phylogenetic analysis showed that the SnRK2 gene family was clustered into three well-supported clades (Groups I, II, and III) and that all the members of the PbrSnRK2 family had two conserved kinase domains in the N-terminal and C-terminal regions. In addition, synteny analyses revealed that SnRK2 genes underwent a purifying selection pressure during the evolution of Rosaceae and that WGD and dispersed duplication played key roles in the expansion of the SnRK2 gene family in Rosaceae. The relative expressions of the 10 SnRK2 genes varied among tissues and abiotic stresses, with prolonged ABA treatments showing that PbrSnRK2s are expressed in different tissues and respond to various abiotic stresses via the ABA signaling pathway. Our results represent a foundation for further studies that will dissect the structures and functions of the SnRK2 gene family in Rosaceae.

Materials And Methods

Identification of SnRK2 gene family members in Rosaceae

To identify the SnRK2 genes in Rosaceae species, multiple database searches were performed. The amino acid sequences of AtSnRK2s were downloaded from The *Arabidopsis* Information Resource (TAIR) (http://www.arabidopsis.org/) and the rice SnRK2 amino acid sequences were downloaded from Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html#). These amino acid sequences were used as queries in BLAST algorithm-based searches against pear (http://peargenome.njau.edu.cn/) and other Rosaceae genome databases (http://phytozome.jgi.doe.gov/pz/portal.html). In addition, the Chinese plum genome sequence was downloaded from the *Prunus mume* Genome Project (http://prunusmumegenome.bjfu.edu.cn/index.jsp). Furthermore, the seed alignment file for the SnRK2 domain (PF00069.25) obtained from the Pfam database was used to build an HMM file using the HMMER3 software package [38]. HMM searches were then performed against the local protein databases of Rosaceae using HMMER3. Finally, all obtained SnRK2 amino acid sequences were checked for the presence of SnRK2 family domains using Pfam (http://pfam.xfam.org) and SMART (http://smart.embl-heidelberg.de/); subsequently, sequences lacking the SnRK2 domain or with E-values of $>e^{-10}$ or redundant sequences were removed.

Sequence alignment and phylogenetic and structural analyses

Multiple sequence alignment of SnRK2 proteins in pear and *Arabidopsis* was performed using DNAMAN software. Program SCANPROSITE (http://www.prosite.expasy.org/scanprosite/) was used to detect the protein kinase conserved domains. Phylogenetic trees were constructed based on the SnRK2 amino acid sequences from *Arabidopsis*, rice, and Rosaceae species using the maximum likelihood method in MEGA6.0 (http://www.megasoftware.net/) [39], with amino acid sequences aligned using MUSCLE. The bootstrap test was performed with 1,000 replicates, and p-distance and pairwise deletion option parameters were selected. Furthermore, based on the cDNA sequences and their corresponding genomic

DNA sequences, the structures of the SnRK2 genes were analyzed using the Gene Structure Display Server v2.0 (http://gsds.cbi.pku.edu.cn/).

Chromosomal location, synteny analysis, and calculation of the Ka and Ks values

The information of chromosomal localization of SnRK2 genes was obtained from genome annotation documents. A method similar to that developed for PGGD (http://chibba.agtec.uga.edu/duplication/) was then performed for synteny analysis [40]. Initially, BLASTP was used to search for potential homologous gene pairs ($E < 1\ e^{-6}$) across multiple genomes. Subsequently, MCScanX was used to identify syntenic chains using homologous pairs as input [41]. In addition, MCScanX was used to identify tandem, whole-genome/segmental, proximal, and dispersed duplications in the SnRK2 gene family. The data were then plotted using Circos software [42]. The Ka and Ks substitution rates of syntenic gene pairs were identified by the MCScanX downstream analysis tools. The mean Ks values of orthologous SnRK2 gene pairs among Rosaceae species were calculated using all homologous gene pairs located in the same syntenic block. KaKs Calculator 2.0 was used to confirm the Ka and Ks values [27].

Plant treatments and the qRT-PCR assays

To examine the expression patterns of *PbrSnRK2* genes in response to abiotic stresses, the leaves of 3-month-old pear seedlings were sprayed with 50 μM ABA solutions. Samples were harvested from leaves at 0, 3, 6, and 9 h after treatment. All samples were frozen immediately in liquid nitrogen and maintained at −80°C for total RNA extraction. Total RNA was isolated independently from pear roots, stems, leaves, flowers, fruits, styles, pollen grains, and pollen tubes cultured for 5 h as well as samples for ABA treatment using the RNA Extraction Kit (Invitrogen™ TRIzol® Reagent, Thermo Scientific, Nanjing, China), according to the manufacturer's instructions. Subsequently, the first-strand cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa, Biotech, Shanghai, China). Specific primers for *PbrSnRK2s* and the housekeeping actin gene *Pbr035825.1* in pear were designed using Primer Premier 5.0 (Table S1). Three biological and three technical replicates were used for qRT-PCR assays, and each PCR reaction included 10 μL of LightCycler 480 SYBRGREEN I Master Mix (Roche, Basel, Switzerland), 100 ng of cDNA, and 200 nM of each primer in a final volume of 20 μl. All reactions were conducted in the CFX96 Real-Time System (Roche), the results were analyzed using Office 2010, and statistical analyses were performed with SPSS17.0 using Duncan's multiple-range test at a significance level (P) of <0.05.

