Research article

Identification of flowering genes in strawberry, a perennial SD plant

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Abstract

Background: We are studying the regulation of flowering in perennial plants by using diploid wild strawberry (Fragaria vesca L.) as a model. Wild strawberry is a facultative short-day plant with an obligatory short-day requirement at temperatures above 15°C. At lower temperatures, however, flowering induction occurs irrespective of photoperiod. In addition to short-day genotypes, everbearing forms of wild strawberry are known. In 'Baron Solemacher' recessive alleles of an unknown repressor, SEASONAL FLOWERING LOCUS (SFL), are responsible for continuous flowering habit. Although flower induction has a central effect on the cropping potential, the molecular control of flowering in strawberries has not been studied and the genetic flowering pathways are still poorly understood. The comparison of everbearing and short-day genotypes of wild strawberry could facilitate our understanding of fundamental molecular mechanisms regulating perennial growth cycle in plants.

Results: We have searched homologs for 118 Arabidopsis flowering time genes from Fragaria by EST sequencing and bioinformatics analysis and identified 66 gene homologs that by sequence similarity, putatively correspond to genes of all known genetic flowering pathways. The expression analysis of 25 selected genes representing various flowering pathways did not reveal large differences between the everbearing and the short-day genotypes. However, putative floral identity and floral integrator genes AP1 and LFY were co-regulated during early floral development. AP1 mRNA was specifically accumulating in the shoot apices of the everbearing genotype, indicating its usability as a marker for floral initiation. Moreover, we showed that flowering induction in everbearing 'Baron Solemacher' and 'Hawaii-4' was inhibited by short-day and low temperature, in contrast to short-day genotypes.

Conclusion: We have shown that many central genetic components of the flowering pathways in Arabidopsis can be identified from strawberry. However, novel regulatory mechanisms exist, like SFL that functions as a switch between short-day/low temperature and long-day/high temperature flowering responses between the short-day genotype and the everbearing 'Baron Solemacher'. The identification of putative flowering gene homologs and AP1 as potential marker gene for floral initiation will strongly facilitate the exploration of strawberry flowering pathways.
Background

Transition from vegetative to reproductive growth is one of the most important developmental switches in plant's life cycle. In annual plants, like *Arabidopsis*, flowering and consequent seed production is essential for the survival of the population until the following season. To assure timely flowering in various environments, *Arabidopsis* utilizes several genetic pathways that are activated by various external or internal cues. Light and temperature, acting through photoperiod, light quality, vernalization and ambient temperature pathways, are the most important environmental factors regulating flowering time [1].

Moreover, gibberellin (GA) and autonomous pathways promote flowering by responding to internal cues [2,3]. In contrast to annual plants, the growth of perennials continues after gestation, and the same developmental program is repeated from year to year. Regulation of generative development in these species is even more complex, because other processes like juvenility, winter dormancy and chilling are tightly linked to the control of flowering time.

In *Arabidopsis* photoperiodic flowering pathway, phytochrome (phy) and cryptochrome (cry) photoreceptors perceive surrounding light signals and reset the circadian clock feedback loop, including TOC1 (TIMING OF CAB EXPRESSION), CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL) [4-7]. The central feature in the photoperiodic flowering is the clock generated evening peak of CO (CONSTANS) gene expression [8]. In long-day (LD) conditions, CO peak coincidences with light resulting in accumulation of CO protein in the leaf phloem and consequent activation of the expression of FT (FLOWERING LOCUS T) [9]. FT protein, in turn, moves to the shoot apex, and together with FD triggers floral initiation by activating floral identity gene AP1 (APETALA 1) [10,11]. FT, together with SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1) and LFY (LEAFY) form also convergence points for different flowering pathways, and therefore are called flowering integrator genes [12].

In winter-annual ecotypes of *Arabidopsis*, MADS-box gene FLC (Flowering Locus C) prevents flowering by repressing FT and SOC1, and vernalization is needed to nullify its function [13]. The major activator of FLC is FRI (FRIGIDA) [14], but several other proteins, including for example FRL1 (FRIGIDA-LIKE 1) [15], PIE (PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1) [16], ELF7 and ELF8 (EARLY FLOWERING 7 and 8) [17], and VIP3 (VERNALIZATION INDEPENDENCE 3) [18] are also needed to maintain high FLC expression. During vernalization, FLC is down-regulated by VRN2-PRC2 (Vernalization 2 - Polycomb Repressive Complex 2) protein complex containing low temperature activated VIN3 (VERNALIZATION INSENSITIVE3), allowing plants to flower [19,20].

Autonomous and GA pathways respond to endogenous cues to regulate flowering time. The role of the autonomous pathway is to promote flowering by lowering the basal level of FLC transcription [3]. Autonomous pathway consists of few sub-pathways, which include for example RNA processing factors encoded by FCA, FPA, FLK (FLOWERING LOCUS K), FY and LD (LUMINDEPENDENS) [21], putative histone demethylases LDL1 and LDL2 (LSD1-LIKE 1 and 2) [22], and deacetylases FLD (FLOWERING locus D) and FVE [23,24]. GA pathway is needed to induce LFY transcription and flowering in short-day (SD) conditions [25].

Strawberries (*Fragaria* sp.) are perennial rosette plants, belonging to the economically important Rosaceae family. Most genotypes of garden strawberry (*Fragaria × ananassa* Duch.) and wild strawberry (*F. vesca* L.) are Junebearing SD plants, which are induced to flowering in decreasing photoperiod in autumn [26,27]. In some genotypes, flowering induction is also promoted by decreasing temperatures that may override the effect of the photoperiod [27,28]. In contrast to promotion of flowering by decreasing photoperiod and temperature, these "autumn signals" have opposite effect on vegetative growth. Petiole elongation decreases after a few days, and later, around the floral transition, runner initiation ceases and branch crowns are formed from the axillary buds of the crown [29,30]. Crown branching has a strong effect on cropping potential as it provides meristems that are able to initiate inflorescences [31].

In addition to SD plants, everbearing (EB) genotypes are found in garden strawberry and in wild strawberry [29,32]. Environmental regulation of induction of flowering in EB genotypes has been a topic of debate for a long time. Several authors have reported that these genotypes are day-neutral [29,33]. Recent findings, however, show that long-day (LD) accelerates flowering in several EB *Fragaria* genotypes [34,35]. Interestingly, in wild strawberry genotype 'Baron Solemacher' recessive alleles of SFL gene locus (SEASONAL FLOWERING LOCUS) have been shown to cause EB flowering habit [36]. SFL has not been cloned, but it seems to encode a central repressor of flowering in wild strawberry. Consistent with the repressor theory, LD grown strawberries have been shown to produce a mobile floral inhibitor that is able to move from mother plant to the attached runner plant [37]. GA is one candidate corresponding to this inhibitor, since exogenously applied GA has been shown to repress flowering in strawberries [38,39].

