Genome-wide identification, characterization, and evolutionary analysis of flowering genes in radish (*Raphanus sativus* L.)

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Abstract

**Background:** Radish (*Raphanus sativus* L.) belongs to the family Brassicaceae, and is an economically important root crop grown worldwide. Flowering is necessary for plant propagation, but it is also an important agronomic trait influencing *R. sativus* fleshy taproot yield and quality in the case of an imbalance between vegetative and reproductive growth. There is currently a lack of detailed information regarding the pathways regulating the flowering genes or their evolution in *R. sativus*. The release of the *R. sativus* genome sequence provides an opportunity to identify and characterize the flowering genes using a comparative genomics approach.

**Results:** We identified 254 *R. sativus* flowering genes based on sequence similarities and analyses of syntenic regions. The genes were unevenly distributed on the various chromosomes. Furthermore, we discovered the existence of *R. sativus* core function genes in the flowering regulatory network, which revealed that basic flowering pathways are relatively conserved between *Arabidopsis thaliana* and *R. sativus*. Additional comparisons with *Brassica oleracea* and *Brassica rapa* indicated that the retained flowering genes differed among species after genome triplication events. The *R. sativus* flowering genes were preferentially retained, especially those associated with gibberellin signaling and metabolism. Moreover, analyses of selection pressures suggested that the genes in vernalization and autonomous pathways were more variable than the genes in other *R. sativus* flowering pathways.

**Conclusions:** Our results revealed that the core flowering genes are conserved between *R. sativus* and *A. thaliana* to a certain extent. Moreover, the copy number variation and functional differentiation of the homologous genes in *R. sativus* increased the complexity of the flowering regulatory networks after genome polyploidization. Our study provides an integrated framework for the *R. sativus* flowering pathways and insights into the evolutionary relationships between *R. sativus* flowering genes and the genes from *A. thaliana* and close relatives.

**Keywords:** *Raphanus sativus* L., Genome-wide, Flowering genes, Regulatory pathway networks, Evolution

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**Background**

Flowering is a necessary part of plant propagation, and the process from bolting to blooming is a crucial period for the transition of Brassicaceae plants from vegetative to reproductive growth. Comprehensively characterizing the regulatory mechanisms underlying bolting and blooming may enable researchers to influence the balance between vegetative and reproductive growth, which may ultimately affect the yield and quality of Brassicaceae crops.

Approximately 174 genes are believed to regulate flowering in the model plant *Arabidopsis thaliana*, which are involved in six major pathways [i.e., vernalization, photoperiod and circadian clock, ambient temperature, gibberellin (GA), age, and autonomous pathways] influencing the bolting or blooming process [1]. Although different genes are responsible for different internal and environmentally mediated flowering pathways, the different pathways appear coordinated primarily by a few floral integrator genes, including FLOWERING LOCUS T (FT), LEAFY (LFY), and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) [1]. The recent completion of genome sequences and the development of novel computational analysis techniques have enabled the genome-wide identification and characterization of flowering genes in economically important plants. For example, 900 and 275 putative flowering genes in *Triticum aestivum* and *Hordeum vulgare* [2] respectively, 96, 98 and 304 flowering gene homologs in *Lotus corniculatus* var. japonicus, *Medicago truncatula* and *Glycine max* [3] separately, have been identified. The genes regulating bolting and flowering vary among different crops.

*Raphanus sativus* is a member of the family Brassicaceae, and is cultivated worldwide. It has recently undergone tetraploidization events (α and β) with *A. thaliana*, *B. oleracea* and *B. rapa*, as well as a whole genome triplication with *B. oleracea* and *B. rapa* following their divergence from *A. thaliana* [4, 5]. *R. sativus* has similar flowering habits to *A. thaliana*, *B. oleracea*, and *B. rapa*, with a highly variable flowering time and diverse responses to temperature and/or day length. Fifty flowering miRNAs targeting 154 transcripts [6], and 95 flowering genes differentially expressed between the vegetative and reproductive stages [7], have been identified in *R. sativus*. Additionally, 290 flowering genes have been detected in the *R. sativus* genome [8]. However, little attention has been paid to their characteristics or evolution of the genes in *R. sativus* from *A. thaliana* and *B. oleracea* genomes. A full range of flowering genes in *R. sativus* have been systematically identified using BLASTP searches against *R. sativus* proteome and protein sequences using the following conditions: E-value <1e-20, identity >50%, coverage >60%, and match length >60 amino acids. The conserved synteny and gene homology of their flanking genes, was used to identify syntenic *A. thaliana* and *R. sativus* genes [13]. We further identified homologous relationships among the similar and syntenic genes. Multiple gene sequences were aligned using CLUSTALW [14], and phylogenetic trees were constructed using the neighbor-joining method of the MEGA 6.0 software (1000 bootstrap replicates) [15]. Putative homologous genes were manually checked on the phylogenetic trees.