Abbreviations

SnRK2: Sucrose non-fermenting 1-related protein kinase 2; WGD:Whole-genome duplication; ABA:Abscisic acid; PGDD:Plant genome duplication database; qRT-PCR:Quantitative real-time PCR; PG:Pollen grain; PT:Pollen tube; HMM:Hidden Markov Model.

Declarations

Availability of data and materials

All data and materials used in this study are publicly available.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing Interests

The authors have declared that no competing interests exist.

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Author contributions

Guodong Chen designed the research, performed the experiments, analyzed the results, and drafted the manuscript. Jizhong Wang managed the experiments analyzed the results and revising the final manuscript. Xin Qiao analyzed the results and participated in revising the final manuscript. Cong Jin, Weike Duan and Xiaochuan Sun participated in carrying out the experiments and revising the final manuscript. Juyou Wu managed the experiments and revising the final manuscript.

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Not Applicable

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

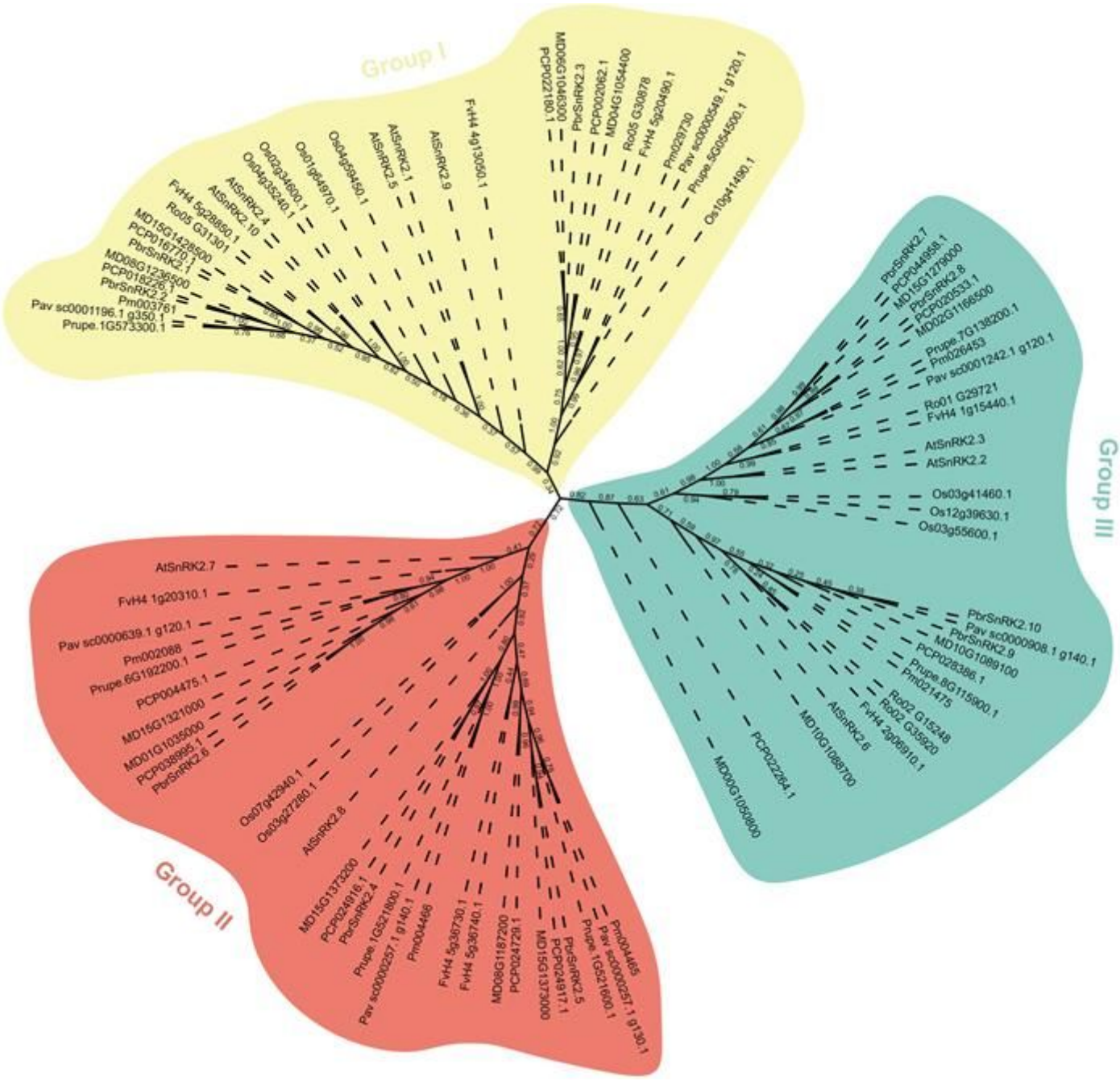

Figure 1

Phylogenetic analysis of SnRK2 proteins from Rosaceae, rice, and Arabidopsis. Full-length protein sequences were aligned with the integrated MUSCLE program and the phylogenetic tree was constructed by the maximum likelihood method using MEGA 6.0 and 1,000 bootstrap replicates. Proteins clustered into three subgroups. The yellow, red, and green regions indicate the three subfamilies of the SnRK2 proteins.

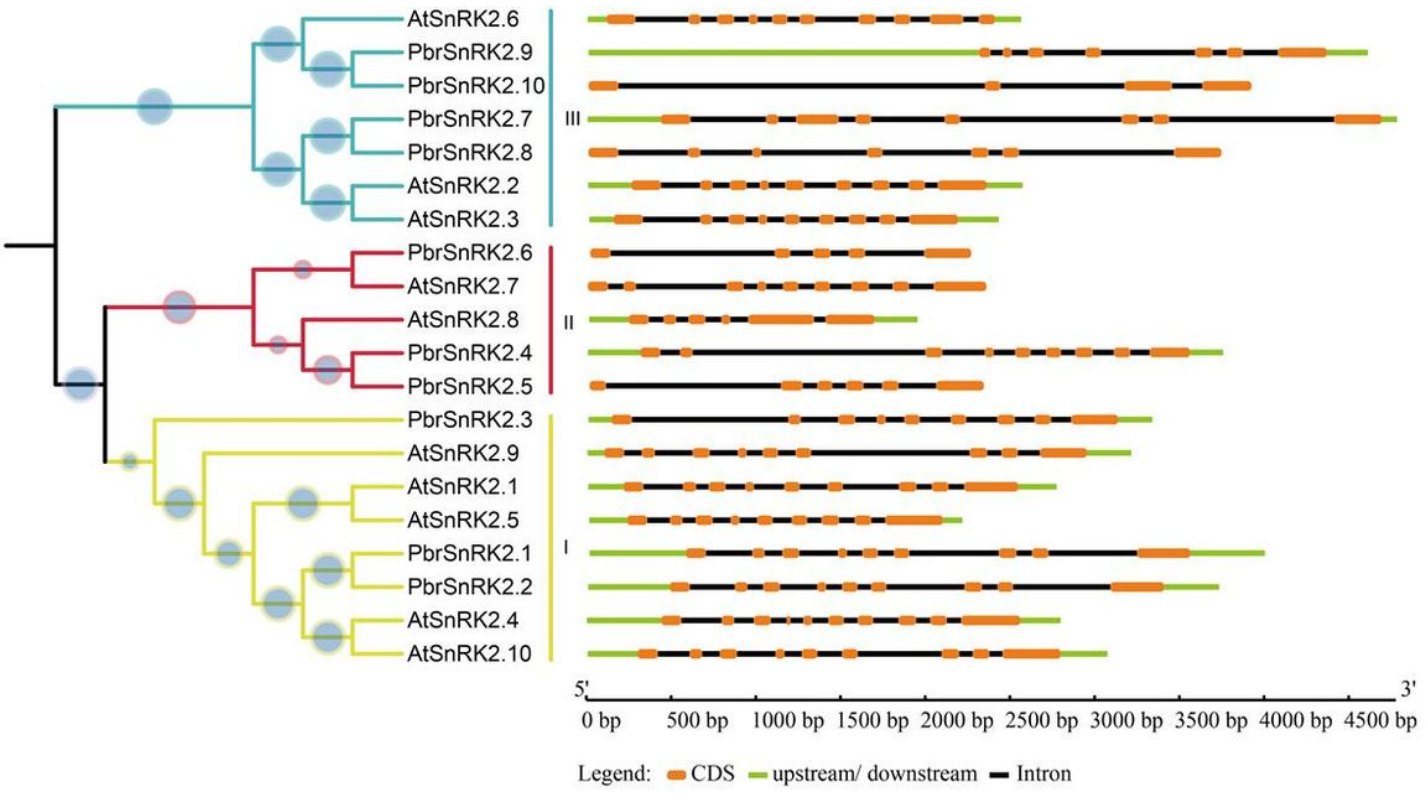

Figure 2

Phylogenetic relationship of the intron/exon structure of SnRK2 genes from pear and Arabidopsis thaliana. A□A phylogenetic tree constructed with CluxtalX2.0 using the full-length amino acid sequences of SnRK2 genes from pear and Arabidopsis. Bootstrap analysis was performed using 1,000 replicates. The species in which SnRK2 proteins were functionally characterized are displayed as icons, and the different colors in the branches represent the different subfamilies. B. The tangerine boxes, black lines, and light-green boxes in the gene structural diagram represent exons, introns, and UTRs, respectively. Gene models are drawn to scale, as indicated at the bottom.