Identification of central genes regulating flowering time and EB flowering habit, as well as those controlling other processes affecting flowering, is an important goal that would greatly accelerate breeding of strawberry and other soft fruit and fruit species of Rosaceae family. In this
paper, we have searched *Fragaria* homologs with the known *Arabidopsis* flowering time genes by EST sequencing and bioinformatics analysis. Dozens of putative flowering genes corresponding to all known genetic pathways regulating flowering time were identified. The expression analysis of several candidate flowering time genes revealed only few differences between the SD and EB wild strawberries, including the presence or absence of AP1 mRNA in the apices of EB and SD genotypes, respectively. Our data provides groundwork for detailed studies of flowering time control in *Fragaria* using transcriptomics, functional genomics and QTL mapping.

**Results**

**Environmental regulation of flowering in two EB genotypes of wild strawberry**

We studied the effect of photoperiod and temperature on flowering time in two EB genotypes, 'Baron Solemacher', which contains recessive alleles in SFL locus [40,41], and 'Hawaii-4'. Flowering time was determined by counting the number of leaves in the main crown before formation of the terminal inflorescence. In SD genotypes of the wild strawberry, SD (<15 h) or, alternatively, low temperature (11°C) is needed to induce flowering [27]. In EB genotypes 'Baron Solemacher' and 'Rugen', instead, LD and high temperature has been shown to accelerate generative development [35], but careful analysis of the environmental regulation of flowering induction has so far been lacking.

Both 'Baron Solemacher' and 'Hawaii-4' produced five to six leaves in LD at 18°C before the emergence of the terminal inflorescence showing that they are very early-flowering in favorable conditions (Figure 1A and 1B). In 'Baron Solemacher', low temperature (11°C) or SD treatment for five weeks at 18°C clearly delayed flowering, but low temperature did not have an additional effect on flowering time in SD. Also in 'Hawaii-4', SD and low temperature delayed flowering, but all treatments differed from each other. Compared to the corresponding LD treatment, SD at 18°C doubled the number of leaves, and low temperature (11°C) delayed flowering time by about three leaves in both photoperiods. Thus, flowering induction in these EB genotypes is oppositely regulated by photoperiod and temperature than previously shown for the SD genotypes [27].

**Construction and sequencing of subtracted cDNA libraries**

We constructed two subtracted cDNA libraries from LD grown EB genotype 'Baron Solemacher' and SD genotype, in order to identify differentially expressed flowering time genes in these genotypes. Plants were grown in LD conditions, where the SD genotype stays vegetative and the EB plants show early flowering. Pooled shoot apex sample covering the floral initiation period was collected from the EB genotype, and vegetative apices of the same age were sampled from the SD genotype. Suppression subtractive hybridization (SSH), the method developed for extraction of differentially expressed genes between two samples [42], was used to enrich either flowering promoting or flowering inhibiting transcripts from EB and SD genotypes, respectively.

A total of 1172 ESTs was sequenced from the library enriched with the genes of the SD genotype (SD library subtracted with EB cDNA) and 1344 ESTs from the library enriched with the EB genes (EB library subtracted with cDNA of the SD genotype). 970 SD ESTs [GenBank:GH202443-GH203412] and 1184 EB ESTs [GenBank:GH201259-GH202442] passed quality checking. Pairwise comparison of these EST datasets revealed that there was very little overlap between the libraries. However, general distribution of the sequences to functional categories (FunCat classification) did not reveal any major differences between the two libraries (Additional file 1).

BLASTx searches against *Arabidopsis*, Swissprot and non-redundant databases showed that over 70% of the ESTs gave a match in one or all of the three databases (Table 1). Moreover, tBLASTx comparison with different genomes
revealed highest number of hits with *Populus trichocarpa* (Table 1). We also performed tBLASTx searches against TIGR plant transcript assemblies of *Malus × domestica*, *Oryza sativa* and *Vitis vinifera* and found hits for 64-76% of ESTs in these assemblies. Finally, the comparison of our sequences with a current *Fragaria* unigene list at the Genome Database for Rosaceae (GDR) showed that 38.2% of our ESTs are novel *Fragaria* transcripts. Taken together, depending on the analysis, 15-22% of sequences from SD genotype and 22-27% of EB sequences encode novel proteins, or originate from untranslated regions of mRNA. Moreover, the high number of novel *Fragaria* sequences in our libraries indicates that SSH method efficiently enriched rare transcripts in the libraries.

**Identification of flowering time genes**

Flowering related genes were identified from our libraries by BLASTx searches as described above and fourteen putative flowering time regulators were identified; four gene homologs were present only in EB library, eight in SD library, and two genes in both libraries. In figure 2, we have summarized the *Arabidopsis* flowering pathways and highlighted the putative homologous genes identified from our EST collection. In general, candidate genes for all major pathways were identified. In addition, 118 *Arabidopsis* flowering time genes were used as a query to search publicly available GDR *Fragaria* EST and EST contig databases using tBLASTn. Sequences passing cut-off value of

|             | WT number | WT average length | EB number | EB average length |
|-------------|-----------|-------------------|-----------|-------------------|
| A) Raw      | 1172      | 946               | 1344      | 965               |
| Poor Quality| 202       | 1037              | 160       | 1066              |
| Singletons/ESTs | 970   | 452               | 1184      | 451               |

**Table 1: The comparison of *F. vesca* ESTs with different databases.**

Average numbers, lengths and percentages of ESTs from EB and SD genotypes. A) numbers and average lengths of raw and poor quality ESTs, and singletons, B) numbers and percentages of BLASTx hits against protein databases, C) numbers and percentages of tBLASTx hits against TIGR plant transcript assemblies of *Malus × domestica*, *Oryza sativa* and *Vitis vinifera* and against *Populus* genome database, D) numbers and percentages of novel ESTs.

![Figure 2](http://www.biomedcentral.com/1471-2229/9/122)

A simplified chart showing *Arabidopsis* flowering pathways and corresponding gene homologs in *Fragaria*. Gene homologs found in cDNA libraries produced from SD and EB genotypes are surrounded by blue and red boxes, respectively. Arrows indicate positive regulation and bars negative regulation.
1e-10 were further analysed by BLASTx algorithm against Arabidopsis protein database, and those returning original Arabidopsis protein were listed. Moreover, sequences that were absent from Fragaria databases were similarly searched from GDR Rosaceae EST database. In these searches, 52 additional Fragaria sequences were identified. Moreover, the total number of 88 homologs of Arabidopsis flowering time genes were found among all available Rosaceae sequences (Additional file 2).

Most genes of the Arabidopsis photoperiodic pathway were found also in Fragaria, and some of the lacking genes were present among Rosaceae ESTs (Table 2, Additional file 2).

