Sex-Biased Temporal Gene Expression in Male and Female Floral Buds of Seabuckthorn (Hippophae rhamnoides)

Aseem Chawla¹, Tsering Stobdan², Ravi B. Srivastava², Varun Jaiswal¹, Rajinder S. Chauhan¹, Anil Kant¹*

¹ Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, India, ² Defence Institute of High Altitude Research, Defence R & D Organisation, Leh, Jammu, and Kashmir, India

* anil_kantv@yahoo.com

Abstract

Seabuckthorn is an economically important dioecious plant in which mechanism of sex determination is unknown. The study was conducted to identify seabuckthorn homologous genes involved in floral development which may have role in sex determination. Forty four putative Genes involved in sex determination (GISD) reported in model plants were shortlisted from literature survey, and twenty nine seabuckthorn homologous sequences were identified from available seabuckthorn genomic resources. Of these, 21 genes were found to differentially express in either male or female flower bud stages. HrCRY2 was significantly expressed in female flower buds only while HrCO had significant expression in male flowers only. Among the three male and female floral development stages (FDS), male stage II had significant expression of most of the GISD. Information on these sex-specific expressed genes will help in elucidating sex determination mechanism in seabuckthorn.

Introduction

Hippophae rhamnoides commonly known as seabuckthorn belongs to the family Elaeagnaceae. Seabuckthorn berries are among the most nutritious and vitamin-rich fruits found in the plant kingdom. In general, the flesh of berries contains a diverse complex of vitamins, mineral substances such as sodium salts, potassium, calcium, carbohydrates, proteins, sugars and amino acids. [1, 2]. Moreover, the oil from the sea buckthorn berry contains on average 35% of the rare and valuable palmitoleic acid (16:1n-7; omega-7 series fatty acid) [3]. Seabuckthorn has a great potential for researchers in the field of biotechnology, nutraceutical and environmental sciences [4]. Various products had been developed from the berries of seabuckthorn such as oil, juice, alcoholic beverages, candies, ice-cream, tea, jam and biscuits. [3]. Thus the demand of seabuckthorn berries has increased in past few years due to their increased use in commercial products [5]. This increase in demand warrants its intensive cultivation, instead of just
collection from wild resources and requires genetic improvement to increase its productivity and quality.

For development of superior seabuckthorn, breeding projects target both females and male cultivars [3]. Moreover, the objectives for breeding male and female plants vary, since there are extra quality criteria to be met in female plants, as berries occur on female plants only [3]. The success of the breeding program in dioecious plants depends upon early identification of progeny’s gender. Unfortunately, gender of seabuckthorn seedlings cannot be determined morphologically until flowering, which usually occurs after 3–4 years in the field [6]. This represents a serious problem for plant breeders who are forced to retain large number of male for several years. Much of the work and money could be saved if large proportion of the males could be discarded at an early stage in evaluation process.

In dioecious plants gender determination is regulated at genetic level by X/Y chromosome system [7]. Many molecular marker based studies like RAPD, SSR, ISSR, SCAR etc. were conducted for past several years for gender identification in seabuckthorn and molecular markers can distinguish male and female plants. [8–12]. However, none of the marker based studies in seabuckthorn related the markers with the mechanism governing sex determination. Therefore the mechanism governing the sex determination in seabuckthorn still remains unknown [3].

Differences between male and female plants are primarily detected in reproductive organs, which occur through differential growth, repression or abortion of sex organs in unisexual flowers [13, 14]. Various category of genes like floral meristem identity genes, floral organ identity genes and flowering time genes play a major role as Genes involved in sex determination (GISD) in development of unisexual flowers [15, 16]. In case of Thalictrum dioicum, floral organ identity genes were differentially expressed in early development stages of male and female flowers. This led to the conclusion that regulation of these homeotic genes resulted in gender determination in this species [17]. Also the role of MADS box homeotic genes was analysed in male and female flowers of Hop (Humulus lupulus). Northern hybridisation in H. lupulus showed that M1 (DEFICIENS homologue) and M2 (Petunia FLORAL BINDING PROTEIN 2 homologue) transcripts were present in the early stages of floral development of both sexes, but at later stages, expression of both genes increased in male flowers and decreased in female flowers [18, 19]. Moreover, apart from floral regulatory genes, sex determination is also dependent upon the regulatory networks which alter sex expression based on environmental cues such as photoperiod and temperature [20].

The genetic control of sex determination is well-known in several model plant systems like Silene latifolia [21–23], Cucumis sativus [24–26], Salix [27, 28], etc. Moreover, molecular and genetic studies showed that the underlying mechanisms controlling flower development are largely conserved in distantly related dicotyledonous plant species [29]. Thus, genomic resources generated from these model plants could be used to identify the potential GISD in seabuckthorn. A possible strategy to identify genes essential in a development process is to screen mRNAs that are present in one sample and absent (or rare) in other ones [30]. In order to identify mRNA transcripts involved in sex determination in dioecious plants like S. latifolia, Rumex acetosa, and Actinidia chinensis, different spatial and temporal development stages of flower were used [31–33]. Numerous flowering genes like APETALA 2, CLAVATA 1 and SEPTALTA 3 showed differential expression among male and female flowers of plants like Z. mays, S. latifolia, A. Officinalis [34–36], which indicated their role in sex determination in the above mentioned plants. Thus for identification of potential seabuckthorn GISD, differential expression of known flowering genes was analysed using quantitative Real Time PCR (qRT-PCR) in three temporal Floral Development Stages (FDS) of both male and female seabuckthorn flowers.
Material and Methods

Plant material, RNA extraction and cDNA synthesis

The flower buds of *Hippophae rhamnoides* collected from Defence Institute of High Altitude Research (DIHAR), J&K, India (Geographic Coordinates—34°08' 236" N, 77° 34' 345" E) were used in this investigation (Permission granted by Director, DIHAR, Leh, Jammu and Kashmir, India). Three different samples of floral buds for current study were collected on the basis of phenological observations on flowering of seabuckthorn in the region of study. Flower buds start developing from the month April and flowers open in the start of May to mid-May. The flower bud samples were collected in the month of April at ten days interval, starting from dormant winter bud to when buds are about to open. This is period when female and male reproductive tissues are formed in the flower buds. Flower buds were immediately frozen in liquid nitrogen and were stored at -80 °C till further use. Male and female flower bud stages were designated as Male Stage I (MST I), Male Stage II (MST II), Male Stage III (MST III) and Female Stage I (FST I), Female Stage II (FST II) and Female Stage III (FST III) respectively as shown in Fig 1. RNA was extracted from flower buds using Bangalore Genei Plant Total RNA extraction kit as per manufacturer instructions. RNA concentration was estimated by U.V. spectrophotometry and integrity was confirmed by electrophoresing samples on a 1.2% denaturing agarose gel. First strand of cDNA was synthesised from 1 \( \mu g \) of total RNA using Verso cDNA Kit (Thermo Scientific). The quality of cDNA was tested by amplifying 26S gene fragment using 26S primers (S1 Table) under following amplification conditions (95°C for 4 min and then 35 cycles at 95°C for 30 s, 55.5°C for 30 s and 72°C for 50 s) and products were electrophoresed in 1.8% agarose gel.

