# MOLECULAR ANALYSIS FOR SEX DETERMINATION IN DIOECIOUS SEABUCKTHORN USING GENOMIC APPROACHES

*Thesis submitted in fulfillment of the requirements for the Degree of*

**DOCTOR OF PHILOSOPHY**

**IN**

**BIOTECHNOLOGY**

By

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b. The work has not been submitted to any other organization for any degree or diploma.

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# Certificate

This is to certify that the thesis entitled, **"MOLECULAR ANALYSIS FOR SEX DETERMINATION IN DIOECIOUS SEABUCKTHORN USING GENOMIC APPROACHES"** which is being submitted by **Aseem Chawla** for the award of degree of Doctor of Philosophy in Biotechnology by the **Jaypee University of Information Technology at Waknaghat**, is a record of the candidate's own work, carried out by his under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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***Place: JUIT, Waknaghat*** ***Aseem Chawla***

***Date:***

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| °C | Degree Celsius |
| % | Percentage |
| mg/g | Microgram per gram |
| Fig | Figure |
| kDa | Kilo- Dalton |
| DNA | Deoxyribonucleic acid |
| PCR | Polymerase chain reaction |
| RAPD | Random Amplified Polymorphic DNA |
| AFLP | Amplified Fragment Length Polymorphism |
| ISSR | Inter Simple Sequence Repeat |
| BSA | Bulk Segregate Analysis |
| SCAR | Sequence Specific Characterised Amplified Region |
| STS | Sequence Tagged Sites |
| CAPS | Cleavage Amplified Polymorphic Sequence |
| GC | Guanine Cytosine |
| EtBr | Ethidium bromide |
| RNA | Ribonucleic Acid |
| RFLP | Restriction Fragment Length Polymorphism |
| ml | Microliter |
| mM | Millimolar |
| CTAB | Cetyl trimethylammonium bromide |
| HCl | Hydrochloric acid |

| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| NaCl | Sodium Chloride |
| V/V | Volume By Volume |
| min | Minutes |
| TE buffer | Tris EDTA buffer |
| NCBI | National Center for Biotechnology Information |
| CDS | Coding DNA Sequences |
| GISD | Genes Involved In Sex Determination |
| FDS | Floral Development Stages |
| qRT-PCR | Quantitative Real Time PCR |
| DIHAR | Defence Institute of High Altitude Research |
| MST I | Male Stage I |
| MST II | Male Stage II |
| MST III | Male Stage III |
| FST I | Female Stage I |
| FST II | Female Stage II |
| FST III | Female Stage III |
| cDNA | Complementary Deoxyribonucleic Acid |
| U.V. | Ultra-violet |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| ETR1 | ETHYLENE RECEPTOR 1 |
| ERS | ETHYLENE RESPONSE SENSOR 1 |
| DAD1 | DEFFECTIVE IN ANTHER DEHISCENCE 1 |
| ER | ERECTA |
| FIM | FIMBRIATA |

| | |
|---|---|
| UFO | UNSUSUAL FLORAL ORGANS |
| FPF1 | FLOWERING PROMOTER FACTOR 1 |
| SI | SHORT INTEGUMENTS |
| PHYB | PHYTOCHROME B |
| PHYA | PHYTOCHROME A |
| TF | Transcription factor |
| LFY | LEAFY |
| SPL | SPOROCYTELESS |
| ERF | Ethylene-Responsive Element-Binding Factor |
| ARF | Auxin Response Factors |
| AuxRE | Auxin Response Elements |
| RD | Repression Domain |
| CTD | Carboxy-Terminal Dimerization Domain |
| HD | Homeo Domain |
| CUC2 | Cup-shaped cotyledon |
| NAM | No Apical Meristem |
| BZIP | Basic Region /Leucine Zipper Motif |
| GAI | GIBBERELLIN-ACID INSENSITIVE |
| RGA | REPRESSOR OF GA1 |
| SCR | SCARECROW |
| MYB | Myeloblastosis |
| PHD | Plant Homeo Domain |
| SAP | STERILE APETALA |
| SRF | Serum Response Factor |
| ARID | AT-rich interaction domain |

| | |
|---|---|
| AD | Activation domain |
| DBD | DNA-binding domain |
| GI | GIGANTIA |
| FRI | FRIGADIA |
| FLT | FLOWERING LOCUS T |
| FLD | FLOWERING LOCUS D |
| FLC | FLOWERING LOCUS C |
| SEP | SEPTALATA |
| YAB | YABBY |
| WUS | WUSCHEL |
| TFL 1 | TERMINAL FLOWER 1 |
| SUP | SUPERMAN |
| SVP | SHORT VEGETATIVE PHASE |
| SOC1 | SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 |
| SPL | SPOROCYTELESS |
| RBE | RABBIT EARS |
| PI | PISTILLATA |
| GLO | GLOBOSA |
| NUB | NUBBIN |
| NZZ | NOZZLE |
| FLO | FLORICAULA |
| KNU | KNUCKLESS |
| JAG | JAGGED |
| FIL | FILAMENTOUS FLOWER |

| | |
|---|---|
| ELF1 | EARLY FLOWERING 1 |
| CRY2 | CRYPTOCHROME2 |
| CRY1 | CRYPTOCHROME1 |
| CO | CONSTANS |
| CLV 1 | CLAVATA1 |
| CRC | CRAB'S CLAW |
| CAL | CAULIFLOWER |
| AG | AGAMOUS |
| PLE | PLENA |
| AP3 | APETALA3 |
| DEF | DEFICIENS |
| AP2 | APETALA2 |
| AP1 | APETALA1 |
| SQUA | SQUAMOSA |
| NEF1 | NO EXINE FORMATION 1 |

# ABSTRACT

Seabuckthorn is a dioecious shrub which belongs to family Elaeagnaceae, found mainly in hilly regions of Europe and Asia. It has unisexual flowers on distinct male and female plants. There are seven species of genus *Hippophae*, out of which three are found in India, namely *H. rhamnoides*, *H. salicifolia* and *H. tibetana*. The female plants bears berries that are rich in vitamins, organic acids, fatty acids, flavonoids and antioxidants, thus provides various health related benefits. Early sex determination of dioecious plants is essential for successful genetic improvement and commercial cultivation. Male and female seabuckthorn plants are morphologically similar and cannot be distinguished prior to 3–4 years of growth, i.e., at the time of flowering. Financial resources and valuable time can be saved if undesired male/ female plants can be discarded at an early stage of research trials and commercial plantation. In current study SCAR based sex linked markers *HrX1* and *HrX2* developed for *H. rhamnoides* were validated on *H. salicifolia* and *H. tibetana*. *HrX1* produced female specific amplification in 85% of females in *H. salicifolia* while in case of *H. tibetana* it amplified in 100% of females plants. *HrX1* did not amplify in male plants of either *H. salicifolia* or *H. tibetana*. *HrX2* did not amplify in either male or female plants of *H. salicifolia* or *H. tibetana.* Differences between male and female plants are mainly detected in reproductive organs including flower buds and flowers. It occur through differential growth, repression or abortion of sex organs in flowers. In such systems sex determination is regulated at genetic level by differential expression of various sex determining loci as well as genes involved in floral organ development in both male and female flowers. Previous molecular marker based studies in seabuckthorn failed to identify genetic locus responsible for sex determination, thus mechanism governing gender determination in seabuckthorn still remains unknown. In pursuit to identify such genetic elements for regulating sex determination, literature survey was conducted to identify probable floral regulatory genes as well as transcription factors which may be involved in sex determination in seabuckthorn. The expression pattern of the identified genes and transcription factors was studied three temporal developmental stages of seabuckthorn male and female flowers using qRT-PCR. Out of the 21 probable genes analysed for differential expression, *HrCO* showed male specific expression while the higher expression level of *HrCRY2* was recorded in female flower buds. The expression level of transcription factor HrTF_AP2 was found highest in female flowers. On the other hand transcription factors HrTF_AUX_IAA and HrTF_SRF showed significant expression in male flowers.