We found several genes encoding putative Fragaria photoreceptor apoproteins including phyA, phyC, cry2, ZTL (ZEITLUPE) and FKF1 (FLAVIN BINDING KELCH REPEAT F-BOX 1) [43]. Of the central circadian clock genes, homologs of LHY and TOC1 [5,7] were present in our EST libraries and GDR, respectively, but CCA1 [6] was lacking from both Fragaria and Rosaceae databases. Furthermore, a putative Fragaria CO from the flowering regulating output pathway has been cloned earlier [44]. Among the regulators of CO transcription and protein stability, GI (GIGANTEA) [45] was identified from Rosaceae and putative COP1, SPA3 and SPA4 [46,47] from Fragaria. In addition to genes of the photoperiodic pathway,

Table 2: The list of genes belonging to the photoperiodic flowering pathway.

| Gene | AT gene locus | Biological function | Act./Repr. +/- | Reference | Fragaria | E-value |
|------|---------------|---------------------|---------------|-----------|----------|---------|
| **Photoreceptors and clock input** |
| PhyA | AT1G09570 | Red light photoreceptor | + | [78] | VES-002-C06 | 5E-33 |
| PhyB | AT2G18790 | Red light photoreceptor | - | | | |
| CRY1 | AT4G08920 | Blue light photoreceptor | + | [79] | | |
| CRY2 | AT1G04400 | Blue light photoreceptor | + | [79] | DY669844 | 2E-110 |
| ZTL | AT5G57360 | F-box protein/blue light photoreceptor | + | [80] | EX668764 | 2E-97 |
| FKF1 | AT1G68050 | F-box protein/blue light photoreceptor | + | [65] | DY671170 | 2E-54 |
| ELF3 | AT2G25920 | Unknown | - | [60] | DY675323 | 3E-33 |
| FYP3 | AT1G03070 | Ser/Thr-specific protein phosphatase 2A | - | [81] | BAR-009-A02 | 1E-56 |
| SRR1 | AT5G59560 | Unknown | - | [92] | CO817759 | 1E-10 |
| **Circadian clock** |
| LHY | AT1G01060 | Myb domain TF | - | [7] | VES-005-E09 | 9E-19 |
| CCA1 | AT2G46830 | Myb domain TF | - | [6] | | |
| TOC1 | AT5G61380 | Pseudo-response regulator | - | [5] | DY673134 | 1E-75 |
| LUX | AT3G46640 | Myb TF | - | [83] | DY668516 | 3E-43 |
| ELF4 | AT2G40080 | Unknown | - | [84] | EX674323 | 2E-25 |
| GI | AT1G22770 | Unknown | + | [45] | | |
| PRR5 | AT5G24470 | Pseudo-response regulator | + | [85] | DY676242 | 3E-56 |
| PRR7 | AT5G02810 | Pseudo-response regulator | + | [85] | VES-013-D12 | 5E-52 |
| ELF6 | AT5G04240 | Jumonji/zinc finger-class TF | - | [86] | VES-002-F05 | 1E-45 |
| **Output pathway** |
| CO | AT5G15840 | putative zinc finger TF | + | [8] | DY672035 | 2E-45 |
| CDF1 | AT5G62430 | | - | [65] | | |
| FT | AT1G65480 | Phosphatidylethanolamine binding | + | [11] | | |
| TFL1 | AT5G03840 | Phosphatidylethanolamine binding | - | [87] | | |
| FD | AT4G35900 | bZIP TF | + | [10] | EX675574 | 2E-14 |
| COPI | AT2G32950 | E3 ubiquitin ligase | - | [46] | DY667888 | 1E-94 |
| SPA1 | AT2G46340 | WD domain protein | - | [47] | | |
| SPA3 | AT3G15354 | WD domain protein | - | [47] | DY671873 | 3E-24 |
| SPA4 | AT1G30900 | WD domain protein | - | [47] | DY671245 | 2E-83 |
| RFI2 | AT2G47700 | Ring domain zinc finger | - | [88] | | |
| HAP3b | AT5G47640 | CCAAT-binding TF | + | [89] | EX658204 | 2E-60 |

The most important genes belonging to the photoperiodic pathway in Arabidopsis and their biological function are presented. Floral activators and repressors are indicated by + and - marks, respectively. Moreover, the presence or absence of homologous sequence in Fragaria sequence databases and E-value of BLASTx comparison against Arabidopsis are indicated. Sequences found in our libraries are named BAR and VES for everbearing genotype 'Baron Solemacher' and short-day genotype, respectively. Other ESTs and EST contigs are found from Genome Database for Rosaceae [http://www.bioinfo.wsu.edu/gdr/]. More complete list is available in Additional file 2.
homologs for both known sequences belonging to light quality pathways, PFT1 (PHYTOCHROME AND FLOWERING TIME 1) and HRB1 (HYPERSENSITIVE TO RED AND BLUE 1) [48,49], were found from our EST libraries.

For the vernalization pathway, we were not able to find FLC-like sequences from our EST libraries or public Fragaria or Rosaceae EST databases by tBLASTn searches although we used the FLC and FLC-like sequences from Arabidopsis (MAF1-MAF5, MADS AFFECTING FLOWERING 1-5) and several other plant species as query sequences [13,50,51]. Similarly, also FRI [14] was lacking from Rosaceae ESTs but putative FRL (FRIGIDA-LIKE) [15] sequences were identified in Fragaria. In addition, we identified several gene homologs belonging to the FRI complex as well as other regulatory complexes (SWR1, PAf) involved in promoting the expression of FLC (Table 3, Additional file 2) [17,52,53]. Also putative members of FLC repressing PRC2 complex, were present in strawberry ESTs. These include putative VIN3 (VERNALIZATION INSENSITIVE 3) [19,20] that has been identified earlier [54], and putative SWN1 (SWINGE 1), FIE (FERTILIZATION INDEPENDENT ENDOSPERM), VRN1 (VERNALIZATION 1) and LHP1 (LIKE HETEROCHROMATIN PROTEIN 1) [19,55,56], which were found in this investigation (Table 3, Additional file 2). However, putative VRN2 that is needed for the repression of FLC by PRC2 was not found [19].