**Localization of flowering genes in the *Raphanus sativus* genome**

To construct physical maps indicating the distribution of flowering genes, genome localization details for the predicted *R. sativus* flowering genes were collected from the annotation information. The MG2C (http://mg2c.iask.in/mg2c_v2.0/) program was used to visualize the putative flowering genes on nine pseudo-molecular chromosomes [16].

**Flowering gene expression analysis based on RNA-seq data**

We analyzed the transcriptomes of six different tissues (i.e., flowers, siliques, leaves, stem, callus, and roots) collected from *R. sativus* inbred line XYB36–2 [11]. Transcript abundance was calculated according to the FPKM method (fragments per kilobase of exon per million mapped reads) using Cufflinks [17] and TopHat2 [18]. Heatmaps were generated with the R package heatmap.2 [19].
Non-synonymous/synonymous substitution ratios of flowering gene pairs between Arabidopsis thaliana and Raphanus sativus

The non-synonymous/synonymous substitution ratio (Ka/Ks) of homologous gene pairs is related to the evolutionary selection patterns of the corresponding genome. In the calculation of Ka/Ks, the full length of amino acid sequences of the R. sativus and A. thaliana flowering genes underwent pairwise alignments using MUSCLE [20] firstly. Then, the aligned amino acid sequences were translated into the corresponding nucleotides coding sequences using PERL scripts. Finally, the translated nucleotides coding sequences were used as input files in computing Ka/Ks values using the Li-Wu-Luo model [21] integrated in KaKs_Calculator2 software [22]. All variable sites of the alignment pairs were used in the Ka/Ks calculation. To detect selection pressures, Ka/Ks ratios greater than 1, less than 1, and equal to 1 were considered to represent positive selection, negative or stabilizing selection, and neutral selection, respectively.

Results

Identification of Raphanus sativus flowering genes

There are 174 genes, including 24 μ-RNA genes, with known functions affecting A. thaliana flowering time [1]. We focused on the 160 protein-coding genes to identify homologous R. sativus flowering genes. We identified 254
R. sativus flowering genes (Additional file 1), and determined that most of the A. thaliana flowering genes have putative R. sativus homologs (139 out of 160). Homologs in the R. sativus genome were lacking for 21 genes, and most of these genes (15 of 21) have functionally redundant effects on flowering (Additional file 2). Interestingly, all of the lost genes (8 genes) which belonging to photoperiod pathway, circadian clock, and light signaling genes set have function redundant genes retained in R. sativus.

Distribution of Raphanus sativus flowering genes on pseudo-molecular chromosomes

We mapped 247 R. sativus flowering genes onto pseudo-molecular chromosomes, while the remaining seven genes were assigned to unanchored scaffolds (Fig. 1). The distribution of these genes was uneven, with 48 genes localized on chromosome 1, representing 19.43% of the flowering genes. Only 14 flowering genes (5.66%) were detected on chromosome 6, with most located on the bottom half.

Comparison of flowering genes from Arabidopsis thaliana, Brassica oleracea, Brassica rapa, and Raphanus sativus

As Brassicaceae species, B. oleracea and B. rapa have been sequenced and studied in-depth [23–25]. We used the abovementioned method to identify homologous flowering genes in B. oleracea and B. rapa. The fewest number of flowering genes were identified for R. sativus (Fig. 2 and Additional file 3), even though it had the second most annotated genes (43,240) [11], which is between B. oleracea (45,758) [23] and B. rapa (41,174) [24]. There were no significant differences in the number of identified flowering genes among R. sativus, B. oleracea and B. rapa (Chi-squared test = 2.3224, P value = 0.1275).

Except for four genes that could not be categorized, the putative R. sativus, B. oleracea, and B. rapa flowering genes were classified into the following four gene sets: GA signaling and metabolism; vernalization and autonomous pathways; photoperiod pathway, circadian clock, and light signaling; and meristem response and development, according to the classification of A. thaliana genes [1] (Table 1 and Additional file 3). Most of the genes belonged to the photoperiod pathway, circadian clock, and light signaling gene set. There was little difference in the numbers of R. sativus, B. oleracea, and B. rapa genes associated with GA signaling and metabolism.