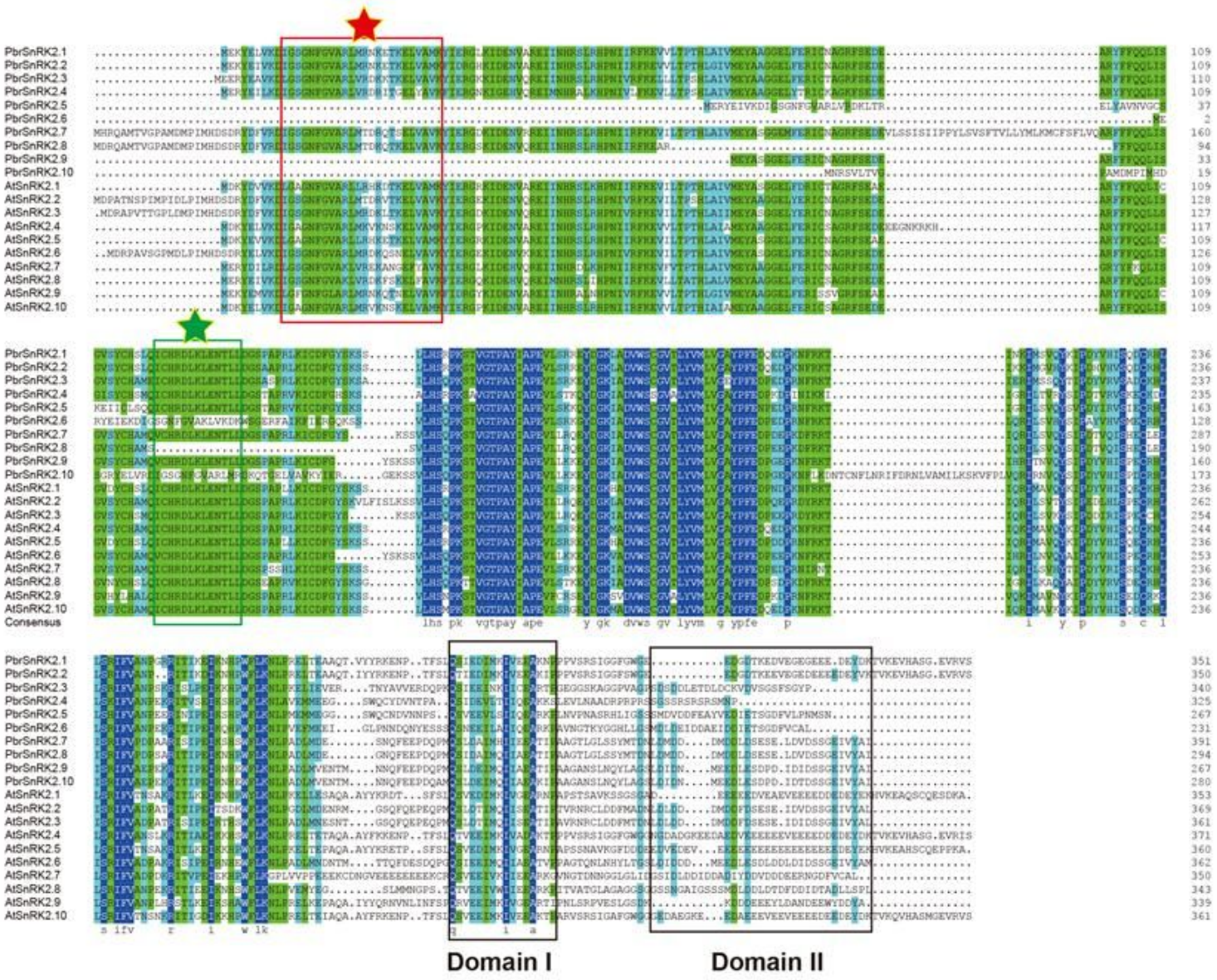

Figure 3

Alignment of the amino acid sequences of PbrSnRK2s and AtSnRK2s. The serine/threonine protein kinase active site signature and the protein kinase ATP-binding region signature are indicated by a green box (a green pentacle) and red box (a red pentacle), respectively. Domain I and Domain II are marked by black boxes at the C-terminus.