### Table 3: The list of genes belonging to the vernalization pathway.

| Gene     | AT gene locus | Biological function                                      | Act./Repr. +/- | Reference                     | Fragaria | E-value |
|----------|---------------|----------------------------------------------------------|----------------|-------------------------------|----------|---------|
| FLC      | AT5G10140     | MADS-box TF                                              | -              | [13]                          | nf       |         |
| MAF1/FLM | AT1G77080     | MADS-box TF                                              | -              | [50]                          | nf       |         |
| **Fri complex** |              |                                                          |                |                               |          |         |
| FRI      | AT4G00650     | Unknown, enhancer of FLC                                  | -              | [14]                          | nf       |         |
| FRL1     | AT5G16320     | Unknown, enhancer of FLC                                  | -              | [15]                          | EX686406 | 4E-45   |
| FRL2     | AT1G31814     | Unknown, enhancer of FLC                                  | -              | [15]                          | Contig 4768 | 6E-49 |
| FES1     | AT2G33835     | CCCH zinc finger protein                                  | -              | [53]                          | nf       |         |
| SUF4     | AT1G30970     | Putative zinc finger containing TF                        | -              | [53]                          | BAR-003-F06 | 5E-46 |
| **Swr complex** |              |                                                          |                |                               |          |         |
| PIE      | AT3G12810     | ATP-dependent chromatin-remodelling factor                | -              | [16]                          | nf       |         |
| SEF1/SWC6| AT5G37055     | Component of chromatin remodelling complex                | -              | [52]                          | DY670674 | 4E-70   |
| ARP6/ESD1| AT3G33520     | Component of chromatin remodelling complex                | -              | [52]                          | nf       |         |
| ATX1     | AT2G31650     | Putative SET domain protein                               | -              | [90]                          | EX687477 | 4E-71   |
| **PAFL complex** |            |                                                          |                |                               |          |         |
| ELF7     | AT1G79730     | RNA polymerase 2 associated factor 1 -like                | -              | [17]                          | nf       |         |
| ELF8     | AT2G06210     | RNA polymerase 2 associated factor -like                  | -              | [17]                          | BAR-008-H08 | 3E-42 |
| VIP4     | AT5G61150     | RNA polymerase 2 associated factor -like                  | -              | [91]                          | EX660943 | 2E-50   |
| VIP3     | AT4G29830     | RNA polymerase 2 associated factor -like                  | -              | [18]                          | EX675781 | 7E-98   |
| EFS/SGD8 | AT1G77300     | Putative histone H3 methyltransferase                     | -              | [53]                          | nf       |         |
| **VRN2-PRC2 complex** |        |                                                          |                |                               |          |         |
| VRN2     | AT4G16845     | Polycomb group zinc finger                               | +             | [92]                          | nf       |         |
| CLF      | AT2G23380     | Polycomb group protein                                   | +             | [93]                          | nf       |         |
| SWN1/EZA | AT4G02020     | Polycomb group protein                                   | +             | [93]                          | EX687655 | 3E-114  |
| FIE      | AT3G20740     | Polycomb group protein                                   | +             | [93]                          | DY671601 | 1E-112  |
| VIN3     | AT5G57380     | PHD domain protein                                        | +             | [20]                          | CO816801 | 2E-58   |
| LHP1     | AT5G17690     | epigenetic silencing                                      | +             | [56]                          | DY669632 | 2E-40   |
| VRN1     | AT3G18990     | DNA binding protein                                       | +             | [55]                          | DY670727 | 8E-43   |

The most important genes belonging to the vernalization pathway in Arabidopsis and their biological function are presented. Floral activators and repressors are indicated by + and - marks, respectively. Moreover, the presence or absence of homologous sequence in Fragaria sequence databases and E-value of BLASTx comparison against Arabidopsis are indicated. Sequences found in our libraries are named BAR and VES for everbearing genotype ‘Baron Solemacher’ and short-day genotype, respectively. Other ESTs and EST contigs are found from Genome Database for Rosaceae [http://www.bioinfo.wsu.edu/gdr/](http://www.bioinfo.wsu.edu/gdr/). More complete list is available in Additional file 2.
In addition to the photoperiod and the vernalization pathways, we searched candidate genes for the autonomous and GA pathways. Several sequences corresponding to Arabidopsis genes from both pathways were identified suggesting the presence of these pathways also in Fragaria (Table 4, Additional file 2). Among these genes we found homologs for Arabidopsis FVE and SVP which have been shown to control flowering in a specific thermosensory pathway [24,57]. Moreover, some additional flowering time regulators that are not placed to any specific pathway were identified (Table 4, Additional file 2).

Identification of floral integrator genes in Fragaria

Sequencing of our EST collections did not reveal any homologs for the floral integrator or identity genes such as FT, SOC1, LFY or AP1 [12,58]. A full-length cDNA sequence of SOC1 homolog [GenBank:FJ531999] and a 713 bp 3’-end fragment of putative LFY [GenBank:FJ532000] were isolated using PCR. Closest protein homolog of the putative FvSOC1 was 72% identical Populus trichocarpa MADS5, and the putative FvLFY showed highest amino acid identity (79%) to Malus domestica FL2. Comparison to Arabidopsis showed that AtSOC1 and AtLFY, respectively, were 66% and 75% identical with the corresponding wild strawberry protein sequences (Figure 3A and 3B). FT homolog, instead, was not identified in Fragaria despite of many attempts using degenerate PCR and screening of cDNA library plaques and E.coli clones from a variety of tissues and developmental conditions with the Arabidopsis coding sequence (K. Folta, unpublished). However, a putative FT was found in Prunus and Malus protein databases at NCBI. Among the other genes belonging to the same gene family, homologs of MFT (MOTHER OF FT AND TFL1) and ATC (ARABIDOPSIS CENTRORADIALIS) [59] were present in GDR Fragaria EST. Moreover, an EST contig corresponding to the floral identity gene AP1 was found. The length of the translated protein sequence of FvAPI was 284 amino acids, being 30 amino acids longer than the corresponding Arabidopsis sequence. However, FvAPI EST contig contained an

| Gene | AT gene locus | Biological function | Act./Repr. +/- | Reference | Fragaria | E-value |
|------|--------------|---------------------|---------------|-----------|----------|----------|
| Autonomous pathway |
| FCA | AT4G16280 | RRM-type RNA binding domain containing | + | [94] | nf | |
| FPA | AT2G43410 | RRM-type RNA binding domain containing | + | [95] | nf | |
| FLK | AT3G04610 | KH-type RNA binding domain containing | + | [96] | EX668302 5E-52 |
| FY | AT5G13480 | mRNA 3′ end processing factor | + | [97] | EX659635 5E-75 |
| SKB1 | AT4G31120 | Arginine methyltransferase | + | [98] | nf | |
| FVE | AT2G19520 | retinoblastoma associated | + | [24] | VES-001-803 3E-76 |
| LD | AT4G02560 | DNA/RNA binding homeodomain protein | + | [99] | DY670534 3E-49 |
| FLD | AT3G10390 | component of histone deacetylase complex | + | [23] | nf | |
| LDL1/SWP1 | AT1G62830 | Histone H3-Lys 4 demethylase-like | + | [22] | Contig 2573 2E-27 |
| LDL2 | AT3G13682 | Histone H3-Lys 4 demethylase-like | + | [22] | DY669828 1E-42 |
| Gibberellin pathway |
| GAI | AT1G14920 | putative transcriptional repressor | - | [100] | Contig 3276 3E-147 |
| RGA | AT2G01570 | putative transcriptional repressor | - | [100] | DO15503 8E-60 |
| SPY | AT3G11540 | O-linked N-acetylglucosamine transferase | - | [101] | BAR-002-C02 2E-93 |
| DDF1 | AT1G12610 | AP2-like TF | + | [102] | Contig 3158 5E-49 |
| DDF2 | AT1G63030 | AP2-like TF | + | [102] | nf | |
| AaMYB33 | AT3G06100 | MYB TF | + | [25] | DY669997 5E-29 |
| FPF1 | AT3G24860 | Unknown | + | [103] | Contig 4074 7E-38 |
| Other |
| SVP | AT2G22540 | MADS-box TF | - | [57] | VES-013-D05 5E-22 |
| AP2 | AT4G36920 | AP2 TF | - | [104] | VES-008-A07 9E-16 |
| PFT1 | AT1G25540 | vWF-A domain protein | + | [48] | BAR-002-D08 1E-17 |
| HRB1 | AT3G49230 | ZZ type zinc finger protein | + | [49] | VES-012-B01 7E-22 |