Identification of seabuckthorn homologues of potential GISD and phylogenetic analysis

A literature survey was undertaken to short list genes involved in flower development of *Arabidopsis* which could be potential candidates for sex determination in seabuckthorn (Table 1). Nucleotide sequences of floral regulatory genes well-characterized in plants like *Silene latifolia*, *Arabidopsis thaliana*, *Vitis vinifera*, *Cucumis sativa*, etc. were downloaded from NCBI Genbank database in FASTA format (S1 File). The sequence data was manually curated and redundant sequences of the same species were discarded. Quality trimmed and filtered nucleotide sequences of seabuckthorn were retrieved from seed [37], root and leaf [38] transcriptome (NCBI Accession No.SRX118240, SRX131619 and SRX131618 respectively) and ESTs from NCBI EST database. A series of BLASTN analyses with default parameters identified broadly
conserved sequences of potential GISD from seabuckthorn genomic resources showing synten-
omic relationship with known GISD (Table 2 and S2 File). BLASTN reports were analysed man-
ually and the sequences (showing similarity with known GISD sequences) having e-value
greater than $10^{-4}$ and query coverage less than 100bp were discarded. Homologous sequences
of GISD having the lowest e-value were chosen for validation through qRT-PCR (Table 2). To
further confirm the identity of the seabuckthorn sequences, domains and repeats were identi-
fied within the GISD sequences. Nucleotide sequences of putative seabuckthorn GISD were
translated to amino acid coding sequences (S3 File) using ExPASy translate tool (http://web.
expasy.org/translate/). The sequences with longest open reading frame were used for repeats,
domain and protein family identification using EBI Interpro server (http://www.ebi.ac.uk/
interpro/). For Phylogenetic reconstruction of potential GISD in sebuckthorn, protein se-
quencies of known GISD characterized in model plant species were downloaded from NCBI
Genbank database (S4 File). The alignment of the sequences was done with the help of CLUS-
TALX [39] and the final tree was constructed using MEGA 6 (Molecular Evolutionary Genetics
Analysis 6.0) software [40].

Expression analysis of GISD by qRT-PCR

Primers for candidate genes were designed using the Primer3 web application (http://bioinfo.
ut.ee/primer3-0.4.0/), with Tm of 55–60°C and amplicon size between 100 bp and 250 bp (S1
Table). qRT-PCR was performed with duplicate amplifications using SYBR-green-based detec-
tion system (IQ SYBR Green Supermix (Biorad) in the Biorad CFX96 Real-Time PCR Detec-
tion System). The reactions contained 100 ng cDNA template and 0.5µM of primers in total
volume of 13µl. Cycle parameters of reaction were 95°C for 3 min and then 39 cycles at 95°C
for 10, 60°C for 30 s and 72 for 20s. Expression data were analysed with ΔΔCT method [41].
The expression of four internal reference genes namely ubiquitin, β-actin, 26S and GAPDH
was checked on four floral bud samples. 26S and GAPDH genes showed consistent expression
pattern in male and female flower bud stages (Unpublished data) and were used for gene ex-
pression data normalisation. The data presented in the figures and tables are based on the aver-
age of 2 PCR samples used from 3 biological samples. Fold expression of genes was calculated
between the same development stages of male and female flowers. Heat map representing the
gene expression data of GISD in three developmental stages of male and female seabuckthorn
flowers was generated using the GENEX Ver. 6.0 software (http://genex.gene-quantification.
info).

Results

Identification of seabuckthorn homologues of potential GISD and phylogenetic analysis

The current study was focused on 44 Arabidopsis genes that were known to be involved in flo-
ral regulatory pathways (Table 1) and could be probable candidates for sex determination in H.
rhamnoides. Out of 44 Arabidopsis flowering genes, 24 genes had homologous sequences in
available H. rhamnoides genomic resources (Table 2). Arabidopsis genes for which homolo-
gous sequences were not present in the transcriptome data of seabuckthorn include AP3, CAL,
CRC, JAG, KNU, LFY, NZZ, NUB, RBE, SPL, SVP, SUP, WUS, FLC, FLT, UFO, FIM, ER and
DADI. The identified homologous sequences of seabuckthorn GISD were compared with similar
genes of other plants species deposited in NCBI genebank nucleotide database as well as
EST databases of other plant species like Actnidia chinesis [90]. Results of the analysis showed
that the sequences of putative seabuckthorn GISD matched with transcripts of either one plant
Table 1. List of potential genes involved in sex determination in seabuckthorn.

| S. No. | Gene Name | Function in flower development | References |
|--------|-----------|---------------------------------|------------|
| 1      | APETALA1 (AP1) / SQUAMOSA (SQA) | Promotes sepal differentiation, suppresses axillary bud initiation, required in secondary whorl development (CLASS A MADS box gene) | [42–44] |
| 2      | APETALA2 (AP2) | Sepal identity (CLASS A MADS box gene) | [45] |
| 3      | APETALA3 (AP3) / DEFICIENS (DEF) | Petal identity in second whorl of flower, stamen identity in third whorl of flower (CLASS B MADS box gene) | [45] |
| 4      | AGAMOUS (AG) / PLENA (PLE) | Stamen identity in third whorl of flower, carpel identity in fourth whorl of flower. (CLASS C MADS Box gene) | [46, 47] |
| 5      | CAULIFLOWER (CAL) | Floral meristem identity gene. | [29, 48] |
| 6      | CRAB’S CLAW (CRC) | Regulates carpel development | [49] |
| 7      | CLVATA1 (CLV 1) | Encodes putative receptor kinase which controls shoot and floral meristem size | [50] |
| 8      | CONSTANS (CO) | Regulates flowering time in response to day length | [51] |
| 9      | CRYPTOCHROME1 (CRY1) | Blue ultraviolet A receptors. Regulates flowering time | [52] |
| 10     | CRYPTOCHROME2 (CRY2) | Blue ultraviolet A receptors. Regulates flowering time | [52] |
| 11     | EARLY FLOWERING 1 (ELF1) | Regulates FLC. Mutations in ELF1 results in suppression of FLC-mediated delay of flowering and causes early flowering in non-inductive photoperiods independently of FLC | [53] |
| 12     | FILAMENTOUS FLOWER (FIL) | Floral organ polarity | [54] |
| 13     | JAGGED (JAG) | Involved in the formation of lateral organs. JAG promotes distal petal development by suppressing premature cell-cycle arrest. | [55] |
| 14     | KNUCKLESS (KNU) | It encodes a C2H2 zinc-finger protein that regulates development of basal pattern elements along the proximo-distal axis of the developing gynoecium. | [56] |
| 15     | LEAFY (LFY) / FLORICAULA (FLO) | Promotes the expression of meristem identity AP1. Together with other co factors it activates the floral organ identity genes like AP3 and AGM. | [57, 58] |
| 16     | NOZZLE (NZZ) | It has a role in the establishment of the pollen sac and nucellus and possibly an early role in sporogenesis. | [59] |
| 17     | NUDBIN (NUB) | Defines stamen and carpel shape. NUB acts redundantly with JAG to promote the growth of the pollen-bearing microsporangia of the anthers and the carpel walls of the gynoecium, which enclose the ovules. JAG and NUB also act redundantly to promote the differentiation of adaxial cell types in the carpel walls, and in the establishment of the correct number of cell layers. | [60] |
| 18     | PISTILLATA (PI) / GLOBOSA (GLO) | It acts with CLASS B MADS box gene AP3. (CLASS B MADS box gene) | [61] |
| 19     | RABBIT EARS (RBE) | Regulates the petal development by maintaining spatial boundaries within young flowers | [62, 63] |
| 20     | SPOROCYTELESS (SPL) | It is required for the initiation of sporogenesis in male and female organs of the plants. | [64] |
| 21     | SUPPRESSOR OF OVEOREXPRESSION OF CONSTANS 1 (SOC1) | Integrates vernalization and gibberellin signals in Arabidopsis | [65] |
| 22     | SHORT VEGETATIVE PHASE (SVP) | SVP mediates the temperature-dependent functions of FCA and FVE within the thermosensory pathway: SVP controls flowering time by negatively regulating the expression of a floral integrator, FLOWERING LOCUS T (FT), via direct binding to the CArG motifs in the FT sequence. | [66] |
| 23     | SUPERMAN (SUP) | It is involved in controlling cell proliferation in stamen and carpel primordia and in ovules in flower development. | [67–71] |
| 24     | TERMINAL FLOWER 1 (TFL 1) | It is putative regulator gene involved in the control of flowering time and floral architecture | [72, 73] |
| 25     | WUSCHEL (WUS) | WUS promotes central identity in both indeterminate shoot and determinate floral meristems and plays an important role in maintaining their structural and functional integrity. | [74] |
| 26     | YABBY (YAB) | Floral organ polarity | [54] |
| 27     | SEPTALATA (SEP)(SEP1, SEP2, SEP3, SEP4) | MADS box CLASS E genes. Role in ovule formation, required to specify petals, stamens and carpels | [45] |
| 28     | FLOWERING LOCUS C (FLC) | Delays flowering in plants. Represses FLOWERING TIME (FT) gene in the absence of low temperature/ vernalization treatment. | [58] |