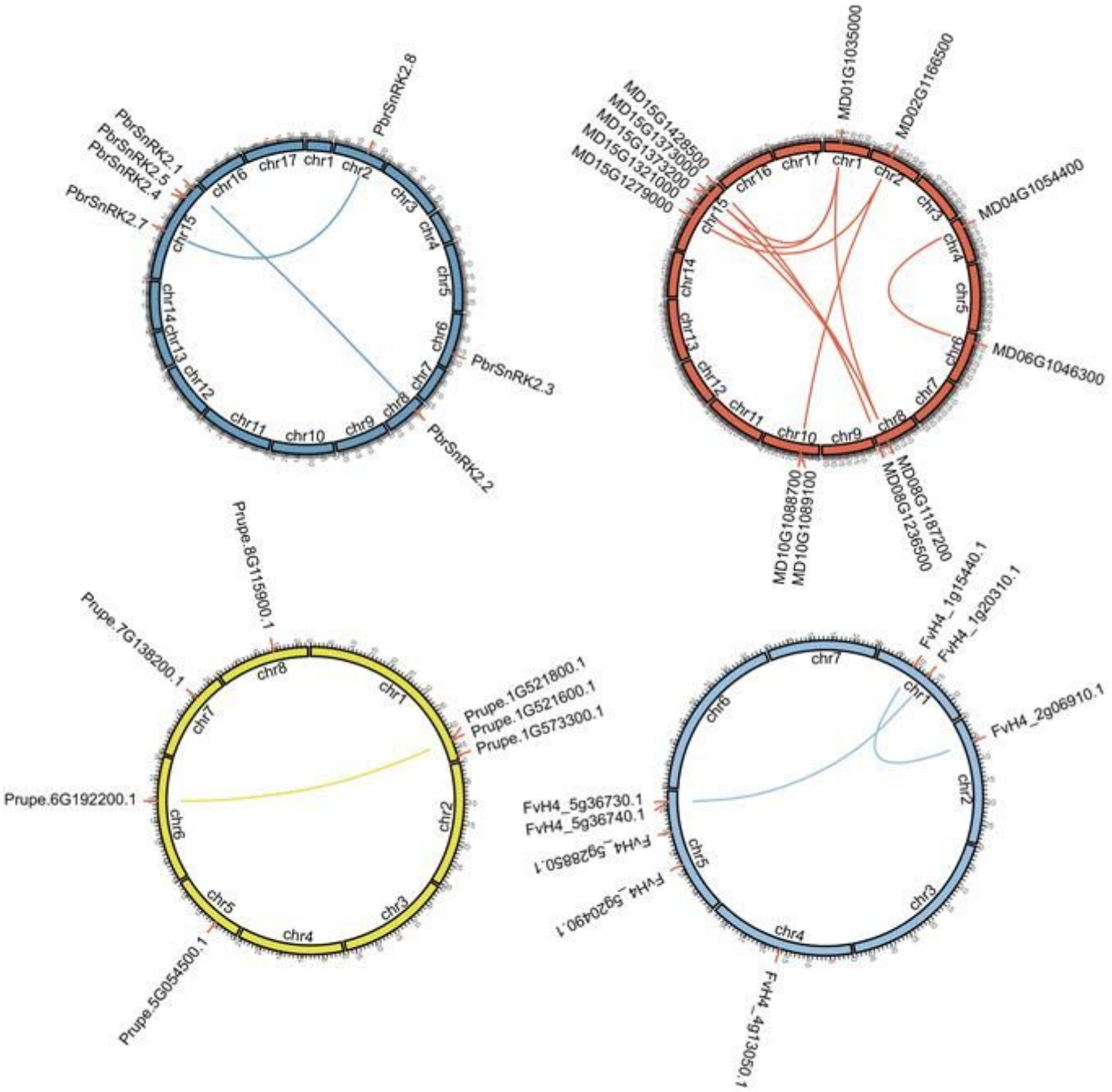

Figure 4

Chromosomal localization and synteny of the SnRK2 genes in Rosaceae genomes. SnRK2 genes in Chinese white pear, apple, peach, and strawberry were mapped onto the different chromosomes. Chromosome number is indicated on the inner side in the inner circle, corresponding to different SnRK2 genes. Gene pairs with a syntenic relationship are joined by a line.