# CHAPTER 1
# INTRODUCTION

Sea buckthorn *(*2n=24, The nuclear 2C DNA (diploid genome size) = 2.66±0.12 pg) is a dioecious multi-branched thorny shrub with yellow or orange berries[1]. The name seabuckthorn is derived from its characteristic of growing near the sea, and from the possession of many spines or thorns that resembles some buckthorn species of the genus *Rhamnus*[2]. It belongs to the family Elaegnaceae and genus *Hippophae.*The benefits of seabuckthorn were known from ancient times since the young leaves of this plant were added to the fodder of horses, which lead to shinning of their coat and instant weight gain[2].This in fact led to the naming of the seabuckthorn genus as "*Hippophae"*. *Hippophae*is originated by combining two latin words, 'Hippo' which means Horse and 'phaos' which means to shine.Seabuckthorn is a fast-growing, frost, wind and heat resistant plant. It is highly adaptable to extreme climatic conditions, including temperature range of -43°C to +40°C,drought, high altitude, salinity, alkalinity and inundation [3].

## 1.1 **Taxonomy**

Numerous studies were conducted to classify seabuckthorn species. The first classification of seabuckthorn consisted of only single species i.e. *H. rhamnoides*, with 3 subspecies, *rhamnoides*, *salicifolia*, and *tibetana*[4]. Then in 1971 Rousi reclassified the genus *Hippophae* recognizing 3 species based on morphological variations: *H. rhamnoides*L., *H. salicifolia*D. Don, and *H. tibetana*Schlecht.*Hippophaerhamnoides*was further sub-divided into 9 subspecies: *carpatica, caucasica, fluviatilis, gyantsensis, mongolica, rhamnoides, sinensis, turkestanica*, and *yunnanensis.* The differences among these subspecies are mainly based on size, shape, number of main lateral veins in the leaves and quantity and colour of stellate hairs. Racial divergence does exist, even within each subspecies. The latest taxonomical classification proposes six species of Seabuckthorn[5]namely *H. rhamnoides, H. salicifolia, H. tibetana, H. goniocarpa, H. neurocarpa*and *H. gyantsensis* (Fig. 1.1).

## 1.2 **Distribution**

The genus *Hippophae*is generally found between the latitude 27–69 N and longitude 7W–122E. It coversaprroximately 14 million hectare area around the world[6, 7]. Although this plant is distributed worldwidebut is majorly found in Asia, Europe and


**Fig. 1.1 Taxonomic classification of Seabuckthorn**

North America as shown in Fig.1.2. *H. rhamnoides* is widely distributed in Europe and Asia particularly in China, India Mongolia, Russia, Kazakhstan, Turkey, Romania, Switzerland, Germany, France and Britain, and northwards to Finland, Sweden and Norway[6, 8] (Fig. 1.2). The remaining species in the genus have a rather limited distribution and occur only in China and some neighbouring countries along the Himalayan Mountains [6, 9, 10].

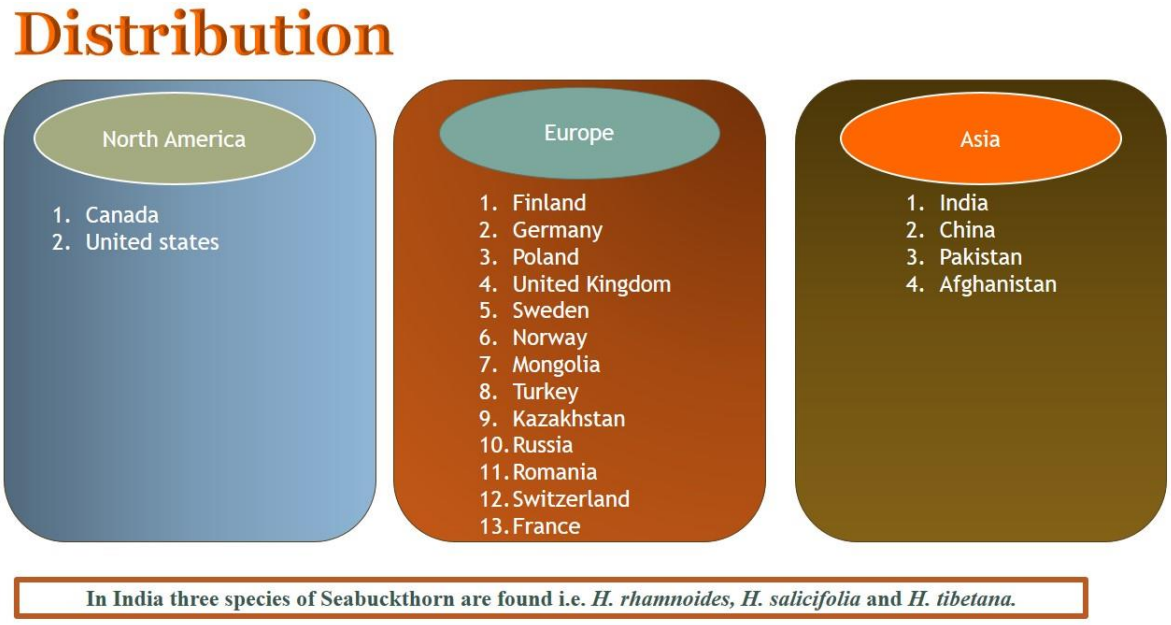


**Fig. 1.2 Geographical distribution of Seabuckthorn across the world.**

### 1.2.1 Seabuckthorn distribution in India

Sea buckthorn is widely spread in different regions of India such as Sikkim, Himachal Pradesh, Ladakh (Jammu and Kashmir), Uttaranchal, and Arunachal Pradesh (Table 1.1). About12,000 hector area is covered under seabuckthornLadakh[11]. It is known as "Charma' or "Charla" in the cold desert areas of Himachal Pradesh such as valleys of Lahaul&Spiti and Kinnaur, "Tsemarang" in Ladakh and "Ames/Chuk" in Uttarakhand.[12]

**Table 1.1 Distribution of seabuckthorn across India (Adapted from Acharya et.al. 2010 [12] )**

| Species | Ladakh | Himachal | Uttarakhand | North –east |
|---|---|---|---|---|
| *H.rhamnoides* | Indus, Nubra, Suru, Changthang Valley | Kukumsari, Lakauk, Kaza, Tabu | - | - |
| *H.salicifolia* | - | Lahaul valley | Buddi, Gori, Kai,Harindun | Dormang, Lachen, Lanchug |
| *H. tibetana* | Zanskar valley | Sangrum, Takcha, Kibbar | Niti, Milan, Nelong, Gomukh | North Sikkim |