The most important genes of Arabidopsis autonomous and gibberellin flowering pathways as well as some other floral regulators are presented. The biological function of the genes is indicated, and floral activators and repressors are marked by + and - marks, respectively. Moreover, the presence or absence of homologous sequence in Fragaria sequence databases and E-value of BLASTx comparison against Arabidopsis are indicated. Sequences found in our libraries are named BAR and VES for everbearing genotype ‘Baron Solemacher’ and short-day genotype, respectively. Other ESTs and EST contigs are found from Genome Database for Rosaceae http://www.bioinfo.wsu.edu/gdr/. More complete list is available in Additional file 2.
Protein alignments of *Fragaria* flowering integrator and identity genes

**Figure 3**

Protein alignments of *Fragaria* flowering integrator and identity genes. Multiple alignments of *Fragaria* protein sequences of full length SOC1 (A), partial LFY (B) and full-length AP1 (C) with closest protein homologs and corresponding protein sequence of *Arabidopsis thaliana*. Alignments were done by ClustalW (A, B) or T-Coffee (C) and modified by Boxshade program. *F. vesca* AP1 protein sequence was translated from GDR *Fragaria* EST contig 4941. PTM5 = *Populus tremuloides* MADS5, AFL2 = Apple FLORICAULA 2, PpAP1 = putative *Prunus persica* AP1.

(A)  

| Protein | Alignment | Length |
|---------|------------|--------|
| FvSOC1  | 5VRGKTQVRRIENATRSQVTFSKRSGLLKRFELESSLDCAEVALIIFSPRGKLYEFASS | 60 |
| PTM5    | 5VRGKTQVRRIENATRSQVTFSKRSGLLKRFELESSLDCAEVALIIFSPRGKLYEFASS | 60 |
| AtSOC1  | 5VRGKTQVRRIENATRSQVTFSKRSGLLKRFELESSLDCAEVALIIFSPRGKLYEFASS | 60 |

(B)  

| Protein | Alignment | Length |
|---------|------------|--------|
| FvLFY   | NGGGCGLGERQREHPFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQNIAKERGEK | 286 |
| AFL2    | NGGGCGLGERQREHPFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQNIAKERGEK | 286 |
| AtLFY   | NGGGCGLGERQREHPFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQNIAKERGEK | 286 |

(C)  

| Protein | Alignment | Length |
|---------|------------|--------|
| FvAP1   | MGRGRVQLKRIENKINQVTFSKRSGLLKRFELESSLDCAEVALIIFSPRGKLYEFASS | 60 |
| PpAP1   | MGRGRVQLKRIENKINQVTFSKRSGLLKRFELESSLDCAEVALIIFSPRGKLYEFASS | 60 |
| AtAP1   | MGRGRVQLKRIENKINQVTFSKRSGLLKRFELESSLDCAEVALIIFSPRGKLYEFASS | 60 |

*FvSOC1, FvAP1, PpAP1, AtSOC1, AtAP1, AFL2, AtLFY, PpLFY, AtLFY, PpLFY, AtLFY*
unknown sequence stretch of 81 bp at nucleotide position 596-677. Putative FvAP1 showed highest overall identity (68%) with putative AP1 from Prunus persica (Figure 3C). Moreover, the 5’ sequence containing 187 amino acids (the sequence before the unknown part) was 73% identical with the Arabidopsis AP1.

**Gene expression analysis revealed few differences between EB and SD genotypes**

We compared the expression of selected flowering time genes (Table 5) corresponding to each flowering pathway in the leaf and shoot apex samples of EB and SD genotypes in order to explore the role of different pathways. Only few of the analysed genes were differentially expressed between the genotypes. Floral integrator gene *LFY* was slightly up-regulated in the shoot apex samples of EB (Table 6). Moreover, PCR expression analysis with two different primer pairs showed that *AP1* was specifically expressed in EB apices correlating with the identity of the meristems. Among the genes from different flowering pathways, only two genes, vernalization pathway gene *ELF8* [17] and photoperiod pathway gene *ELF3* [60], were slightly differentially expressed between the genotypes (Table 6).

**Developmental regulation of floral integrator, floral identity, and GA pathway genes**

We analysed the developmental regulation of *API*, *LFY*, *SOC1*, *GA3ox* and *GA2ox* transcription in the shoot apices of LD grown plants of EB and SD genotype containing one to four leaves. *Ubiquitin*, used as a control gene, was stable between different developmental stages, but was amplified ~1 PCR cycle earlier in SD genotype (Additional file 3). Thus direct comparison between the genotypes is not possible, but the trends during development are comparable. Three genes, *API*, *LFY*, and *GA3ox*, had clear developmental stage dependent expression pattern in EB apices, showing biggest changes after one or two leaf stage (Figure 4). The expression of *API* was detected in EB apices already at one leaf stage, and its mRNA accumulated gradually reaching 6-fold increase at two leaf stage and 50-fold increase at four leaf stage (Figure 4A). In parallel, transcription of *LFY* started to increase at 2-leaf stage, but the change in its expression was much smaller (Figure 4B). A floral integrator gene, *SOC1*, in contrast, did not show clear developmental regulation (Figure 4C). Also GA pathway was co-regulated with *API* and *LFY*, since GA biosynthetic gene *GA3ox* was strongly down-regulated after two leaf stage (Figure 4D). In addition, GA catabolism gene, *GA2ox*, tended to follow changes in the expres-