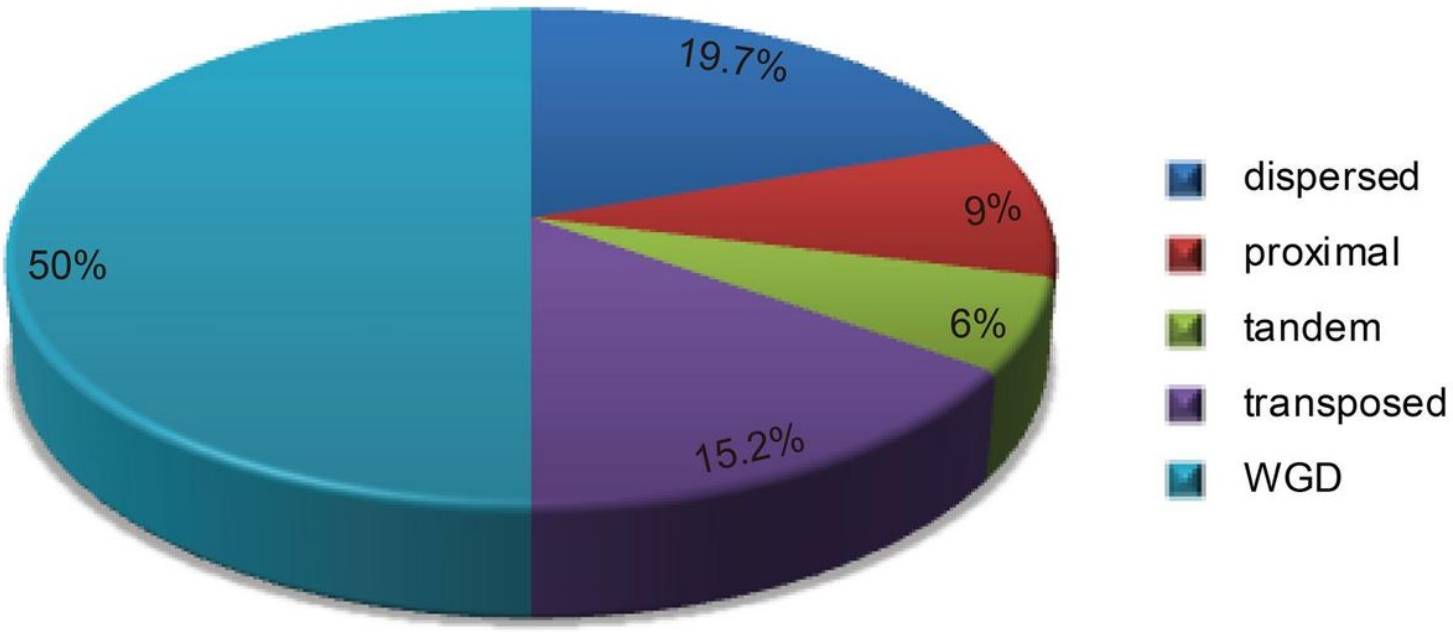

Figure 5

Number of SnRK2 genes from different origins in eight Rosaceae genomes.