## 1.3 Economic Importance

### 1.3.1 Nutraceutical importance

According to Schroeder and Yao (1995)[13], the seabuckthorn berries are the most nutritious and vitamin-rich fruits found in the plant kingdom.Seabuckthorn berries are rich source of carbohydrates, protein and fat soluble vitamins, antioxidants (i.e. vitamins C and E, β-carotene, and lycopene), essential fatty acids, amino acids, phytosterols and flavonoids, in addition to chemical elements (i.e. iron, calcium, etc.). [14-16].The concentration of vitamin C (695 mg/100g) is higher in sea buckthorn fruit as compared to strawberry, kiwi, orange, tomato, carrot and hawthorn [17]. The flesh of berries is rich in vitamins namely A, B, C, D, E, F, K and P. Apart from vitamins, berries contain numerous minerals (sodium salts, potassium, and calcium), fatty acids (oleic acid and palmitoleic acid), sugars, proteins, amino acids and organic acids (malic, quinic, oxalic citric and tartaric acids)[18]. A large number of bioactive compounds like flavonoids (isorhamnetin, quercetin, myricetin, kaempferol and their glycoside compounds), tannins and carotenoids (β and δ- carotene, lycopene) are also present in sea buckthorn fruits[18].One of the major constituent of the berries is the oil, which is extracted from pulp, seeds and the fruit residue.The oil contains an average 35% of the rare and valuable palmitoleic acid (16:1n-7; omega-7 series fatty acid)[2]. The chemical composition of the

seabuckthorn oil extracted from various parts of the berries is listed in Table 1.2[19]. The protein levels are also high in seabuckthorn juice [20]. Due to such nutritional properties a number of food products such as juices, jams, candy, jellies, purees and teas are prepared from seabuckthorn berries (Fig. 1.3)[21]. Sea buckthorn leaves consist of flavonoids [22], carotenoids, free and esterified sterols, triterpenols, and isoprenols.[23]. In Russia, vitamins and flavonoids are extracted from the leaf of seabuckthorn. From air-dried leaves numerous products can be made, but most important are teas and tea powders [24]