(Continued)
Table 1. (Continued)

| S. No. | Gene Name                          | Function in flower development                                                                 | References |
|--------|------------------------------------|-----------------------------------------------------------------------------------------------|------------|
| 30     | FLOWERING LOCUS D (FLD)            | It encodes a plant homologue of a protein found in histone deacetylase complexes in mammals. Lesions in FLD result in hyperacetylation of histones in FLC chromatin, up-regulation of FLC expression, and extremely delayed flowering. | [75]       |
| 31     | FLOWERING LOCUS T (FLT)            | It acts in parallel with the meristem identity gene LEAFY (LFY) to induce flowering of Arabidopsis. | [76]       |
| 32     | FRIGADIA (FRI)                     | Delays flowering in plants. Promotes the expression of FLOWERING LOCUS C (FLC) in the absence of vernalization / low temperature. | [58]       |
| 33     | GIGANTIA (GI)                      | Control of Flowering time in response to day length                                            | [58]       |
| 34     | PHYTOCHROME A (PHYA)               | It encodes a chloroplastic phospholipase A1 that catalyzes the initial step of JA biosynthesis which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. | [77–80]   |
| 35     | PHYTOCHROME B (PHYB)               | Far red light absorbing receptor gene which senses daylight changes to promote flowering. Arabidopsis thaliana PHYA-null mutant plants are insensitive to floral induction by day-length extensions or light treatments for short-day grown plants, both of which mimic long-day growth conditions. Under long-day growth conditions, PHYA-null mutant plants display a late-flowering phenotype when compared with the wild type plants. | [74]       |
| 36     | SHORT INTEGMENTS (SI)              | Controls ovule development and flowering time in Arabidopsis.                                  | [82]       |
| 37     | FLOWERING PROMOTER FACTOR 1 (FPF1) | It is expressed after photoperiodic induction of flowering in A. thaliana. It is involved in GA-dependent signalling pathway and modulates a GA response in apical meristems during the transition to flowering. | [83]       |
| 38     | UNSUSUAL FLORAL ORGANS (UFO)       | Mediator between floral meristem identity genes and floral organ genes.                        | [84]       |
| 39     | FIMBRIATA (FIM)                    | It mediates between floral meristem identity and floral organ genes. Expression and function of FIM depends on the activity of meristem identity genes, and FIM in turn controls the spatial and temporal expression of organ identity genes. | [85]       |
| 40     | ERECTA (ER)                        | It encodes a putative receptor protein kinase. It regulates shape of organs originating from the shoot apical meristem. | [86]       |
| 41     | DEFFECTIVE IN ANther DEHISCENCE 1 (DAD1) | It encodes a chloroplastic phospholipase A1 that catalyzes the initial step of JA biosynthesis which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. | [87]       |
| 42     | ETHYLENE RESPONSE SENSOR 1 (ERS)   | Ethylene receptor genes                                                                       | [88]       |
| 43     | ETHYLENE RECEPTOR 1 (ETR1)         | Ethylene receptor genes                                                                       | [88]       |
| 44     | NO EXINE FORMATION 1 (NEF1)        | Required in exine formation of pollen wall                                                    | [89]       |

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species or the other (Table 2). Also more than one copy of homologous sequences were found for genes CO (3), CRY1 (2), FRI (2) and TFL (2) in H. rhamnoides (Table 2). Domains and repeats found in all the homologues of seabuckthorn except for EF3, GI and NEF1 were similar to those present in Arabidopsis genes (Table 2). Such an outcome signifies that the identified contigs of putative seabuckthorn GISD have similar gene structure as of A. thaliana genes and thus are likely to perform identical functions as performed by respective genes in A. thaliana. Phylogenetic reconstruction of genes (Fig 2) showed that most of the seabuckthorn GISD clustered with similar genes except for HrCRY2, HrFIL, HrAP2 and HrNEF. Clustering of putative seabuckthorn GISD along with characterized genes in model plants further confirms that putative seabuckthorn genes share high homology to well characterized genes in model plants.

Expression analysis of GISD by qRT PCR
The temporal expression of the 24 potential GISD and their additional homologues (Total of 29 candidate GISD) across three developmental stages of male and female flowers of H. rhamnoides was analysed (S2 Table). 21 GISD were analysed for differential expression among temporally corresponding male and female flower developmental stages (FDS). The CT values of
Table 2. List of potential Seabuckthorn GSD retrieved from available seabuckthorn resources.