**Table 5: The list of PCR primers used in real-time RT-PCR.**

| Gene  | Forward primer | Reverse primer |
|-------|----------------|----------------|
| UBI   | CAGACCAGCAGAGGCTTATCTTT | TTCTTGATATTGTAGTCTGCTAGGG |
| LFY   | CGGCATTACGTTCTACTGCTA | CCTGTAACACGCCGCTCATACT |
| SOC1  | CAGGTCGGCAGGAGAGATAGGAA | AGAGCCTTTCTCTGGGAGAGA |
| API   | CGCTCCAGAAGAAGGATAAGG | CATGTGACTGAAGCGCTGCTG |
| API   | TCTGAAGCAGTAAGGTCTCA | ATTCCTGATCATACCTCAG |
| LHY   | AAACGTCGGAAGAAGGAGGAGCAGT | CCAGGAGTAAAGGATTTGCTTGGT |
| ZTL   | TCCAGGGTGGCTAGAAACA | CACCTCCGACAGTACCTT |
| FKF1  | ACCCATCGTCTTCTCTGC | ACATCCGATCAGACCAACAG |
| ELF3  | TCTCCAGAAAGCAGTTAAGAAA | CACTCCGAGACAGCAGTTGAG |
| ELF6  | TTCGAAGGTCCTCCGCAATGG | GCGCCGATAGTTTATCCACAC |
| COL4  | GACCGGAAATTCACCCTGCTG | CTTCCCGTCCGACAAAGTAC |
| CO    | GACATCTCCCTCCGGCAAC | GTGGAGCCCAAGTACTTGT |
| PFT1  | GCGGATCAGCTCAAGGTAGAATT | TCGGCCGCCTACACTTTCAC |
| HRB1  | GAATGTTGGACATCAGACAATCC | CCTCCGAAAGAATTTGCTCAAC |
| FYP33 | ACAAAAATGGCCGCTAAACA | GATGCTATGTGTCATAGT |
| FRL   | CGTATGTTCGAAGGGGAG | CGACCTATCTCCTCAGGCA |
| ELF8  | GCTCAAGAATGTCTTCTGTG | TGAATGTGCAAGCCACTTC |
| VRNS  | AGGCCCTGTATGCTACACTGCT | CCGATGGATGTGCTCTGTAAG |
| MSI1  | TCTCCACACTTTGATTGCACAA | ACCACATCGTACTTCGGCCAG |
| LHP1  | GGAACAGCAAGAACGAAGGAG | CTCCACCTTTCCTCCTCCT |
| FVE   | GATCCAGCAAGCAAACAAAAGTTCTC | CCGATGGATGTGCTCTGTAAG |
| SVP   | CGTCTGAAAGGCAGATGTAAGTG | TGAAGCAGACGTCAGACTTC |
| SPY   | TGGCTGTTCAAGTTGAAATGATC | GCGCAACATCAAGATTGAGTTG |
| GA3ox | CCTGCAACATCACAACCAATCCC | CGCGGATGTTGATCACCA |
| GA2ox | CACCTGCGCCAGAGCTCA | AGGCGCAGAATGTGATGT |
| TFL1  | TGAGAAGCAACACAGATGTTCC | CGAGGACGATCAGTATTGCG |
| AP2   | CCCGAATTCCTTGGATGTTCC | AACACTGCAATCTCAACAG |

T<sub>n</sub> value of the primers is 60 ± 1°C.
Table 6: The expression of selected genes in the wild strawberry.

| Gene       | MSII as a control | FVE as a control |
|------------|-------------------|------------------|
| Shoot apex samples |                   |                  |
| API        | Expressed only in EB | Expressed only in EB |
| LFY        | 1.8 ± 0.4          | 1.9 ± 0.3        |
| ELF8       | 1.5 ± 0.1          | 1.6 ± 0.1        |
| Leaf samples |                   |                  |
| ELF3       | 1.5 ± 0.1          | 1.8 ± 0.0        |

Relative gene expression in the shoot apex or leaf samples of LD grown plants of EB genotype compared to SD genotype. Ct values of genes of interest were normalized against CoVa values of MSII and FVE to get normalized ΔCt values. The expression ratios between genotypes (EB/SD) were calculated from the formula 2^ΔCt one leaf stage. The expression values at one leaf stage were artificially set to 1 separately for both genotypes. Values are mean ± standard deviation. Pooled shoot apex samples and leaf samples at four leaf stage were used.

For comprehensive identification of candidate genes of the strawberry flowering pathways, we searched homologs for 118 Arabidopsis flowering time genes from our own cDNA libraries and from GDR. In total, we were able to identify 66 gene homologs among about 53000 EST sequences. Moreover, gene homologs lacking from Fragaria were further mined from Rosaceae EST collections containing about 410 000 EST sequences. These searches revealed 22 additional putative flowering time genes in Rosaceae. Ongoing genome sequencing projects in apple, peach and wild strawberry will ultimately reveal the currently lacking flowering regulators in these species [63].

Figure 4 Developmental regulation of gene expression in wild strawberry apices. The expression of API (A), LFY (B), SOC1 (C) and GA3ox (D) in the SD and EB ('Baron Solemacher') genotype of the wild strawberry. Triplicate shoot apex samples were collected from LD grown plants at one to four leaf stage. Ct values were normalized against a Ubiquitin [GenBank: DY672326] gene to get normalized ΔCt values. The expression differences between one leaf stage and later developmental stages were calculated from the formula 2^ΔCt later developmental stage/2^ΔCt one leaf stage. The expression values at one leaf stage were artificially set to 1 separately for both genotypes. Values are mean ± SD. Note that Ubiquitin was amplified ~1 cycle earlier in SD genotype, but was stable between different developmental stages. Therefore, expression values between genotypes cannot be directly compared, while the expression levels between the various developmental stages are comparable.

Discussion

Identification of flowering genes in strawberry

Genetic regulation of flowering in strawberry has earlier been studied only by crossing experiments. According to Weebadde et al. [61], everbearing character is a polygenic trait in garden strawberry whereas other studies indicate the presence of a single dominant gene [62]. Different results may arise from different origin of everbearing habit, since at least three different sources have been used in strawberry breeding [32,61,62]. Studies in F. vesca 'Baron Solemacher' have shown that EB flowering habit in this genotype is controlled by recessive alleles of a single locus, called seasonal flowering locus (sfl) [40,41]. Identification of central regulators of flowering, as well as those controlling other processes that affect flowering (runnnering, chilling), is an important goal that would greatly accelerate breeding of strawberry and other soft fruit and fruit species of Rosaceae family.
legumes [66], CCA1 was lacking in Rosaceae, but its redundant paralog, LHY, is represented by few ESTs in Fragaria. CCA1 and LHY are MYB-type transcription factors which repress the expression of TOC1 in the central loop of Arabidopsis circadian clock [67]. Thus, in Fragaria and other species of Rosaceae family, LHY alone may control the expression of TOC1 in the clock core. This contrasts with other species, like Populus, where duplications of the LHY/CCA1 genes contribute to an apparently more complex mode of clock control [68].

Vernalization pathway in Arabidopsis culminates in FLC and FLC-like floral repressors [13,50]. They have been functionally characterized only in Brassicaceae [13,69], although homologous MADS box genes have been recently found from several eudicot lineages by phylogenetic analysis [51]. However, we were not able to identify FLC-like sequences in Rosaceae by using several FLC-like sequences as a query. Similarly, also FRI homologs were lacking from the Rosaceae sequence collections. However, putative homologs of FRI-like genes, FRL1 and FRL2, which are involved in FLC activation in Arabidopsis [15] were found, as well as several other homologs of genes belonging to FLC regulating protein complexes. Despite the presence of these transcripts, the presence of FLC is unclear, since at least PRC2 complex has several target genes [70]. Cloning and characterization of putative FLC-like and FRI genes as well as FT in strawberry would greatly expand our understanding of strawberry flowering pathways, and therefore, it is one of the most important targets of further studies. If these transcripts are present in strawberry, it is likely that the precise control of flowering has placed their expression in specific tissues or contexts where they are not easily detected. However, their presence should be substantiated in analysis of the impending genome sequence. Another important goal is the identification of putative Rosaceae or Fragaria specific flowering time genes. Ultimately, transcriptomics studies and functional analysis of central genes may reveal how different flowering pathways, which may be closely related to Arabidopsis pathways, make seasonal flowering in strawberry.