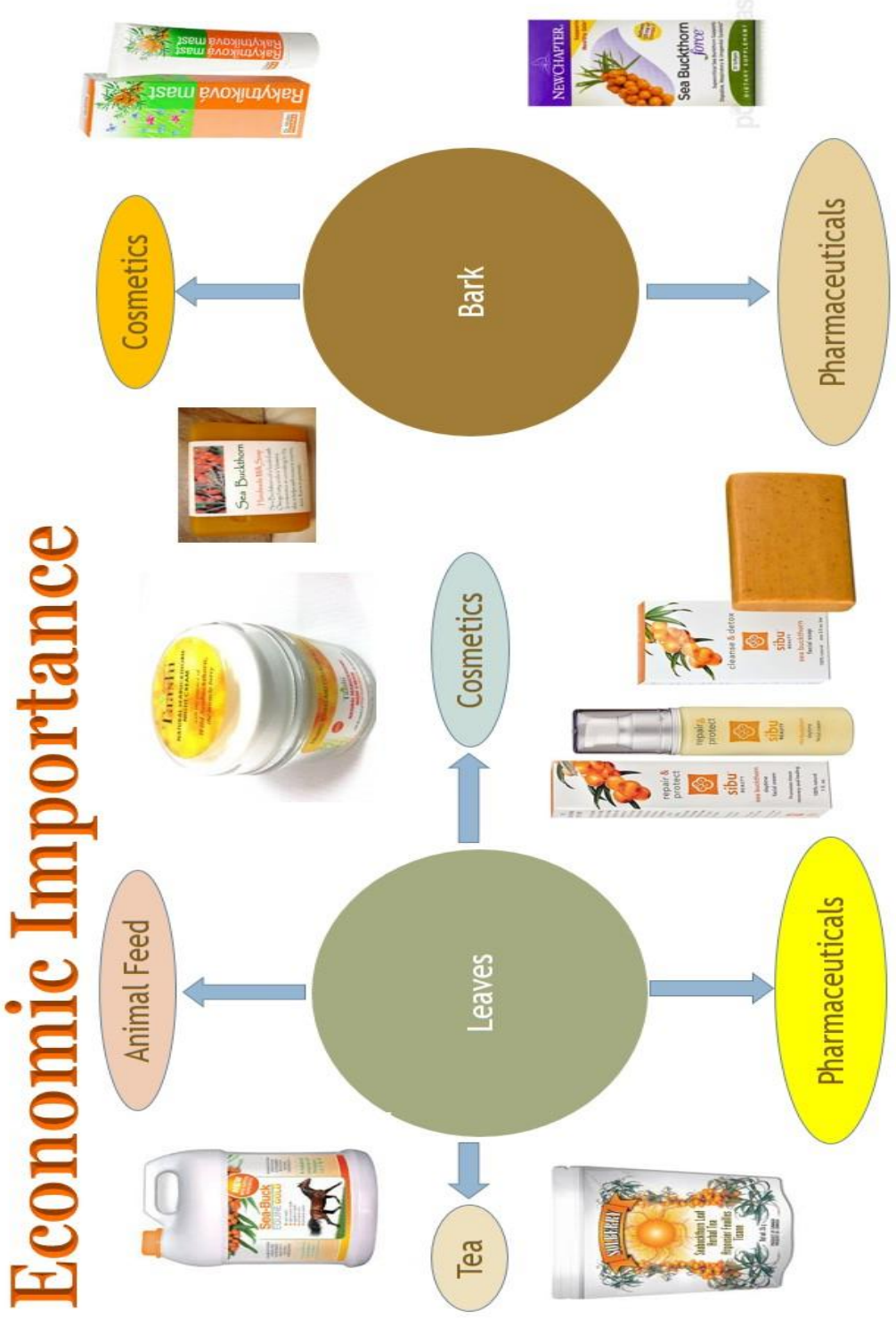


**Fig. 1.3 Economic importance of Seabuckthorn**

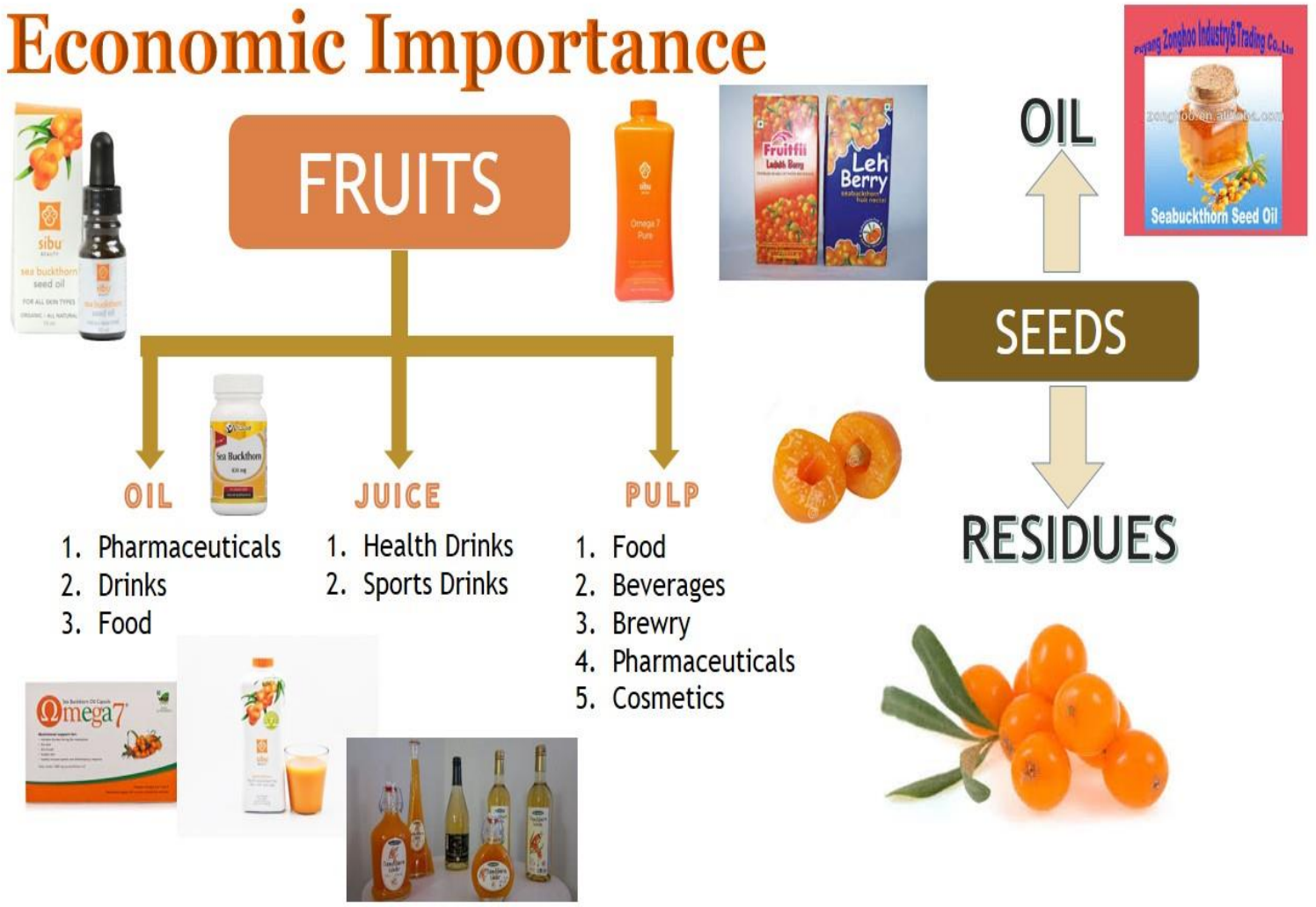


**Fig. 1.3 (contd.) Economic importance of Seabuckthorn**

**Table 1.2: Constituents of the seabuckthorn oil extracted from seed, pulp and fruit residue after removing juice. ( Adapted from Zeb A, 2004 [19] )**

| Ingredients | Seed oil | Pulp oil | Fruit residue oil |
|---|---|---|---|
| Vitamin-E | 207 mg/100g | 171 mg/100g | 300-600 mg/100g |
| Vitamin-K | 110-230 mg/100g | 54-59 mg/100g | - |
| Carotenoids | 30-250 mg/100g | 300-870 mg/100g | 1280-1860 mg/100g |
| Total acids | 11 mg/100g | 38 mg/100g | - |
| Total flavonoids | - | - | 550 mg/100g |
| Total steroids | 1094 mg/100g | 721 mg/100g | - |
| Unsaturated fatty acids | 87 % | 67 % | 70 % |
| Saturated fatty acids | 13% | 33% | 30% |

### 1.3.2 Medicinal importance

Seabuckthorn possesses various medicinal properties like anti-aging, anti-inflammatory, immunomodulatory and antioxidant activities [25].It provides protection against chromium induced oxidative injury with increased free-radical scavenging activities [26]. The chemical compound found in sea buckthorn (i.e. Triterpenoids) has shown remarkable inhibitory effect on nitric oxide production [27]. *In vitro* studies have also shown protection from oxidative stress in glial cells and lymphocytes on treatment with sea buckthorn extracts [18, 28]. The seed oil is found to be very effective in treatment of various skin disorders such as eczema, burns, wounds, wrinkles, dryness and premature skin aging and is used as an anti-ageing agent either in the form of cream or lotion(Fig. 1.3)[29].Seabuckthorn is also used for the treatment of various ailments such as oral mucositis, duodenal ulcers, acid reflux, esophagitis, scalds and gum bleeding. The antioxidant activity in sea buckthorn is mainly attributed to its flavonoids, vitamins E and C [18]. The aqueous and hydro-alcoholic leaf extracts of seabuckthorn has shown antioxidant, cytoprotective, antibacterial and anti-cancer effects [30, 31]. One of the clinical trials has shown that sea buckthorn extracts helps in normalization of serum bile acids, liver enzymes, and immune system markers, which are involved in liver inflammation and degeneration [32]. Moreover it protects the liver from harmful effects by toxic chemicals[33]

### 1.3.3 Environmental importance

Seabuckthorn holds great environmental significance because of its extensive root system, which is ideal for afforestation, soil conservation and waste land reclamation particularly in fragile slopes as it transforms insoluble organic matter in to more soluble state and prevents soil erosion [7, 34]. seabuckthorn is resistant to drought, tolerates soil salinity and low temperatures, it is ideal to grow inwide range ofterrain such as riverbanks, lakeshores, steep slopes and other susceptible terrain. The extensive, sub-terranean rhizomatous type of root system helps in strong soil -binding, soil stabilization and water retention. Also their roots are in symbiotic association with nitrogen fixating organismFrankia. This interaction helps to fix atmospheric nitrogen and hence increases the fertility of soil [7, 35]. This property is assisted by rapid growth and strong coppicing making it efficient for soil conservation, improvement and restoration of degraded land in hilly areas [36].

## 1.4 Monoecious and Dioecious plants

### 1.4.1 Distribution across plant taxa

Ten percent or so of plant species [37] have evolved floral unisexuality as spatial separation of their flowers (Table 1.3). This can be exhibited as monoecy, where the male and female organs are carried on separate flowers on the same plant, or dioecy, where male and female flowers are carried on separate male (staminate) or female (pistillate) individual plants. Unisexual plant species appear to be distributed throughout the flowering plant families (around three quarters of families include dioecious species) suggesting independent evolutionary events. However, the frequency of unisexuality is not evenly spread throughout the plant kingdom; dioecy is particularly prevalent in the familiesMenispermaceae, Myristicaceae, Monimiaceae, Euphorbiaceae, Moraceae, Cucurbitaceae, Anacardiaceae and Urticaceae, and appears to be rather more common among dicot genera than among monocot genera [38].

**Table 1.3: List of few important monoecious and dioecious plant species. (Adapted from Ainsworth, 2000[39])**

| Monoecious species | Dioecious species | |
|---|---|---|
| Castor bean (*Ricinuscommunis*) | Seabuckthorn*(Hippophaerhamnoides)* | Papaya (*Carica papaya*) |
| Fig (*Ficuscarica*) | Asparagus (*Asparagus officinalis*) | Pistachio (*Pistaciavera*) |
| Cucumber (*Cucumissativus*) | Cloudberry (*Rubuschamaemorus*) | Poplar (*Populus spp*.) |
| Hazelnut (*Corylus spp.)* | Date palm (*Phoenix dactylifera*) | Spinach (*Spinaciaoleracea*) |
| Melon(*Cucumismelo*) | Hemp (*Cannabis sativa*) | Willow (*Salix spp*.) |
| Maize (*Zea mays*) | Hop (*Humuluslupulus*) | Yam (*Dioscorea spp*.) |
| Oil palm (Elaeisguineensis) | Kiwifruit (*Actinidiadeliciosa*) | White campion (*Silenelatifolia*) |
| Walnut (*Juglansregia*) | Mistletoe (*Viscum album*) | Sorrel (*Rumexacetosa*) |

### 1.4.2 Unisexual flowers occur in Monoecious and Dioecious plants

The majority of flowering plants (around 90 %) produce flowers which are ‘perfect' i.e. they produce bisexual flowers possessing both male and female gamete producing organs (Fig. 1.4). Bisexual flowers are called perfect because the flower has everything needed to produce a seed by sexual reproduction. The situation in higher plants contrasts strikingly with that in animals where most species are unisexual with male and female gametes produced by different individuals [39]. Some plants have flowers that are not perfect, they do not have both male and female reproductive parts in the same structure. Instead they produce male flowers that have only stamens or female flowers that have only pistils. Monecious plants have both male and female flowers rather that perfect flowers (Fig. 1.4). However, some plant species are more like animals and each plant is either a male or a female. Plants with this situation are called dioecious. As with monoecious plants, flowers produced will have stamens but no pistils or pistils but no stamens. These male or female specific flowers, however, are made on separate plants, not on separate parts of the same plant (Fig. 1.4).