| S. no. | Gene name | Contig No. | Protein family, Domains & Repeats | Origin of Reference Genes | Identity | E-value | Accession no. of Reference Genes |
|--------|-----------|------------|----------------------------------|--------------------------|----------|--------|---------------------------------|
| 1      | HrAP1     | 87601      | MADS box, K-box domain           | R. hybrid^               | 76%      | 7e-67  | FJ970028.1                      |
|        |           |            |                                  | A. thaliana             | -        | -      |                                 |
| 2      | HrAP2     | 31712      | DNA binding domain, AP2/ERF domain | V. vinifera^             | 62%      | 3e-96  | NP_001267881.1                  |
|        |           |            |                                  | A. thaliana             | 78%      | 5e-55  | NP_195410.1                     |
| 3      | HrCLV1    | 30543      | Protein Kinase domain, Leucine rich repeats | M. notabolis^         | 76%      | 0.0    | EXC25022.1                      |
|        |           |            |                                  | A.thaliana              | 69%      | 0.0    | AAB58929.1                      |
| 4      | HrFLD     | 20188      | SWIRM, NADP, amine oxidase domain | P. mume^                | 85%      | 0.0    | NP_00833274.1                   |
|        |           |            |                                  | A.thaliana              | 81%      | 0.0    | NP_187650.4                     |
| 5      | HrCO      | 32194      | Zinc Finger B-box, CCT domain    | P. deltoids^             | 72%      | 8e-178 | AAS00054.1                      |
|        |           |            |                                  | A.thaliana              | 54%      | 1e-114 | NP_197088.1                     |
| 6      | HrCOLK4   | 24698      | Zinc Finger B-box, CCT domain    | P. mume^                | 74%      | 1e-167 | NP_008220621.1                  |
|        |           |            |                                  | A.thaliana              | 63%      | 2e-140 | Q940T9.2                        |
| 7      | HrCOLK9   | 13913      | Zinc Finger B-box, CCT domain    | F. vesca^               | 68%      | 0.0    | XP_004303586.1                  |
|        |           |            |                                  | A.thaliana              | 56%      | 2e-139 | NP_187422.1                     |
| 8      | HrCRY1    | 12695      | Rossmann-like alpha/beta/alpha sandwich fold, DNA photolyase, N-terminal, DNA photolyase, FAD-binding/ Cryptochrome, C-terminal, Cryptochrome C-terminal | Populus trichocarpa^    | 82%      | 0.0    | XP_002307379.1                  |
|        |           |            |                                  | A.thaliana              | 76%      | 0.0    | NP_567341.1                     |
| 9      | HrCRY1LK  | 12696      | Rossmann-like alpha/beta/alpha sandwich fold, DNA photolyase, N-terminal, DNA photolyase, FAD-binding/ Cryptochrome, C-terminal, Cryptochrome C-terminal | Populus trichocarpa^    | 82%      | 0.0    | XP_002307379.1                  |
|        |           |            |                                  | A.thaliana              | 78%      | 0.0    | NP_567341.1                     |
| 10     | HrCRY2    | 7867       | Rossmann-like alpha/beta/alpha sandwich fold, DNA photolyase, N-terminal, DNA photolyase, FAD-binding/ Cryptochrome, C-terminal | Theobroma cacao^        | 74%      | 0.0    | XP_007035111.1                  |
|        |           |            |                                  | A.thaliana              | 68%      | 0.0    | NP_171935.1                     |
| 11     | HrEF1     | 34677      | Helicase/SANT-associated, HAS subgroup | G. max^                | 51%      | 1e-88  | XP_003518059.1                  |
|        |           |            |                                  | A.thaliana              | 64%      | 7e-62  | NP_187887.3                     |
| 12     | HrEF3     | 30075      | N.D.                             | Citrus sinensis^        | 46%      | 0.0    | XP_006466166.1                  |
|        |           |            |                                  | A.thaliana              | -        | -      |                                 |
| 13     | HrFIL     | 7258       | YABBY protein, High mobility group box domain | V. vinifera^             | 80%      | 7e-84  | XP_002266233.1                  |
|        |           |            |                                  | A.thaliana              | 55%      | 7e-48  | NP_566037.1                     |
| 14     | HrFRI     | 20160      | Frgadia protein family           | V. vinifera^             | 72%      | 0.0    | NP_002282465.1                  |
|        |           |            |                                  | A.thaliana              | 61%      | 0.0    | NP_566709.1                     |
| 15     | HrFRIK    | 84388      | Frgadia protein family           | V. vinifera^             | 77%      | 0.0    | NP_002266233.1                  |
|        |           |            |                                  | A.thaliana              | 69%      | 0.0    | NP_566709.1                     |
| 16     | HrGl      | 30943      | N.D.                             | P. mume^                | 83%      | 0.0    | XP_008323480.1                  |
|        |           |            |                                  | A.thaliana              | 77%      | 0.0    | ABP96488.1                      |
| 17     | HrPHYB    | 1355       | PHY A/B/C/D/E protein family, PAF, GAF domain | V. vinifera^             | 85%      | 0.00   | NP_002278263.1                  |
|        |           |            |                                  | A.thaliana              | -        | -      |                                 |
| 18     | HrSl      | 20174      | P-loop, helicase, Dicer, Ribonuclease III, PAZ, DS RNA binding domain | V. vinifera^             | 87%      | 0.0    | XP_002268369.1                  |
|        |           |            |                                  | A.thaliana              | 80%      | 0.0    | NP_171612.1                     |
| 19     | HrTFL1    | 8067       | PEBP superfamily                | Citrus trifoliate^       | 87%      | 8e-108 | ABY91243.1                      |
|        |           |            |                                  | A.thaliana              | -        | -      |                                 |

(Continued)
eight GISD which showed values greater than 35, were not considered for further investigation. Seven GISD showed elevated expression in female FDS while fourteen GISD showed higher expression in male FDS, (Fig 3 and Table 3) details of which are given below.

**Floral meristem identity genes.** As data presented in Table 3 and Fig 3A demonstrates, meristem identity gene *HrAP1* showed female specific expression. The expression of this gene was notably 1347 fold higher in FST II as compared to MST II. On the contrary, expression of gene *HrAP2* was higher in all the male flower developmental stages with the maximum differential expression being in MST II (7.70 fold) as compared to FST II. *HrLFY* and *HrCLV1* showed stage specific expression in male and female FDS. The expression of *HrLFY* was notably higher in MST I (32.16 fold) and FST II (10.15 fold) as compared to their corresponding stages. *HrCLV1* was significantly expressed in MST II (9.11 fold) and FST III (4.15 fold). On the basis of this data it is concluded that expression of gene *HrAP1* is female specific while that of *HrAP2* is male specific. However, the expression of gene *HrLFY* and *HrCLV1* in male and female flowers was stage dependent.

| S. no. | Gene name | Contig No. | Protein family, Domains & Repeats | Origin of Reference Genes | Identity | E-value | Accession no. of Reference Genes |
|--------|-----------|------------|-----------------------------------|---------------------------|----------|---------|----------------------------------|
| 20     | *HrNEF1*  | 18354      | N.D.                              | *Theobroma cacao*          | 84%      | 9e-87   | XP_007043754.1                   |
| 21     | *HrSOC1*  | 8883       | MADS box, K-box domain            | *A. thaliana*              | 72%      | 1e-76   | NP_196843.1                      |
| 22     | *HrYAB5a* | 21143      | YABBY protein superfamily, HMG domain | *V. vinifera*              | 71%      | 1e-98   | ABF56527.1                       |
| 23     | *HrYAB5b* | 70948      | YABBY protein superfamily, HMG domain | *A. thaliana*              | 70%      | 1e-56   | NP_850080.1                      |
| 24     | *HrYAB4*  | 7257       | YABBY protein superfamily, HMG domain | *A. thaliana*              | 72%      | 1e-77   | NP_850080.1                      |
| 25     | *HrSEP3*  | 15336      | MADS box, K-box domain            | *G. max*                  | 82%      | 3e-117  | XP_003549900.1                   |
| 26     | *HrACC*   | 8293       | PPD transferase, Amino transferase CLASS I/II domain | *M. notabilis*              | 80%      | 0.0     | EXB37292.1                      |
| 27     | *HrETR1*  | 23688      | Signal transduction histidine kinase, GAF domain | *A. thaliana*              | -        | -       | -                                |
| 28     | *HrERS*   | 11717      | Signal transduction histidine kinase, GAF domain | *P. domestica*              | 86%      | 0.0     | CAI64505.1                      |
| 29     | *HrX1*    | 27099      | AMP-dependent synthetase / ligase, AMP-binding enzyme C-terminal domain | *T. cacao*                 | 77%      | 0.0     | XP_007034413.1                  |

*Contigs were obtained from the assembled unigenes of leaf and root transcriptome of *H. rhamnoides*.[35]. Sequences of unigenes could be downloaded from [http://www.plosone.org/article/fetchSingleRepresentation.action?uri = info:doi/10.1371/journal.pone.0072516.s004](http://www.plosone.org/article/fetchSingleRepresentation.action?uri = info:doi/10.1371/journal.pone.0072516.s004). For sequence analysis the nucleotide sequence was translated to protein sequence using Expasy translate.

Sequence of *HrX1* was obtained from Chawla et al, 2014 (NCBI Accession No.KF359497).