Dominant pathways and key families of Raphanus sativus flowering genes

Photoperiod pathway, circadian clock, and light signaling

Plants can sense day length changes and use them to control the onset of flowering. We identified 101 R. sativus genes that were homologous to 58 A. thaliana genes of photoperiod pathway, circadian clock, and light signaling (Additional file 1).

CONSTANS (CO), which acts as a point of integration of the internal circadian clock and the external day-night cycles, plays a central role in photoperiodic flowering control of plants [26]. In R. sativus, one copy was identified to be homologous of CO. Significantly, the genes involved in circadian clock including CCA1, LHY, TOC1, GI, CDF1 and LKP2 [27–30] were identified in R. sativus. LKP2 belongs to a family of F-box proteins, which also include ZTL and FKF1 [27]. ZTL and FKF1 are both lost and LKP2 have three tightly linked copies in R. sativus, that are similar to that of B. rapa [25], suggesting the lose of ZTL and FKF1 and the local triplication event of LKP2 may have taken place in the common ancestor of R. sativus and B. rapa. In addition, CRY1, CRY2, PHYA, PHYB, PHYC, and PHYE being implicated in plant light signaling pathways [31] were identified in R. sativus.

Vernalization and autonomous pathways

Many plants growing in temperate climates require vernalization (i.e., prolonged exposure to low temperatures), which involves the silencing of FLC, to initiate or accelerate the flowering process [32]. Similar to the genes of the vernalization pathway, genes in the autonomous pathway

Table 1 Number of flowering genes in Arabidopsis thaliana, Raphanus sativus, Brassica oleracea, and Brassica rapa

| Flowering pathways and their gene sets | A. thaliana | R. sativus | B. oleracea | B. rapa |
|--------------------------------------|------------|-----------|-------------|--------|
| Photoperiod pathway, circadian clock, light signaling | 67 | 101 | 107 | 114 |
| Gibberellin signaling and metabolism | 21 | 35 | 33 | 34 |
| Vernalization and autonomous pathways | 49 | 72 | 72 | 81 |
| Meristem response and development | 19 | 38 | 44 | 42 |
| Other | 4 | 8 | 8 | 7 |
| Total | 160 | 254 | 264 | 278 |
normally indirectly promote flowering by repressing the floral repressor FLC [33]. FLC, which is a MADS-box gene, is the major flowering repressor in the vernalization pathway [34]. Three FLC homologs were identified in R. sativus. As expected, most of vernalization-response genes including VIN3, VRN1, VRN2, FRI were also identified in this study [35–38]. Furthermore, in the FLC-independent vernalization pathway, prolonged exposure to cold conditions can elevate AGL19 and AGL24 expression levels, which can activate LFY and AP1 expression and eventually leads to flowering [39]. The R. sativus contained three copies of AGL19 and two copies of AGL24.

Moreover, we also identified homologous genes in autonomous pathway, including LD, FCA, FY, FPA, FVE, FLD, and FLK. All autonomous genes are indirectly involved in inducing early flowering through the repression of FLC [40].

**Gibberellin signaling and metabolism**

The initiation of flowering in A. thaliana under non-inductive short-day conditions can be promoted by GA. There are 35 genes likely related to the GA pathway in R. sativus. The GA pathway genes are classified as those associated with GA biosynthesis (e.g., GA2ox, GA3ox, GA20ox [41]) and those acting as key signal transduction factors (e.g., SLY1, RGA, and GID1 [42]). Except for GAI, homologs of all GA biosynthesis genes and transduction factors were retained in R. sativus. The GAI and RGA genes are members of the DELLA family, which repress GA-induced vegetative growth and floral initiation [43].

**Meristem response and development**

The onset of flowering is largely dependent on the expression of a relatively small number of central floral pathway factors that integrate signals from several related pathways.

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**Fig. 3** Number of flowering gene homologs retained as syntenic genes in Raphanus sativus. The retained homologs among the four gene sets of flowering genes and their immediate neighbors, all A. thaliana genes, and 459 core eukaryotic genes were included in the analysis. **a** Ratios of the retained genes in different gene sets. **b** Number of retained homologous genes in different gene sets.
During floral transitions [44]. In our study, 38 *R. sativus* genes were identified as floral integrators or were associated with the flower meristem, including *SOC1, FT, API, LFY,* and *FD.* Jung et al. (2016) did not detect *R. sativus LFY* through transcriptomic analysis [45], while, we detect two copies, although they both little expressed, which indicated *LFY* may expressed in specific periods and tissues.