What is the SFL gene?

SFL is a single dominant locus that enforces seasonal flowering habit in wild strawberry, and homozygous mutation in this locus leads to continuous flowering habit in at least one genotype, ‘Baron Solemacher’ [36]. In SD genotypes of wild strawberry, SD or low temperature induce flowering [27] probably by overcoming the function of SFL repressor gene. We showed here that EB genotypes ‘Baron Solemacher’ and ‘Hawaii-4’ produce only 5-6 leaves to the main crown before the formation of the terminal inflorescence in LD at 18°C. Hence, flowering induction in these conditions occurs soon after germination. In SD (12 h) or at low temperature (11°C) instead, plants formed several leaves more before the inflorescence. This finding shows that, in contrast to SD genotypes, both SD and low temperature restrain flowering induction in these genotypes, confirming earlier suggestions that EB genotypes of wild strawberry are in fact LD plants [35]. Most simple explanation for these opposite environmental responses is that the lack of flowering inhibitor, produced by active SFL gene, unmasks LD induced flowering promotion pathway in ‘Baron Solemacher’ and possibly in other EB genotypes. Given that both SD and low temperature repress SFL, analogous flowering regulating pathway has not yet been characterized at molecular level.

Our gene expression analysis did not give any hints of the putative location of SFL in wild strawberry flowering pathways. However, homologs of certain flowering repressors can be considered as candidates for SFL, including the rice CO homolog HD1 (HEADING DATE 1), or Arabidopsis vernalization pathway genes FLC and FRI [13,14,71]. In strawberry, the role of vernalization pathway remains unclear until the presence or absence of FRI or FLC function is confirmed or other targets for this pathway are found. Strawberry CO, instead, has been cloned and mapped in Fragaria reference map, but its position does not match with the genomic location of SFL showing that CO itself is not SFL [44,72]. However, the possibility that some regulator of CO transcription or protein stability could be SFL cannot be ruled out and should be studied further.

Exogenously applied GA inhibits flowering in wild strawberry, and therefore, GA has been suggested to be a floral repressor [38,39]. Similar patterns have been observed and delineate differences between recurrent and non-recurrent roses [73]. However, we did not find clear differences in the expression of GA biosynthetic and catabolism genes, GA3ox and GA2ox, in the shoot apex samples of EB and SD genotypes before the floral initiation had occurred. In contrast, GA3ox was strongly repressed in EB apices after floral initiation and GA2ox showed similar trend. The fact that these changes in GA pathway occurred after two leaf stage suggests that GA signal was regulated during early flower development rather than during floral transition. These data does not support the role of endogenous GA as the regulator of flowering induction, indicating that SFL is not situated in the GA pathway. However, quantitative analysis of GA levels is needed to show whether the observed changes in the expression of GA pathway genes are reflected at the metabolic level.

**AP1 is a potential marker of floral initiation in strawberry**

Gene expression analysis revealed that two putative flowering genes, **AP1** and **LFY**, were co-regulated during floral development in EB wild strawberry. The homolog of floral
identity gene \textit{API} was expressed in the EB apex already at one leaf stage, and its expression was strongly enhanced during later developmental stages. Also \textit{LFY} mRNA accumulated along with \textit{API} during floral development in EB genotype, whereas \textit{SOC1} did not show a clear trend. The mRNA of \textit{SOC1} and \textit{LFY} were present also in SD genotype, but \textit{API} transcription was not detected. In \textit{Arabidopsis}, \textit{LFY} and \textit{API} activate each other’s expression constituting a feedback loop [12,58]. Moreover, \textit{API} is activated by FT-FD heterodimer shortly after flowering induction [10]. Thus, the expression patterns of \textit{API} and \textit{LFY} in the meristems of EB genotype suggest that flowering induction in these plants occurs before two leaf stage in LD conditions. Consistent with this conclusion, flower initials were clearly visible by stereomicroscope in the meristems at three or four leaf stage, and plants flowered after producing on average 4.7 leaves in the main crown. Based on our results, \textit{API} can be used as a marker for floral initiation in wild strawberry. However, functional studies are needed to confirm the role of \textit{API}, \textit{LFY} and \textit{SOC1} as floral integrator and identity genes, and this approach is currently going on.

\textbf{Conclusion}

We have explored putative components for the genetic flowering pathways in perennial SD plant wild strawberry by identifying 66 homologs of \textit{Arabidopsis} flowering time genes. Although few central genes are lacking, these data indicate that all known genetic flowering pathways may be present in \textit{Fragaria}. This is consistent with the finding that EB genotypes, ‘Hawaii-4’ and ‘Baron Solemacher’, show similar environmental regulation of flowering than \textit{Arabidopsis} summer-annuals. We also studied the expression of selected candidate genes and found that few genes were co-regulated in the shoot apex of the EB genotype during early floral development. Most strikingly, the mRNA of \textit{API} specifically accumulated in EB genotype, but was absent in SD genotype, showing its usefulness as a marker of floral initiation. Finally, identification of putative flowering time genes reported here enables their transcriptional and functional characterization, as well as genetic mapping, which may give answers for the relative importance of each genetic flowering pathway and lead to cloning of the central repressor gene, \textit{SFL}. Ultimately, these genetic resources could be utilized in cultivar breeding of various species of Rosaceae family through genetic transformation and marker assisted selection breeding.

\textbf{Methods}

\textit{Plant materials, growing conditions and sampling}

Seeds of SD and EB (‘Baron Solemacher’) genotypes of the wild strawberry (NCGR accession numbers [PI551792] and [PI551507], respectively) were sown on potting soil mixture (Kekkilä, Tuusula, Finland) and grown in a greenhouse under LD conditions (day length min. 18 h), provided by 400 W SON-T lamps (Airam, Kerava, Finland) and natural sunlight. After two to three leaves had developed per plant, shoot apex samples (tip of the shoot containing the meristem as well as two to three leaf initials) were collected under a stereomicroscope at ten different time points with three days intervals. Samples from each time point were pooled and used for the construction of cDNA libraries and real-time RT-PCR. WT samples contained shoot apices of the main crown, collected from 50 plants per time point. Also in EB genotype, shoot apices of the main crown were collected until the sepals initials became visible in the meristems. After this time point, the apices from one to three side shoots per plant were collected, altogether from 40 plants per sampling. In addition, leaf samples were collected from the same plants at four leaf stage for real-time RT-PCR analysis. Moreover, separate shoot apex samples were collected from WT and EB genotypes at one, two, three and four leaf stages. Control plants were grown in LD and their flowering time was determined by counting the number of leaves in the main crown before the terminal inflorescence. All samples were collected in July - August 2006 - 2008.