^ Plant species with maximum identity and minimum E-value.
Floral organ identity genes. Among floral organ identity genes the expression of floral organ polarity gene HrFIL was higher in all FDS of female flowers (Fig 3B). The differential expression was notably wider in FST III vs MST III (53.88 fold). On the contrary the expression of HrYAB5 and HrSEP3 was higher in all male FDS with highest differential expression of 250 fold and 1000 fold were recorded in MST II respectively. Stamen and carpel identity gene AGAMOUS (HrAG) showed stage dependent expression pattern which was higher in FST I (12.55 fold) and MST II (6.34 fold) as compared to their corresponding stages. Thus from the data recorded it can be concluded that expression of floral organ identity gene HrSEP3 and HrYAB5 was higher in male FDS and that of HrFIL was higher in female FDS. Also the relative expression of gene HrAG was flower developmental stage dependent rather than sex of flower.

Flowering time regulation genes. The expression of Blue-Ultraviolet A receptor gene CRYPTOCHROME2 (HrCRY2) was higher across all the female FDS as compared to male FDS. The expression of this gene was 129.3 fold higher. CRYPTOCHROME1 (HrCRY1) was relatively expressed higher in all male FDS with MST II and MST III showing 6.6 fold and 2.33 fold higher expression as compared to corresponding female FDS (Fig 3C). Similarly the expression of far red light receptor gene PHYTOCHROME B (HrPHYB) was higher in all male FDS notably MST II and MST III, which showed 25 fold and 7.5 fold higher expression with respect to FST II and FST III respectively (Fig 3C). The expression of CONSTANS (HrCO) responsible for flowering in long days was higher in all male FDS (Fig 3C) (9.91 fold in MST I with respect to FST I, 30 fold in MST II with respect to FST II and 113 fold in MST III with respect to FST III). The second homologue of CO (HrCOLK) showed similar pattern of expression but relative difference in a expression level was less pronounced in male and female FDS as compared to HrCO. FRIGADIA (HrFRI) and its second homologue HrFRILK responsible for delayed flowering in absence of cold temperatures, were also found to have elevated expression in male FDS as compared to their corresponding female FDS (HrFRI 17.31 fold higher in MST II; HrFRILK 9.78 fold higher in MST II) (Fig 3C). The relative expression of genes HrGI and
HrEF1 was stage dependent. Thus it is concluded that expression of most of flowering time genes including HrCRY1, HrPHYB and HrCO was higher in all male FDS while that of HrCRY2 was higher in all female FDS.

**Phytohormone ethylene response pathway genes.** The expression of seabuckthorn homologues of ethylene response pathway genes ETHYLENE RESPONSE SENSOR 1 (HrERS1) and ETHYLENE RECEPTOR 1 (HrETR1) was higher in male flowers. HrERS1 (Fig 3D) showed 10.86 fold higher expression in MST II with respect to FST II while expression of HrETR1 (Fig 3D) was recorded 9.78 fold higher in MST II with respect to FST II.

**Pollen exine formation genes.** HrX1 is the female specific SCAR marker which was found to show high level of similarity to Acyl CoA synthetase and other related plant ligases on the basis of BLASTn and tBLASTx analysis of sequence [8]. HrX1 (Fig 3E) expressed 43.47 fold higher in MST II with respect to FST II. On the contrary, expression of HrNEF1 (Fig 3E) was observed to be 19.01 fold higher in FST I as compared to MST I.

**Floral development stage (FDS) specific expression of GISD**

In stage I of flower development the expression of genes HrAP1, HrCRY2, HrEF1, HrNEF and HrAG was higher in female flowers while expression of genes HrAP2, HrLFY, HrFRI, and HrGI was higher in male flowers (Fig 4). The expression of all putative GISD except for HrCRY2 and HrLFY was higher in 2nd developmental stage of male flowers (Fig 5). In STAGE III FDS female flowers had higher expression of HrAP1, HrCRY2, HrEF1 and HrFILF while male flowers had...
higher expression levels of HrCRY1, HrCO and HrPHYB (Fig 6). Moreover, the heat map of putative GISD (Fig 7) shows that male flowers have maximum GISD with higher level of expression as compared to female flowers in 2nd floral developmental stage.

**Discussion.** The identified putative GISD of seabuckthorn shared the sequence similarity with plant species like R. hybrid, V. vinefera, M. notabilis, P. trichocarpa, etc. and had similar repeats and domains as Arabidopsis floral regulatory genes. Most of these genes also clustered along with well characterised genes of model plants. Identification of homologous flowering genes in seabuckthorn reflects that flowering pathways of seabuckthorn share similarity with Arabidopsis as well as other model dioecious plants. Thus as in the case of S. latifolia, R. acetas
and *A. chinensis*, genes involved in these flowering pathways could be potential candidates of sex determination in seabuckthorn.

Expression pattern of MADS box genes in male and female flowers of sorrel (*Rumex acetosa*) suggested that these genes could play an important role in sex determination [31]. CLASS A MADS box gene *HrAP1* (Fig 3A) showed female specific expression while *HrAP2* (Fig 3A), expressed particularly in male flowers (Fig 3A). AP2 plays an important role for sex determination in maize [35]. It suppresses carpel in tassel of male flowers by targeting *TASSELESEED 4* (*TSL4*). Similarly expression of another floral meristem identity gene *HrCLV1* (Fig 3A) was recorded highest in MST II. In case of *S. latifolia*, *CLV1* triggers carpel suppression in male flowers [36]. Thus *HrAP2* and *HrCLV1* may be involved in determining meristem identity in male flowers while *HrAP1* could trigger meristem development in female flowers of seabuckthorn.

Expression of floral organ identity gene *HrSEP3* (Fig 3B) was recorded highest in MST II. Higher expression of *SEP3* homologue was also observed in male flowers of *Asparagus officinalis* [34]. On the other hand *HrAG* (Fig 3B) showed significant expression in FST II. Thus *HrSEP3* and *HrAG* may have a crucial role in establishing floral organ identity in male and female flowers respectively.

The expression of cryptochrome receptor gene *HrCRY2* (Fig 3C) was higher in female flowers as compared to male flowers. On the other hand, level of expression of cryptochrome gene *HrCRY1* (Fig 3C), phytochrome gene *HrPHYB* (Fig 3C) and circadian pathway gene *HrCO* (Fig 3C) was higher in all male flower development stages. In dioecious plants like *S. latifolia* and *Populus tomentosa*, male and female flowers develop at different time. The photoreceptor encoding genes like *CRY1*, *CRY2*, *PHYA* and *PHYB* regulate circadian pathway genes like *CO*, *GI* and *FT* and could alter flowering time depending upon external cues [91].
expression of CRY1, CRY2, CO and GI was observed among male and female flowers of *P. tomentosa* and was correlated with asynchronous development of male and female flowers [92]. Thus expression pattern of flowering time genes showed that *HrCRY2* could influence time-dependent development of female flowers while *HrCRY1, HrPHYB* and *HrCO* may affect temporal development of male flowers in seabuckthorn.

Phytohormone ethylene response genes *HrERS1* and *HrETR1* differentially expressed in all the stages of male and female flower but without bias of expression towards particular gender. Such an outcome could be expected because, in case of dioecious plants genetic variations have a more prominent role in gender determination than internal environment and environment variation. Expression of gene containing the female specific SCAR marker *HrX1* was higher in male flowers as compared to female flowers. *HrX1* shares sequence similarity with known plant ligases such as acyl Coa synthatase [8]. In *A. thaliana* knocking out of acyl CoA synthatase led to production of unviable pollen, which in turn produced male sterile plants [93]. Another pollen exine gene *HrNEF1* showed higher expression in female flowers as compared to male flowers. Disruption of *NEF1* in *A. thaliana* affected lipid accumulation in the plastids of tapetum as well as exine formation of pollen, thus resulted in male sterility in *A. thaliana* [89]. Thus expression pattern of *HrX1* and *HrNEF1* suggested that these genes could play an important in sex determination in seabuckthorn.