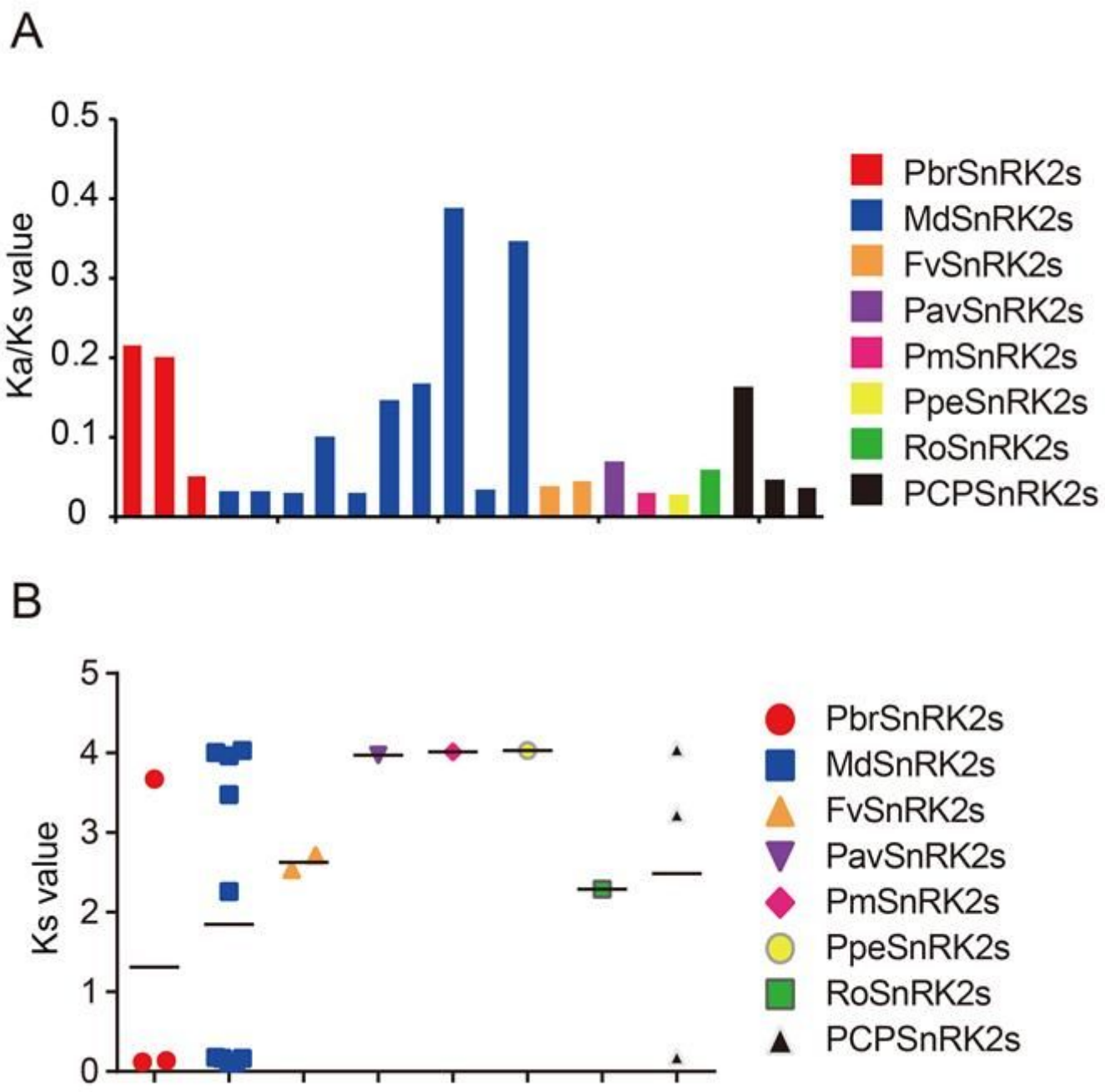

Figure 6

Distribution of mean Ka/Ks and Ka values of SnRK2 genes in Rosaceae. A. Ks values indicating the times of SnRK2 gene evolution in Rosaceae. B. Ka/Ks ratios indicating the driving forces of SnRK2 gene evolution in Rosaceae.