\textit{Preparation and sequencing of subtracted cDNA libraries}

Total RNA from pooled shoot apex samples was extracted with a pine tree method for RNA isolation [74]. The cDNA was synthesized with BD SMART cDNA Synthesis kit (Clontech, Palo Alto, US), amplified with PCR as instructed for subtraction, purified with Chroma Spin-1000 DEPC-H2O Columns (Clontech), extracted with chloroform:isoamylalcohol (24:1) using Phase Loch Gel Heavy 1.5 ml tubes (Eppendorf, Hamburg, Germany), digested with RsaI (Boehringer Mannheim, Mannheim, Germany), and purified with High Pure PCR Product Purification kit (Roche Diagnostics, Indianapolis, US). The cDNAs were subtracted using BD PCR-Select cDNA Subtraction Kit (Clontech) in both forward and reverse directions. The forward and reverse PCR mixtures were digested with RsaI (Boehringer Mannheim) and purified with High Pure PCR Product Purification Kit (Roche). After digestion, A-tailing was done as instructed in the technical manual of pGEM-T and pGEM-T Easy Vector Systems and PCR mixtures were ligated to pGEM-T Easy Vector (Promega, Wisconsin, US), and electroporated to TOP10 cells. The libraries were sequenced at the Institute of Biotechnology, University of Helsinki, as described earlier [75].
Bioinformatics analysis

Raw EST sequences were quality checked before annotation. Base calling, end clipping and vector removal were performed by CodonCodeAligner-software (CodonCode Corporation, US). After this the ESTs were manually checked and sequences that contained poly-T in the beginning followed by short repetitive sequences were removed. BLASTx was performed against functionally annotated Arabidopsis protein database (v211200, MIPS), Swissprot and non-redundant protein database (NCBI), and Populus trichocarpa genome of DOE Joint Genome Institute [76] using cut-off value 1e-10. tBLASTx was performed against TIGR plant transcript assemblies of Malus domestica, Oryza sativa and Vitis vinifera [77], and GDR Fragaria and Rosaceae Contigs using cut-off value 1e-10. For MIPS BLAST hits corresponding functional classes and Gene Ontology classes were obtained from Functional Classification Catalogue (Version 2.1) and GO annotation for Arabidopsis thaliana (Version 1.1213).

Homologs of Arabidopsis flowering time genes were searched from GDR Fragaria contig and EST databases using tBLASTx algorithm and Arabidopsis protein sequences as a query. Homologous sequences passing a cut-off value 1e-10 were further analysed by BLASTx algorithm against Arabidopsis protein database, and sequences showing highest sequence homology with the corresponding Arabidopsis genes were selected. The sequences lacking from Fragaria were similarly searched from GDR Rosaceae EST database and from Rosaceae protein database at NCBI.

Photoperiod and temperature treatments

For the analysis of environmental regulation of flowering in EB genotypes, seeds of 'Baron Solemacher', and 'Hawaii-4' were germinated in 18 h LD at 18°C. During germination, plants were illuminated using 400 W SON-T lamps (Airam) for 12 h daily (90 ± 10 μmol m⁻² s⁻¹ at plant height plus natural light) and incandescent lamps were used for low-intensity daylength extension (5 ± 1 μmol m⁻² s⁻¹ at plant height). After opening of the cotyledons plants were moved to four treatments, SD and LD (12/18 h) at low or high temperature (11/18°C), for five weeks. In LD, incandescent lamps were used for low-intensity daylength extension (5 ± 1 μmol m⁻² s⁻¹ at plant height) after 12 h main light period. Also photoperiods of 8 and 8 + 8 h (SD/LD) were tested, but because of very slow growth in these light treatments, longer photoperiods were selected (data not shown). SD treatments were carried out at the greenhouse using darkening curtains, while LD treatments (photoperiod 18 h) were conducted in a similar greenhouse compartment without curtains. The experiments were carried out during winter 2007 - 2008, when the natural day length was under 12 h. After treatments, plants were potted to 8 x 8 cm pots, moved to LD (18 h), and flowering time was determined as described above.

Gene expression analysis

Total RNA from leaf and shoot apex samples was extracted with a pine tree method [74], and cDNAs were synthesized from total RNA using Superscript III RT kit (Invitrogen, Carlsbad, US) and dT₁₈VN anchor primers. LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Indianapolis, US) was used to perform 15 μl real-time RT-PCR reactions in 384-well plates according to manufacturer’s instructions by using Light Cycler 480 real-time PCR system (Roche Diagnostics). PCR primers with Tₘ value of 60°C were used (Table 5). Three biological replicates were analysed for shoot apex samples from different developmental stages (Figure 4), and two biological replicates were used for pooled shoot apex and leaf samples (Table 6).

Authors’ contributions

TH, KM and PE designed all experiments. PE coordinated the study and helped to draft the manuscript. TH run the real-time PCR analysis, performed flowering gene searches from sequence databases, and drafted the manuscript together with KM. KM constructed the subtracted cDNA libraries and performed bioinformatics analysis together with KF. KF also helped to draft the manuscript. MR participated in flowering time analysis and sampling of shoot apices. PA and LP were responsible for the EST sequencing. All authors read and approved the final manuscript.

Additional material

Additional file 1

Functional classification of ESTs from EB and SD genotypes. The percentage of gene hits in different FunCat classes in two cDNA libraries prepared in this study is shown. Same gene may be classified in one or several classes. WT and EB libraries were prepared from SD and everbearing genotypes, respectively.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2229-9-122-S1.PDF]

Additional file 2

Complete list of flowering time genes searched. Genes belonging to different flowering pathways are listed in separate sheets of .xls file. Homologous sequences found in Fragaria are indicated. Moreover, corresponding Rosaceae sequences were searched, if Fragaria sequence was not found. GenBank EST sequence number or Genome Database for Rosaceae contig number is given for Fragaria and Rosaceae ESTs and contigs, respectively.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2229-9-122-S2.XLS]
Additional file 3

The stability of control genes used in this study. Ct values of FVE, MSI and UBI in the leaf samples collected at four leaf stage (a) and in the pooled shoot apex samples (b). Same plant material of SD and EB ('Baron Solemacher') genotypes was used than in Table 6. Panel c: Ct values of UBI in the shoot apex samples of SD and EB genotypes at different developmental stages. Values are means (± standard deviation) of two (a, b) or three (c) biological and three technical replicates. One g of total RNA was used for cDNA synthesis for each sample. Different Ct values of UBI in shoot apex images in figures b and c are due to different cDNA dilutions used for PCR.

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