The expression of genes varied throughout the development of flowers in both male and female flowers of seabuckthorn. Out of the three developmental stages, 2nd stage had the maximum number of genes with expression biased towards male flowers (Fig 4 and Fig 5 & Fig 6). Thus stage II of male and female flowers require further investigation to justify the tilt of GISD expression towards male flowers.

In conclusion, the current study showed differential expression of putative seabuckthorn GISD in all the three floral developmental stages of both male and female flowers. The expression level of *HrCO* gene was observed higher in the developmental stages of male flowers as compared to female flowers. Whereas *HrCRY2* gene significantly showed higher expression levels in the female floral developmental stages only. Further investigation is required to understand the role of *HrCO* and *HrCRY2* genes in development of male and female flowers respectively.

**Supporting Information**

**S1 File.** Nucleotide sequences of the known flowering pathway genes in model plants.

(RAR)

**S2 File.** Nucleotide sequences of seabuckthorn putative GISD retrieved from seabuckthorn genomic resources.

(TXT)
S3 File. Protein sequences of seabuckthorn putative GISD.
(TXT)

S4 File. Protein sequences of the known flowering pathway genes in model plants.
(TXT)

S1 Table. List of primers used in qRT PCR analysis of putative GISD.
(DOCX)

S2 Table. Normalized expression values of seabuckthorn putative GISD in three temporal developmental stages of male and female flowers.
(DOCX)

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Author Contributions
Conceived and designed the experiments: AC TS RBS RSC AK. Performed the experiments: AC. Analyzed the data: AC VJ AK. Contributed reagents/materials/analysis tools: AC TS RBS AK. Wrote the paper: AC AK. In Silico analysis of genes: AC AK. Mining of putative GISD from seabuckthorn transcriptome: AC VJ.

References
1. Fan J, Ding X, Gu W. Radical-scavenging proanthocyanidins from sea buckthorn seed. Food chemistry. 2007; 102(1):168–77.
2. Li C, Xu G, Zang R, Korpelainen H, Berninger F. Sex-related differences in leaf morphological and physiological responses in Hippophae rhamnoides along an altitudinal gradient. Tree Physiology. 2007; 27(3):399–406. PMID: 17241981
3. Kalia RK, Singh R, Rai MK, Mishra GP, Singh SR, Dhawan A. Biotechnological interventions in sea buckthorn (Hippophae L.): current status and future prospects. Trees. 2011; 25(4):559–75.
4. Stobdan T, Angchuk D, Singh SB. Seabuckthorn: An emerging storehouse for researchers in India. Bangalore, India: Indian Academy of Sciences 2008.
5. Zubarev YA. Commercial cultivation of Seabuckthorn in Western Siberia, Russia. Seabuckthorn (Hippophae L): A Multipurpose Wonder Plant. 2008; 3:49–60.
6. Gupta SM, Grover A, Pandey P, Ahmed Z. Female plants of Hippophae salicifolia D. Don are more responsive to cold stress than male plants. Physiology and Molecular Biology of Plants. 2012; 18(4):377–80. doi:10.1007/s12298-012-0133-7 PMID: 24082501
7. Charlesworth D. Plant sex determination and sex chromosomes. Heredity. 2002; 88(2):94–101. PMID: 11932767
8. Chawla A, Kant A, Stobdan T, Srivastava RB, Chauhan R. Cross-species application of sex linked markers in H. salicifolia and H. tibetana. Scientia Horticulturae. 2014; 170:281–3.
9. Jain A, Ghangal R, Grover A, Raghuvanshi S, Sharma PC. Development of EST-based new SSR markers in seabuckthorn. Physiology and Molecular Biology of Plants. 2010; 16(4):375–8. doi: 10.1007/s12298-010-0037-3 PMID: 23572988
10. Korekar G, Sharma R, Kumar R, Meenu, Bisht N, Srivastava R, et al. Identification and validation of sex-linked SCAR markers in dioecious Hippophae rhamnoides L. (Elaeagnaceae). Biotechnol Lett. 2012; 34(5):973–8. doi:10.1007/s12298-012-0952-4 PMID: 22245920
11. Persson HA, Nybom H. Genetic sex determination and RAPD marker segregation in the dioecious species sea buckthorn (Hippophae rhamnoides L.). Hereditas. 1998; 129(1):45–51.
12. Sharma A, Zinta G, Rana S, Shirko P. Molecular identification of sex in Hippophae rhamnoides L. using isozyme and RAPD markers. Forestry Studies in China. 2010; 12(2):62–6.
13. Chuck G. Molecular mechanisms of sex determination in monoecious and dioecious plants. Advances in botanical research. 2010; 54:53–83.

14. Matsunaga S. Sex chromosome-linked genes in plants. Genes and Genetic Systems. 2006; 81(4):219. PMID: 17038793

15. Iovene M, Yu Q, Ming R, Jiang J. Evidence for Emergence of Sex-Determining Gene (s) in a Centromeric Region in Vasconcellea parviflora. Genetica. 2014; genetics. 114.173021.

16. Miko I. Sex chromosomes and sex determination. Nature Education. 2008; 1(1):108.

17. Yang M, Yang H, Zhang Y. Sex determination in hop (Humulus lupulus L. and H. japonicus Sieb. et Zucc.). floral morphology and sex chromosomes. Sexual Plant Reproduction. 1999; 8:139–50.

18. Shepard H, Parker J, Darby P, Ainsworth CC. Sex expression in hop (Humulus lupulus L. and H. japonicus Sieb. et Zucc.)– floral morphology and sex chromosomes. Sex Determination in Plants. 1999; 8:139–50.

19. Shepard HL, Ainsworth CC. MADS box gene expression during flower development in hop (Humulus lupulus L.) 1998 [updated 22-7-2014; cited 2014 14 October]. Available: http://extras.springer.com/1999/978-3-642-64248-7/edo1998/meeting/york98/p09.pdf.

20. Zhao H, Li Y, Duan B, Korpelainen H, Li C. Sex-related adaptive responses of Populus cathayana to photoperiod transitions. Plant, cell & environment. 2008; 32(10):1401–11.

21. Delph LF, Amzt AM, Scotti-Saintagne C, Scotti I. The genomic architecture of sexual dimorphism in the dioecious plant Silene latifolia. Evolution. 2010; 64(10):2873–86. doi: 10.1111/j.1558-5646.2010. 01048.x PMID: 20850675

22. Lebel-Hardenack S, Hauer E, Law TF, Schmid J, Grant SR. Mapping of sex determination loci on the white campion (Silene latifolia) Y chromosome using amplified fragment length polymorphism. Genetics. 2002; 160(2):717–25. PMID: 11861573

23. Matsunaga S, Kawano S, Takano H, Uchida H, Sakai A, Kuroiwa T. Isolation and developmental expression of male reproductive organ-specific genes in a dioecious campion, Melandrium album (Silene latifolia). The Plant Journal. 1999; 10(4):679–89. PMID: 8993544

24. Adhikari S, Bandyopadhyay TK, Ghosh P. Hormonal control of sex expression of cucumber (Cucumis sativus L.) with the identification of sex linked molecular marker. The Nucleus. 2012; 55(2):115–22.