**Differential retention of flowering genes in various species**

The gene dosage hypothesis predicts that genes whose products are dose-sensitive or interact with other proteins or in networks are over-retained, [46]. We compared the retention of genes from each of the above-mentioned four gene sets and three other gene sets: all *A. thaliana* genes, 2780 genes flanking the flowering genes (10 on either side), and 459 core eukaryotic genes. Overall, 80.95% of the GA signaling and metabolism genes, 76.12% of the photoperiod pathway, circadian clock, and light signaling genes, 73.68% of the meristem response and development genes, and 65.31% of the vernalization and autonomous pathway genes were retained as syntenic genes. In contrast, 65.79% of the core eukaryotic genes, 56.74% of the neighboring genes, and 45.67% of all *A. thaliana* genes were retained as syntenic genes (Chi-squared test = 232.5112, \( P < 0.001 \)) (Fig. 3a). Most (57.89%) of the meristem response and development genes and 65.31% of the vernalization and autonomous pathway genes were retained as syntenic genes. In contrast, 65.79% of the core eukaryotic genes, 56.74% of the neighboring genes, and 45.67% of all *A. thaliana* genes were retained as syntenic genes (Chi-squared test = 232.5112, \( P < 0.001 \)) (Fig. 3a). Most (57.89%) of the meristem response and development genes were retained as two or three copies (Fig. 3b).

**Selection pressure on flowering pathway gene sets**

The Ka/Ks ratios for homologous gene pairs were estimated to determine the direction and magnitude of natural selection acting on the *R. sativus* flowering genes. The mean Ka/Ks ratios of different flowering gene sets ranged from 0.18 to 0.25 (Fig. 4 and Additional file 4), suggesting that negative selection had acted against extreme polymorphic variants in flowering genes. In particular, genes of the vernalization and autonomous pathways appear to have been subjected to less negative selection pressures than the genes from other pathways.

**Raphanus sativus** Flowering gene expression analysis

To characterize the divergence in the expression patterns of homologous genes and confirm their involvement in flowering, we analyzed the expression of the putative *R. sativus* flowering genes. By comparing transcript abundances in roots, stem, leaves, flowers, siliques, and callus, we determined that the expression of 16 putative flowering genes was undetectable in all tissues (Additional file 5). Furthermore, transcripts for most of the expressed genes (183 of 254) accumulated in flowers (Additional file 5), with seven genes that were preferentially or specifically expressed in flowers. Four of these seven genes (i.e., *LMI1, SPLA, SLP5,* and *TFL1*) were related to meristem response and development. Besides, it was found that *COPI* and *VIL1,* only have one copy in *R. sativus* and not expressed in all tissues. It seems that these genes have lost function in *R. sativus.*

Duplicated genes can undergo non-functionalization, neo-functionalization, or sub-functionalization [47]. We chose flowering genes with more than three copies in the *R. sativus* genome to analyze the divergence of the homologous gene expression patterns. Although some genes exhibited similar expression patterns, we also observed considerable differences, suggesting that some homologs are functionally similar, while others are functionally diverse (Fig. 5).

**Discussion**

In contrast to the phenotypic effects of vernalization, photoperiod, and GA on *R. sativus* flowering, which have been well studied [48], little is known about the mechanisms mediating the effects. To address this deficiency, we used a bioinformatics approach to analyze the *R. sativus* genes potentially involved in flowering. Based on studies of *A. thaliana* flowering genes, we identified 254 putative *R. sativus* flowering-like genes through a genome-wide comparative analysis. The number of flowering genes in *R. sativus* was slightly less than that in *B. oleracea* and *B. rapa,* which is reasonable considering the genome sizes of the three species [23, 24]. While, homologs for 21 *A. thaliana* flowering genes were not detected in the *R. sativus* genome and many of them have function redundant genes retained in *R. sativus.* The loss of these functionally redundant genes might be due to gene dosage imbalances [49, 50]. However, exactly why certain functionally redundant genes are lost during evolution is unclear. The lost flowering-related genes likely do not affect the core flowering pathways, considering *R. sativus*
Fig. 5 Heatmap of *Raphanus sativus* flowering gene expression profiles. The analyzed tissues are indicated at the bottom of each column. The *R. sativus* gene codes to the right of the expression bar in the same color are homologs of the corresponding genes indicated on the right side with a colored line. The color scale bar at the bottom left of the figure represents log2 transformed FPKM values.
can still receive various endogenous and environmental cues that facilitate flowering.