The basic flower pattern has evolved in a number of ways such that sexual reproduction involves outcrossing, and the consequent flow of genetic variation derived from mutation, is favoured [40]. The mechanisms in plants which promote outcrossing include the

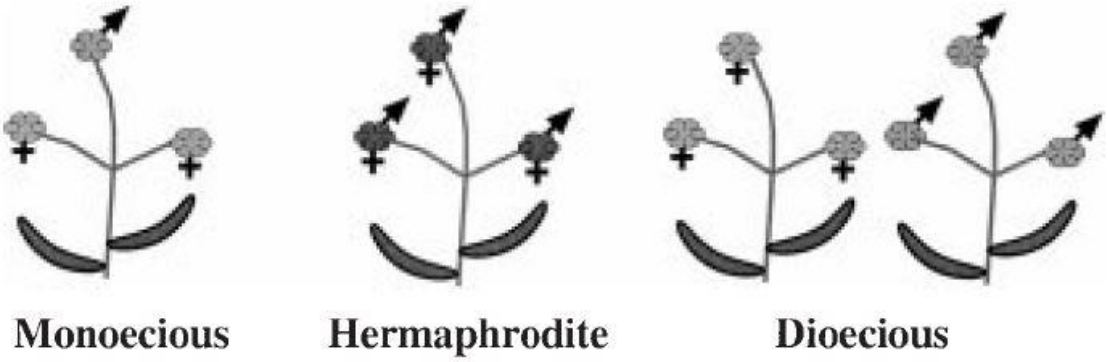


**Fig. 1.4: Pictorial representation of Monoecious, Dioecious and hermaphrodite plants. Bisexual 'perfect' flowers exist on hermaphrodite plants. Monoecious plants have unisexual flowers i.e. male and female flowers within the same individual plant. Dioecious plants have male and female flowers on separate individual plants. Adapted from Ainsworth, 2000[39].**

temporal separation of the maturation of the male and female organs within an otherwise perfect flower (dichogamy), self-incompatibility mechanisms, both sporophytic and gametophytic, where there is genetic control over the possible fertilization events, and dioecy, the spatial separation of the sexual organs on separate plants. Among these plant development processes, dioecy is considered as an extreme mechanism which avoids the deleterious effects of inbreeding among plants [39]. Wind pollinated plants appear to have higher correlation for dioecy since the structure of the flower is clearly important in relation to pollen distribution and reception. Moreover sexual specialization of male and female flowers is common in wind pollinated plants[38]. In case of unisexual flowers staminate flowers are often borne on long, pendulous and flexible inflorescences which aid pollen dispersal, while female flowers and inflorescences are generally more rigid and have feathery stigmas. The distribution of flowers is also important since male flowers tend to be borne on slender branches while female flowers bear on large, more rigid branches of the plant [41].

### 1.4.3 Morphological Difference between male and female plants of dioecious species

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 [42, 43]. In case of *Asparagus officinalis*mature flowers are unisexual [44]. The first difference between the two sexes is visible when the style starts developing in female flowers.Stamens are present in flowers of both sexes, but in mature female flowers they are collapsed and reduced to a sort of vestigial organ.In female flowers ovary is tricarpellate and

trilocular and it has a rather short style with a lobate stigma. In male flowers, the ovary remains very small and the style generally does not develop. [44]. In *Silenelatifolia* the first gender specific differences are detected when stamen primordial appear in both the male and female flowers. The undifferentiated space left in the centre of the floral meristem after the stamen primordia emerge is much smaller in male flowers as compared to female flowers. The stamen development is arrested after some time in female flowers and it degenerates when gynoecium reaches to its maturity. In contrast to female flowers gynoecium continues to grow in male flower[45].

### 1.4.3.1 Structure of male and flowers of seabuckthorn

Seabuckthorn is a dioecious species with male and female flowers on separate trees.Its flowers are formed mainly on 2-year-old wood (or third leaf) differentiated during the preceding growing season [17]. The sex of seedlings cannot be determined until the setting of flower buds in 3–4 years after seeding [46]. Female plants begin to set fruit in 4–6 years from seeds, 2–3 years from cuttings. Male buds are larger than female buds with 6–8 covering scales. The smaller female buds are more elongated and have only two covering scales. The male inflorescence consists of 4–6 apetalous flowers. The female inflorescence usually consists of a single apetalous flower consisting of a pistil, a hypanthium and 2-lobed perianth, and occur in small racemes in the leaf axils(Fig. 1.5)[6]. The male flower hassomewhat longer, oblong perianth leaves and the stamens are all of about equal length. Both male and female flower buds within the same species open at the same time, about 1 week prior to the leaves, in mid to late May. Pollen is released in large quantities when air temperatures reach 6–10°C. Female flowers are receptive for approximately 10 days. Neither the male nor the female flowers produce nectar, which do not attract insects; thus, pollination of female flowers depends entirely on the wind to spread pollen from male flowers [20]