25. Foucart C, Boualem A, Lasseur B, Eleblu J, Fahraj I, Bendahmane A. Sex determination in cucurbits. Biologie aujourd’hui. 2012; 206(1):57. doi: 10.1051/jbio/2012005 PMID: 22463996

26. Wu T, Qin Z, Zhou X, Feng Z, Du Y. Transcriptome profile analysis of floral sex determination in cucumber. Journal of plant physiology. 2010; 167(11):905–13. doi: 10.1016/j.jplph.2010.02.004 PMID: 20303197

27. Liu J, Yin T, Ye N, Chen Y, Yin T, Liu M, et al. Transcriptome Analysis of the Differentially Expressed Genes in the Male and Female Shrub Willows (Salix suchowensis). PloS one. 2013; 8(4):e60181. doi: 10.1371/journal.pone.0060181 PMID: 23560075

28. Liu J, Yin T, Ye N, Chen Y, Yin T, Liu M, et al. Sex-specific genes in a dioecious campion, Melandrium album (Silene latifolia). Evolution. 2010; 64(10):2873–86. doi: 10.1111/j.1558-5646.2010. 01048.x PMID: 20850675

29. Yanofsky MF. Floral meristems to floral organs: genes controlling early events. Annual review of plant biology. 1995; 46(1):167–88.

30. Perl-Treves R. Male to female conversion along the cucumber shoot: approaches to studying sex and floral development in Cucumis sativus: Oxford, UK: Bios Scientific Publishers; 1999.

31. Ainsworth C, Crossley S, Buchanan-Wollaston V, Thangavelu M, Parker J. Male and female flowers of the dioecious plant sorrel show different patterns of MADS box gene expression. The Plant Cell Online. 1994; 6(12):1775–87. PMID: 7866023

32. Ainsworth C, Crossley S, Buchanan-Wollaston V, Thangavelu M, Parker J. Male and female flowers of the dioecious plant sorrel show different patterns of MADS box gene expression. The Plant Cell Online. 1994; 6(12):1775–87. PMID: 7866023

33. Kim HB, Jun S-S, Choe S, Cho JY, Choi S-B, Kim S-C. Identification of differentially expressed genes from male and female flowers of kiwifruit. African Journal of Biotechnology. 2010; 9(40):6684–94.

34. Caporali E, Spada A, Losa A, Marziani G. The MADS box gene AOM1 is expressed in reproductive meristems and flowers of the dioecious species Asparagus officinalis. Sexual Plant Reproduction. 2000; 13(3):151–6.

35. Chuck G, Meiley R, Irish E, Sakai H, Hake S. The maize tasselseed6 microRNA controls sex determination and meristem cell fate by targeting Tasselseed6/indeterminate spikelet1. Nature genetics. 2007; 39(12):1517–21. PMID: 18026103
36. Koizumi A, Yamanaka K, Nishihara K, Kazama Y, Abe T, Kawano S. Two separate pathways including SICLVL1, SISTM and SICUC that control carpel development in a bisexual mutant of Silene latifolia. Plant and cell physiology. 2010; 51(2):282–93. doi: 10.1093/pchp/pcp187 PMID: 20064843

37. Fatima T, Snyder CL, Schroeder WR, Cram D, Datla R, Wishart D, et al. Fatty Acid Composition of Developing Sea Buckthorn (Hippophae rhamnoides L.) Berry and the Transcriptome of the Mature Seed. PLoS One. 2012; 7(4):e34099. doi: 10.1371/journal.pone.0034099 PMID: 22558083

38. Ghangal R, Chaudhary S, Jain M, Purty RS, Sharma PC. Optimization of De Novo Short Read Assembly of Seabuckthorn (Hippophae rhamnoides L.) Transcriptome. PLoS One. 2013; 8(8):e72516. doi: 10.1371/journal.pone.0072516 PMID: 23991119

39. Larkin MA, Blackshields G, Brown N, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007; 23(21):2947–8. PMID: 17846036

40. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution. 2013; 30(12):2725–9. doi: 10.1093/molbev/mst197 PMID: 24132122

41. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. methods. 2001; 25(4):402–8. PMID: 11846609

42. Huijser P, Klein J, Lönning W, Meijer H, Saedler H, Sommer H. Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in Antirrhinum majus. The EMBO journal. 1992; 11(4):1239. PMID: 1563342

43. Irish VF, Sussex IM. Function of the apetala-1 gene during Arabidopsis floral development. The Plant Cell Online. 1990; 2(8):741–53. PMID: 1983792

44. Mandel MA, Gustafson-Brown C, Savidge B, Yanofsky MF. Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. 1992.

45. Krizek BA, Fletcher JC. Molecular mechanisms of flower development: an armchair guide. Nature Reviews Genetics. 2005; 6(9):688–98. PMID: 16151374

46. Bradley D, Carpenter R, Sommer H, Hartley N, Coen E. Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum. Cell. 1993; 72(1):85–95. PMID: 8093684

47. Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM. The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. Nature. 1990; 346(6279):35–9. PMID: 1973265

48. Bowman JL, Alvarez J, Weigel D, Meyerowitz EM, Smyth DR. Control of flower development in Arabidopsis thaliana by APETALA1 and interacting genes. DEVELOPMENT-CAMBRIDGE-. 1993; 119:721–.

49. Alvarez J, Smyth DR. CRABS CLAW and SPATULA, two Arabidopsis genes that control carpel development in parallel with AGAMOUS. Development. 1999; 126(1):2377–86. PMID: 10225997

50. Clark SE, Williams RW, Meyerowitz EM. The CLAVATA1 Gene Encodes a Putative Receptor Kinase That Controls Shoot and Floral Meristem Size in Arabidopsis. Cell. 1997; 89(4):575–85. PMID: 9160749

51. Samach A, Onouchi H, Gold SE, Schwarz-Sommer Z, Yanofsky MF, et al. Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science. 2000; 288(5471):1613–6. PMID: 10834834

52. Guo H, Yang H, Mockler TC, Lin C. Regulation of flowering time by Arabidopsis photoreceptors. Science. 1998; 279(5355):1360–3. PMID: 9478998

53. Erle H, Ventzki R, Voss H, Rechmann S, Benes V, Stegemann J, et al. Sequence and analysis of chromosome 3 of the plant Arabidopsis thaliana. Nature. 2000; 408(6814):820–2. PMID: 11130713

54. Nole-Wilson S, Krizek BA. AINTEGUMENTA contributes to organ polarity and regulates growth of lateral organs in combination with YABBY genes. Plant Physiology. 2006; 141(3):977–87. PMID: 16714408

55. Dinneny JR, Yadegari R, Fischer RL, Yanofsky MF, Weigel D. The role of JAGGED in shaping lateral organs. Development. 2004; 131(5):1101–10. PMID: 14973282

56. Payne T, Johnson SD, Kolturnow AM. KNUCKLES (KNU) encodes a C2H2 zinc-finger protein that regulates development of basal pattern elements of the Arabidopsis gynoecium. Development. 2004; 131(15):3737–49. PMID: 15240552

57. Coen ES, Romero J, Doyle S, Elliott R, Murphy G, Carpenter R. Floricaula: A homeotic gene required for flower development in Antirrhinum majus. Cell. 1990; 63(6):1311–22. PMID: 1702033

58. Reeves PH, Coupland G. Response of plant development to environment: control of flowering by day-length and temperature. Current opinion in plant biology. 2000; 3(1):37–42. PMID: 10679453