Whole genome duplications and triplications are typically followed by a considerable loss of genes. However, the gene dosage hypothesis assumes that genes whose products participate in macromolecular complexes, signaling networks, or transcription in a dose-sensitive manner are over-retained, because an imbalance associated with the loss of one member of a complex or network is likely to decrease fitness [49–51]. Many *B. rapa* circadian clock genes have exhibited preferential retention [25]. However, in this study, we observed that genes related to GA signaling and metabolism were preferentially retained over genes from other pathways. Additionally, genes from the photoperiod pathway, circadian clock, and light signaling gene sets or meristem response and development gene sets were also preferentially retained.

The results of our study suggest that basic flowering pathways are likely relatively conserved between *A. thaliana* and *R. sativus*. Three *R. sativus* FLC homologs were identified in this study, which is consistent with the findings of a transcript-level analysis by Yi et al. (2014), and their functions in transgenic *A. thaliana* have been examined [52]. Jung et al. (2016) also did not detect *R. sativus* FRI homologue through RNA-seq, while we identified two copies and both expressed [45]. The existence and expression of FLC and FRI in *R. sativus* indicate that the FLC/FRI mode of action on vernalization is conserved, as are the components of the autonomous pathway [53].

Previous study reported that the genes in the vernalization pathway are not conserved between dicotyledoneous and monocotyledonous plant species [2, 54]. Based on the Ka/Ks ratios, we determined that the sequences of genes related to vernalization pathway were more variable than that of other pathways between *R. sativus* and *A. thaliana*, which indicated vernalization gene sequences also exhibit great sequence diversity among dicotyledon plants. The variation of the vernalization gene sequences may contribute to the rapidly evolutionary capacity in changing thermal requirement to flowering in *R. sativus* [55].

Two FT homologs and one copy of CO were identified in *R. sativus*. This suggests a CO-FT module exists in *R. sativus*, which implies the photoperiod pathway control over flowering evolutionarily conserved in *R. sativus* and *A. thaliana* to a certain extent. In the dark, CO would be efficiently ubiquitinated by the COP1 E3 ligase complex and degraded, which contributed to late flowering in short days [56, 57]. The lost function of COP1 may suggest that CO would not be degraded in dark in *R. sativus*, which seems to be the reason for that *R. sativus* can flower in both short and long day.

Conclusions

We identified 254 putative flowering genes during a comparative genome analysis, and classified them into four flowering regulatory pathway gene sets in *R. sativus*. We also comprehensively analyzed the loss, presence, and variation of different pathway genes as well as the expression patterns of the flowering genes in *R. sativus*. Our results reveal that the flowering regulatory network is conserved between *R. sativus* and *A. thaliana* to a certain degree. The flowering-related genes were preferentially retained, especially those associated with GA signaling and metabolism. Furthermore, most of the *R. sativus* flowering genes lost during evolution were functionally redundant, possibly because of gene dosage imbalances.

Moreover, analysis of selection pressures indicated that the vernalization and autonomous pathway genes are the most variable in *R. sativus*. Besides, The function loss of COP1 seems that photoperiod pathway can promote flowering in both short and long day in *R. sativus*.

In summary, our results further systematic and comprehensive understanding of the flowering regulatory molecular networks that evolved after a whole genome triplication event in *R. sativus*, which will be beneficial for breeders aiming to improve and regulate these processes in *R. sativus* and other Brassicaceae species.

Additional files

Additional file 1: Raphanus sativus flowering genes. (XLS 46 kb)

Additional file 2: Raphanus sativus flowering genes lost during evolution as well as functionally redundant genes. ‘− ’ indicates a lack of functionally redundant genes. (XLS 26 kb)

Additional file 3: Flowering genes identified in *Raphanus sativus*, *Brassica oleracea*, and *Brassica rapa*. (XLS 64 kb)

Additional file 4: The Ka/Ks value of flowering-related genes of *Raphanus sativus* and *Arabidopsis thaliana* in different flowering gene sets. (XLS 54 kb)

Additional file 5: The FPKM (fragments per kilobase of exon per million mapped reads) value of the flowering genes in *Raphanus sativus* flowers, siliques, leaves, stems, callus, and roots. (XLS 70 kb)

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Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information files. The raw reads of RNA-seq have been deposited in the NCBI Sequence Read Archive (SRA) database (BioProject PRJNA413464). The *R. sativus* genome data can be downloaded from BRAD database (http://brassicadb.org/brad/).

Authors’ contributions

XL conceived the project and revised the manuscript. JW designed and performed experiments, analyzed the data and wrote the manuscript. YQ, FC, XC, XZ, HW, JS, MD, and HY participated in experiments and analyzed the data. All authors read and approved the final manuscript.
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