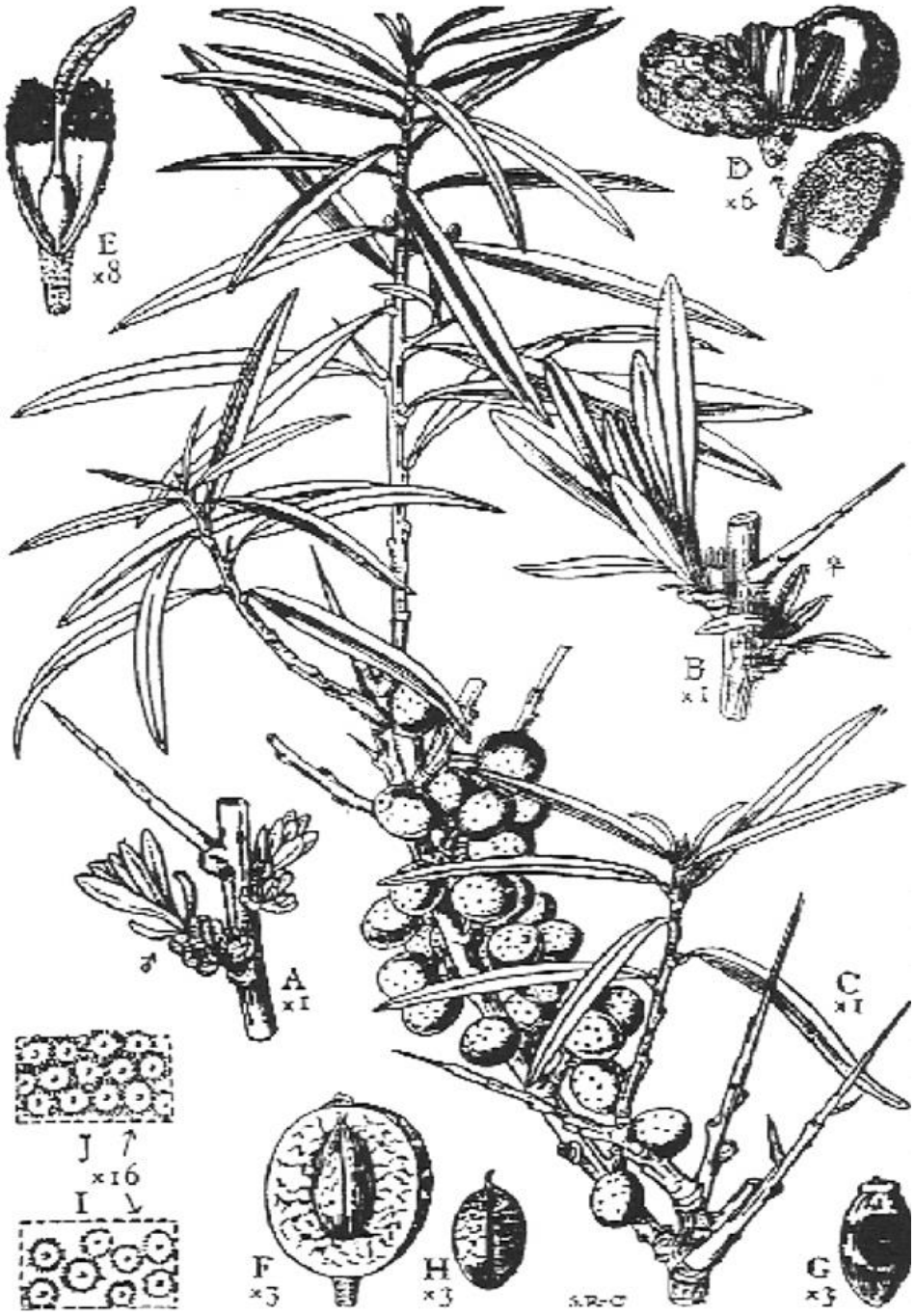


**Fig. 1.5 Diagrammatic representation of flowering and reproductive parts in seabuckthorn (*Hippophaerhamnoides*L). A) Male flowering branches B) Female flowering branches C) Fruiting branch with young leafy shoots D) Male flower E) Female flower F) Drupe — partly cut away to show the stone G) Stone – part of the membranous covering cut away to show the seed H) Seed I) Upper surface of leaf J) Lower surfaces of leaf. Adapted from Li and Beveridge, 2003 [20]**

## 1.5 Gender identification in Dioecious Plants

One of the vital area of research in agricultural domain is gender identification of commercially important dioecious plant at seedling stage because sexual phenotypes determine how the species would be cultivated. In several dioecious crops, like *Simmondsiachinensis*[47, 48], *Hippophaerhamnoides*[49, 50] the female plant produces the commercial harvest, while in few others such as *A. officinalis*[51, 52], *Cannabis sativa* males are preferred over female plants[53, 54]. Contrarily, hermaphrodites are favoured over the male or female plants in some cases as Carica papaya[55]. But in such perennial dioecious species, reproductively mature stages (vary from 1 to 20 years), are the only way to determine the gender of the plant. Determination of the sex of such economically important dioecious crops just by the external morphology of their embryogenic and juvenile forms is difficult prior to flowering. Even extensive cytogenetic studies conducted on such dioecious taxa revealed the rare occurrence of sex chromosomes in such crops, if otherwise they are present, methodology for histocytological studies are not that much of user friendly[56, 57]. Therefore, an early diagnosis of gender is of paramount importance in such commercial crops. Methods to determine the gender in such crops at the very early stages would tackle the difficulties faced by the breeders particularly when all superior parental selections are unknowingly composed of the commercially unwanted gender. It would help in reducing the effort of breeders and cultivators in saving field space, time and other useful resources that get wasted in maintaining undesired plants till flowering[58]. Realising these underlying problems, several approaches have been attempted to resolve the problems associated with sex identification. One such approach is the development of marker system to identify sex at a very early stage of the plant life cycle. In this progression, several marker types such as morphological cytological, physiological, biochemical and molecular markers (Fig. 1.6) have been developed and characterized to a certain extent that proved beneficial to delineate male from female plants in several dioecious crops.