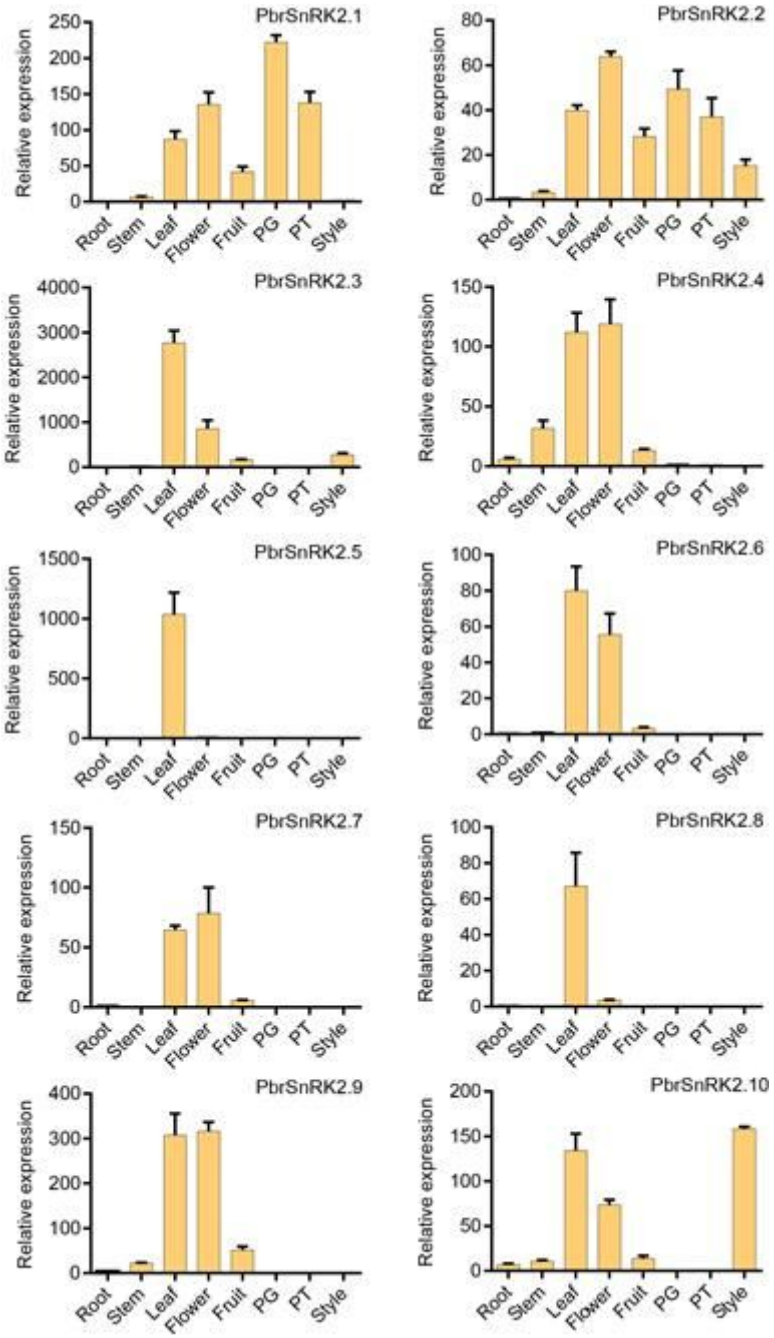

Figure 7

Expression analyses of 10 PbrSnRK2 genes in different tissues. The relative transcript abundances of PbrSnRK2 genes were examined by qRT-PCR. Total RNA was extracted from roots, stems, leaves, flowers, fruits, styles, pollen grains, and pollen tubes cultured for 5 h. Regarding each gene, the relative expression levels were obtained by normalization to that of pear UBQ. The error bars indicate standard deviations. Data are expressed as mean ± standard deviation.

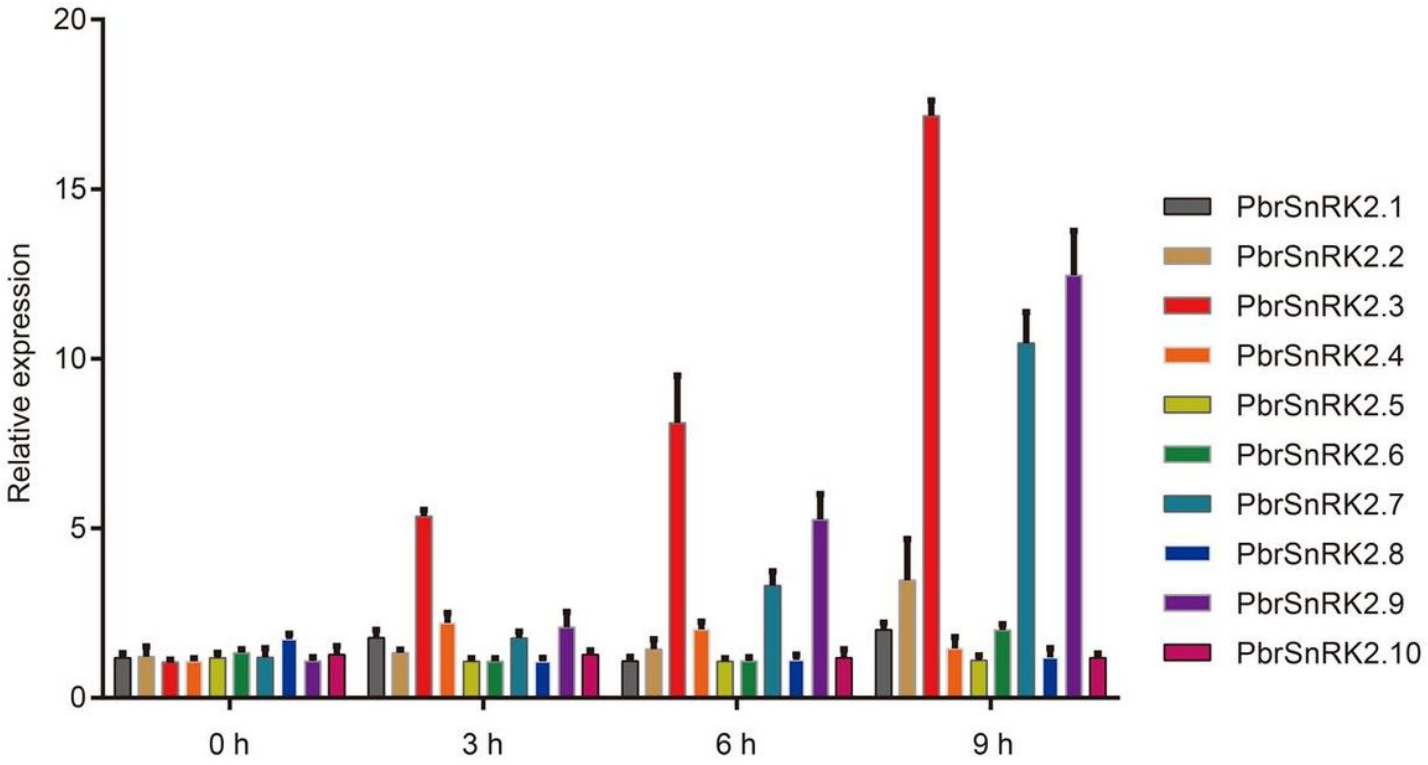

Figure 8

Expression patterns of PbrSnRK2 genes under abiotic treatments. The relative transcript levels of PbrSnRK2.1–PbrSnRK2.10 were examined by qRT-PCR, and the samples were harvested at 0, 3, 6, and 9 h after foliar spraying in leaves from 3-month-old seedlings under 50 μM ABA stress treatments. Regarding each gene, the relative expression levels were obtained by normalization to that of pear actin. The error bars indicate standard deviations. Data are expressed as mean ± standard deviation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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