59. Reeves PH, Coupland G. Response of plant development to environment: control of flowering by day-length and temperature. Current opinion in plant biology. 2000; 3(1):37–42. PMID: 10679453
59. Schiefthaler U, Balasubramanian S, Sieber P, Chevalier D, Wisman E, Schneitz K. Molecular analysis of NOZZLE, a gene involved in pattern formation and early sporogenesis during sex organ development in Arabidopsis thaliana. Proceedings of the National Academy of Sciences. 1999; 96(20):11664–9. PMID: 10500234
60. Dinnye JR, Weigel D, Yanofsky MF. NUBBIN and JAGGED define stamen and carpel shape in Arabidopsis. Development. 2006; 133(9):1645–55. PMID: 16554365
61. Tröbner W, Ramirez L, Motte P, Huijser P, Lönnig W, et al. GLOBOSA: a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. The EMBO Journal. 1992; 11(13):4693. PMID: 1361166
62. Krizek BA, Lewis MW, Fletcher JC. RABBIT EARS is a second-whorl repressor of AGAMOUS that maintains spatial boundaries in Arabidopsis flowers. The Plant Journal. 2006; 45(3):425–34. PMID: 16412084
63. Takeda S, Matsumoto N, Okada K. RABBIT EARS, encoding a SUPERMAN-like zinc finger protein, regulates petal development in Arabidopsis thaliana. Development. 2004; 131(2):425–34. PMID: 14681191
64. Yang W-C, Ye D, Xu J, Sundaresan V. The SPOROCYTELESS gene of Arabidopsis is required for initiation of sporogenesis and encodes a novel nuclear protein. Genes & Development. 1999; 13(16):2108–17.
65. Moon J, Suh SS, Lee H, Choi KR, Hong CB, Paek NC, et al. The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. The Plant Journal. 2003; 35(5):613–23. PMID: 12940954
66. Lee JH, Yoo SJ, Park SH, Hwang I, Lee JS, Ahn JH. Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. Genes & Development. 2007; 21(4):397–402.
67. Bowman JL, Sakai H, Jack T, Weigel D, Mayer U, Meyerowitz EM. SUPERMAN, a regulator of floral homeotic genes in Arabidopsis. Development. 1992; 114(3):599–615. PMID: 1352237
68. Gaiser JC, Robinson-Beers K, Gasser CS. The Arabidopsis SUPERMAN gene mediates asymmetric growth of the outer integument of ovules. The Plant Cell Online. 1995; 7(3):333–45. PMID: 12242374
69. Sakai H, Krizek BA, Jacobsen SE, Meyerowitz EM. Regulation of SUP expression identifies multiple regulators involved in Arabidopsis floral meristem development. The Plant Cell Online. 2000; 12(9):1607–18. PMID: 11006335
70. Sakai H, Medrano LJ, Meyerowitz EM. Role of SUPERMAN in maintaining Arabidopsis floral whorl boundaries. 1995.
71. Schultz EA, Pickett FB, Haughn GW. The FLO10 gene product regulates the expression domain of homeotic genes AP3 and PI in Arabidopsis flowers. The Plant Cell Online. 1991; 3(11):1221–37. PMID: 12324589
72. Alvarez J, Guli CL, Yu XH, Smyth DR. terminal flower: a gene affecting inflorescence development in Arabidopsis thaliana. The Plant Journal. 1992; 2(1):103–16.
73. Esumi T, Tao R, Yonemori K. Isolation of LEAFY and TERMINAL FLOWER 1 homologues from six fruit tree species in the subfamily Maloideae of the Rosaceae. Sexual plant reproduction. 2005; 17(6):277–87.
74. Laux T, Mayer K, Berger J, Jurgens G. The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development. 1996; 122(1):87–96. PMID: 8565856
75. He Y, Michaels SD, Amasino RM. Regulation of flowering time by histone acetylation in Arabidopsis. Science. 2003; 302(5651):1751–4. PMID: 14593187
76. Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, et al. Activation tagging of the floral inducer FT. Science. 1999; 286(5446):1962–5. PMID: 10583961
77. Johnson E, Bradley M, Harberd NP, Whelam GC. Photoreponses of light-grown phyA mutants of Arabidopsis (phytochrome A is required for the perception of daylength extensions). Plant Physiology. 1994; 105(1):141–9. PMID: 12232194
78. Neff MM, Chory J. Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. Plant Physiology. 1998; 118(1):27–35. PMID: 9733523
79. Reed JW, Nagatani A, Elich TD, Fagan M, Chory J. Phytochrome A and phytochrome B have overlapping but distinct functions in Arabidopsis development. Plant Physiology. 1994; 104(4):1139–49. PMID: 12232154
80. Sandhu KS, Hagely K, Neff MM. Genetic interactions between brassinosteroid-inactivating P450s and photomorphogenic photoreceptors in Arabidopsis thaliana. G3: Genes| Genomes| Genetics. 2012; 2(12):1585–93. doi: 10.1534/g3.112.004580 PMID: 23275881
81. Halliday KJ, Koornneef M, Whitelam GC. Phytochrome B and at least one other phytochrome mediate the accelerated flowering response of Arabidopsis thaliana L. to low red/far-red ratio. Plant Physiology. 1994; 104(4):1311–5. PMID: 12232170

82. Ray A, Lang JD, Golden T, Ray S. SHORT INTEGUMENT (SIN1), a gene required for ovule development in Arabidopsis, also controls flowering time. Development. 1996; 122(9):2631–8. PMID: 8787738

83. Kania T, Russenberger D, Peng S, Apel K, Melzer S. FPF1 promotes flowering in Arabidopsis. The Plant Cell Online. 1997; 9(8):1327–38. PMID: 9286110

84. Ingram GC, Goodrich J, Wilkinson MD, Simon R, Haughn GW, Coen ES. Parallels between UNUSUAL FLORAL ORGANS and FIMBRIATA, genes controlling flower development in Arabidopsis and Antirrhinum. The Plant Cell Online. 1995; 7(9):1501–10. PMID: 8589630

85. Simon R, Carpenter R, Doyle S, Coen E. Fimbriata controls flower development by mediating between meristem and organ identity genes. Cell. 1994; 78(1):99–107. PMID: 8033217

86. Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, et al. The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. The Plant Cell Online. 1996; 8(4):735–46. PMID: 8624444

87. Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K. The DEFECTIVE IN ANther DEHISCENCE1 gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. The Plant Cell Online. 2001; 13(10):2191–209. PMID: 11595796

88. Yamazaki S, Fujii N, Takahashi H. The ethylene-regulated expression of CS-ETR2 and CS-ERS genes in cucumber plants and their possible involvement with sex expression in flowers. Plant and Cell Physiology. 2000; 41(5):608–16. PMID: 10929944

89. Ariizumi T, Hatakeyama K, Hinata K, Inatsugi R, Nishida I, Sato S, et al. Disruption of the novel plant protein NIF1 affects lipid accumulation in the plastids of the tapetum and exine formation of pollen, resulting in male sterility in Arabidopsis thaliana. The Plant Journal. 2004; 39(2):170–81. PMID: 15225283

90. Fraser LG, Tsang GK, Datson PM, De Silva HN, Harvey CF, Gill GP, et al. A gene-rich linkage map in the dioecious species Actinidia chinensis (kiwifruit) reveals putative X/Y sex-determining chromosomes. BMC genomics. 2009; 10(1):102.

91. Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science. 2004; 303(5660):1003–6. PMID: 14963328

92. Song Y, Ma K, Ci D, Chen Q, Tian J, Zhang D. Sexual dimorphic floral development in dioecious plants revealed by transcriptome, phytohormone, and DNA methylation analysis in Populus tomentosa. Plant molecular biology. 2013; 83(6):559–76. doi: 10.1007/s11103-013-0108-2 PMID: 23860796

93. de Azevedo Souza C, Kim SS, Koch S, Kienow L, Schneider K, McKim SM, et al. A novel fatty Acyl-CoA Synthetase is required for pollen development and sporopollenin biosynthesis in Arabidopsis. The Plant Cell Online. 2009; 21(2):507–25. doi: 10.1105/tpc.108.062513 PMID: 19218397