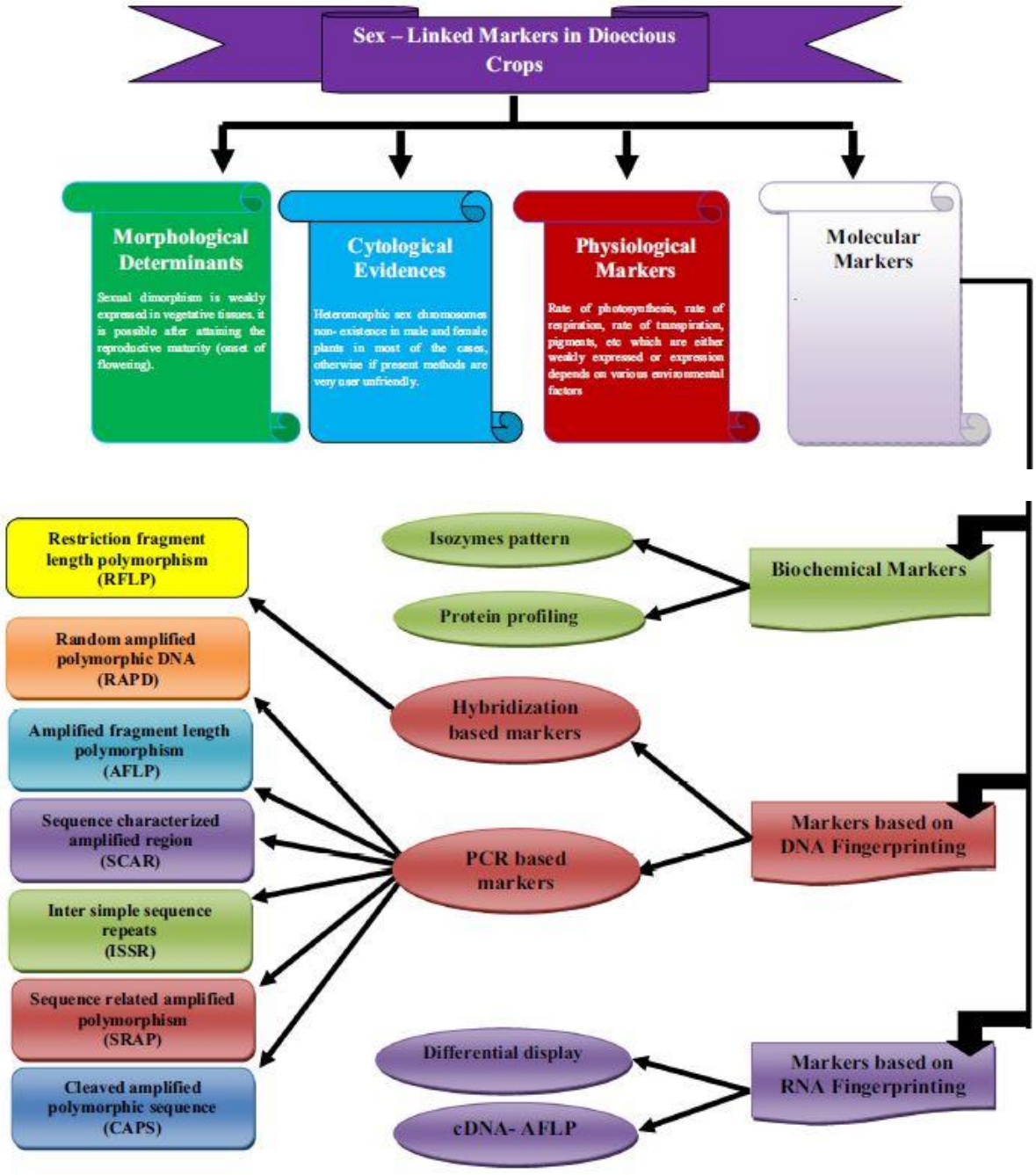


**Fig. 1.6: Various strategies employed for identification of male and female plants (Adapted from Heikrujjam*et. al.*, 2015 [59] )**

### 1.5.1 Markers based on morphological, physiological and biochemical aspects

#### 1.5.1.1Morphological markers

Morphological differences between the male and female in dioecious plantsin terms of size of leaves, stems, branches, inflorescences, flowers, canopies, etc.[60, 61] can be seen only after its reproductive maturity but complete reliability on these morphological features remains doubtful. Morphologically, it is impossible to determine male and female plants at the vegetative stage in almost all the dioecious crops.

#### 1.5.1.2 Physiological markers

Since male and female individuals are destined to perform different reproductive functions, it is pertinent that the physiological aspects between male and female may differ[59]. Physiological studies show that the sex ratio is more male biased when plants are grown in less favourable habitats. Bierzychudek and Eckhart in 1988 [62]found that out of the 32 dioecious species studied, 17 species had sex raito biased towards males. Male and female individuals of dioecious plants differ in many physiological aspects such as photosynthetic activity, respiration rate, transpiration rate, water efficiency, phenolic content, etc. [59].

*Salix arctica,* a dwarf willow, was the focus of one of the first comprehensive studies of sexual dimorphism in physiology. The sex ratio of *A. arctica*changed from female biased to male biased as the habitats changing from wet meadows to dry ridge crests [63].In wet meadows, females have higher rates of gas exchange than males during large portions of the day and of the growing season when soil temperatures fall below 4-5 °C (67% of the growing season). In contrast, in xeric habitats, females show greater sensitivity to water stress than males; females close their stomata whenever soil water potential falls below -1.0 MPa.

*Acer negundo,* a dioecious riparian tree and the only fully dioecious member of the maple genus, exhibits sex specific separation of the unisexual flowers along soil moisture gradients[64, 65]. Males are typically more common in drier sites away from streams while females are more common in wet sites along streams. Male *A. negundo*are consistently more conservative in their water use and are better able to avoid drought stress compared to females. The leaf stomata of males exhibit much greater sensitivity to changes in the vapor pressure gradient and to fluctuations in soil water potential. In the field, females are less likely to close their stomata relative to males even though they experience more negative leaf water potentials

and hence greater levels of water stress. As a result of these differences in water use, rates of leaf photosynthesis and transpiration are lower in males than in females. In dry sites, the sex ratio becomes increasingly male biased as tree size (and age) increases, whereas in wet sites, the sex ratio is significantly more female biased in the largest as compared to the smallest trees [66].

Physiological parameters are liable to change throughout the developmental stages. Furthermore, availability of limited differences and strong environmental impacts on various physiological processes have made this strategy unreliable, thereby halting their applicability in sex identification at an early stage of development.

#### 1.5.1.3 Biochemical markers

Khukhunaishvili and Dzhokhadze in 2006 [67]studied the electrophoretic patterns of leaf proteins to detect specific proteins marking the sex of two *Actinidia* species i.e. *A. kolomikta*and *A. chinensis*. The electrophoretic pattern of male plants had an intense band with a molecular weight of approximately 18 kDa, which was absent in the pattern of female plants. The electrophoretic pattern of female plants contained a distinct band of the polypeptide with a molecular weight of ≈ 45 kDa and two less intense bands with molecular weights of ≈ 67 kDa. Presumably, the latter two components are polypeptides of one polymorphic protein, probably encoded by a multiallelic gene specific for female plants. These components were absent in the male pattern or gave minor bands.

In case of *Asparagus officinalis*[68]extracts from phylloclads were electrophoreticallyanalyzed for isozyme polymorphism. Fourteen enzyme systems were used in the study.Isozyme polymorphism was studied in seven pairs of male and female doubled haploids and in their male $F_1$s. In case of isocitrate dehydrogenase females showed three bands while in males only the slowest and the fastest migrating bands were present. Males showed eight bands pattern in case of Malate dehydrogenase while females showed six bands. For 6-phosphogluconate dehydrogenase: males showed eight banding pattern; females showed seven banding pattern.

Peroxidase and esterase isoenzymes in leaves of the two sexual phenotypes of hemp were studied [69]. Significant differences in isoperoxidase and isoesterase patterns were found between male and female plants, both in the number and stain intensity of bands. For both esterase and peroxidase, the isoenzymatic spectrum were richer for staminate plants. Also, some

differences are obvious between the two sexes concerning catalase and peroxidase activities, as well as the level of soluble protein. The quantitative analysis of flavones, polyholozides and polyphenols emphasized differences depending not only on sex, but also on tested organ. In leaves, polyphenols and flavones level was high in females while male leaves contained no polyphenols and very less flavones. Polyphenols were absent in males while present in high amounts in females.

Sharma *et. al.* in 2010 [70] studied four isozymes, viz. peroxidase, esterase, malate dehydrogenase and catalase of seabuckthorn to demarcate the male and female plants.The peroxidase enzyme system produced three anodal bands at RM values of 0.23, 0.32 and 0.47. The two slower moving bands at RM values of 0.23 and 0.32 were found to be monomorphic, since they were observed in all the male and female genotypes, whereas the fastest moving band at RM value of 0.47 was observed only in five female genotypes. Thus a female specific sex marker was identified, which could successfully differentiate between the staminate and pistillate genotypes of *H. rhamnoides*L.

Isozyme markers suffer from certain disadvantages. These are usually affected by environmental conditions, their expression varies from tissue to tissue, plant phenological stages and post transcriptional modification. Hence the results from the different laboratories may not be compared [70]. Because of these disadvantages, the use of protein profiles as marker systems to study phylogeny or diversity or gender determination is limited.

### 1.5.2 DNA based markers

Determination of gender at pre-reproductive stages of dioecious plants using morphological features, biochemical and cytological evidences had certain limitations.The advancements of the DNA/RNA fingerprinting techniques made in the recent past have been helpful in development of gender specific DNA and RNA markers with greater reliability and accuracy. Restriction digestion of genomic DNA or PCR amplification of male DNA and female DNA with short oligonucleotides primers, electrophoretic separation of the amplified fragments followed by gel documentation and image analysis are various steps involved in this process[59].

### 1.5.2.1 Hybridization based markers

#### 1.5.2.1.1 Restriction Fragment Length Polymorphism (RFLP)

DNA polymorphism in RFLP is detected by hybridizing a chemically/ radioactively labelled DNA probe to a southern blot of DNA pre-digested by restriction endonucleases which results in differential DNA fragment profile. This differential profile is generated due to point mutation, insertion/deletion, translocation, inversion and duplication [47]. Although, RFLP markers offer various advantages such as high reproducibility, codominant inheritance and good transferability between laboratories but the technology has not been adopted fully for sex determination.

In *A. officinalis*probe δ47 was utilised for gender identification studies [71]. The DNA of the progeny from various crosses were subjected to single restriction digestion with numerous enzymes. Out of all the enzymes *HIND III* showed the highest level of polymorphism with the probe δ47 in all the four crosses. Probe δ47 gave 168 correct predictions out of 181 for the gender of the progeny.

In *Rumexacetosa*[72] DNA from male and female leaf samples was digested with different restriction enzymes, electrophoresed on a 3 per cent Nusieveagarose gel and stained with ethidium bromide. After EcoR I digestion one band of 180 bp was cut from the gel and cloned in pUC19. This 180 bp obtained during RFLP analysis was more intense or prominent in males as compared to female plants.

The Hybridization technique has certain limitations like it is tedious to perform, cost ineffective and requires a large quantity of high quality DNA along with radioactive/toxic reagents. Due to such limitations hybridization based analysis of detecting DNA level variations like RFLP was therefore replaced by the Polymerase Chain Reaction (PCR) based techniques.

### 15.2.2 PCR based markers

PCR based markers are advantageous over the hybridization based ones in the terms of practicality and affordability. PCR based techniques require less amount of DNA and avoids DNA blotting and radioactive compounds. Initially techniques like Random Amplified Polymorphic DNA (RAPD) [73, 74], Amplified Fragment Length Polymorphism (AFLP) [75] and Inter Simple Sequence Repeat (ISSR) [76] were used along with Bulk Segregate Analysis (BSA) [77] to develop sex linked markers in dioecious plant taxa. The above mentioned

markers could be transformed into locus specific Sequence Characterised Amplified Region (SCAR)[78]Sequence Tagged Sites (STS) [79]or Cleavage Amplified Polymorphic Sequence (CAPS) [80] markers making them more helpful in elucidating gender in dioecious plants.

For successful gender identification the PCR based sex-linked markers should fulfil the following criteria

1. The sex linked marker should amplify gender specific band.
2. Additional bands could be amplified in all the individuals of both the gender which could act as 'Positive Control'.
3. The difference in the sizes of the amplified bands should be large enough to identify sex using standard agarose gels and should not require Post-PCR specialised techniques like DNA sequencing.

PCR based sex linked markers have been successfully applied in various dioecious plant species like *S. latifolia*[81, 82], *P. vera*[83], *Atriplexgarrettii*[84], *S. viminalis*[85], *C. sativa*[86], *Carica papaya*[87], and *S. chinensis*[48, 58], etc.

**15.2.2.1 Random Amplified Polymorphic DNA (RAPD)**

PCR based techniqueRandom amplified polymorphic DNA (RAPD) was introduced by Welsh and McClelland (1990) and Williams et al. (1990)[73, 74]. In this technique random decamer oligonucleotide primers are used for differential PCR amplification of random genomic DNA fragments. DNA polymorphisms identified in this technique are the result of rearrangements or deletion at or between oligonucleotide primer binding sites in the genome. The designing of the random primers requires at least 60 % GC content with no palindromic sequences. The RAPD primers amplify a set of differing size DNA fragments ranging from 0.5 to 5 kb at several different loci of a genome. The difference in pattern of amplified bands is observed by presence and absence of amplified bands through ethidium bromide stained agarose gels[88, 89]. A numbers of reports describe identification of RAPD markers for sex discrimination in plants some of which have been given in table 1.4.

**Table 1.4List of few gender specific RAPD markers in Dioecious Plants (With inputs from Milewicz and Sawicki, 2013 [90] and Herikrujam*et. al.*, 2015 [59].**

| Species name | Primer name | Gender specificity | References |
|---|---|---|---|
| *Actinidiachinensis* | SmX | Female | [91] |
| | SmY | Mmale | |
| *Asparagus officinalis* | T35R54 | Male &Supermale | [92] |
| *Cannabis sativa* | No. 8, No. 11 | Male | [93] |
| *Humuluslupulus* | OPA-07, OPJ-09, OPU-08 | Male | [94] |
| *Piper betle* | OPA04,OPN-02 | Male | [95] |
| | OPC-06 | Female | |
| *Pistaciavera* | OPO-08 | Female | [83] |
| *Simmondsiachinensis* | F1 | Female | [96] |
| | F10 | Male | |
| *Trichosanthesdioica* | OPC-07, OPC-14 | Female | [97, 98] |
| | OPC-05 | Male | |
| *Hippophaerhamnoides* | OPD-15, OPD-20 | Male | [99] |
| *Hippophaesalicifolia* | OPF-11 | Female | [100] |

The first RAPD based markers for gender determination were reported byMulcahy et al. (1992) [81]in a dioecious plant species, *S. latifolia* using BSA analysis. The genomic DNA from 18 female plants and 18 male plants separately pooled and were screened with 60 RAPD primers. Four out of the 60 primers amplifiedone fragment each that were present in all the males but absent in all the female siblings.

In *P. vera*DNA was also extracted from 14 cultivars and from 94 open-pollinated, few weeks-old *P. vera*seedlings of unknown sex (Table 1.4)[83]. Seven hundred different decamer oligonucleotide primers were used to perform DNA amplification, with 1 of these (OPO08) producing a 945 bp amplification band that was present only in the bulked female samples and absent in the bulked male samples of the two crosses.

Polley*et al*. (1997)[94]compared pools of male and female plants derived from a hop cross to identify molecular markers associated with the Y or male-specific chromosome.Of 900 functional RAPD primers, 32 revealed fragments specific for male plants that were absent in female plants of this cross. Subsequently, the 32 positive primers were tested on unrelated male and female plants. Three of these 32 primers were specific for the Y chromosome in all lines. The Y-specific product derived from one of these primers (OPJ9) was of low copy in hybridization experiments and predominantly present in male plants (Table 1.4).

In *C. sativa* two out of 15 primers yielded fragments of 500 and 730 bp which were detected in all male plants but not in any of the female plants tested (Table 1.4)[93]. When the male and female DNAs were allowed to hybridize with the 500-bp probe, no differences in patterns were observed between male and female plants. By contrast, when these DNAs were allowed to hybridize with the 730-bp probe, much more intense bands specific to male plants were detected, in addition to less intense bands that were common to both sexes. The 730-bp DNA fragment was named MADCl (male-associated DNA sequence in *Cannabis sativa*).

The advantages of the RAPD based molecular markers include cost efficiency, simplicity in assaying, requirement for less sophisticated equipment, no prior knowledge of sequence information as well as no need for cloning and radioactive probes. However, despited these advantages RAPD has certain limitations like low reproducibility due to lower annealing temperature of the short primers (decamer) and their sensitivity to experimental conditions which make them unreliable for gender indentification studies

#### 1.5.2.2.2 Amplified Fragment Length Polymorphism (AFLP)

The Amplified Fragment Length Polymorphism (AFLP) technique was developed byZabeau and Vos in 1993[101] and introduced by Vos et al. (1995)[75]. It is an effective tool for analyzing genomic variations by means of PCR amplification. It involves double restriction digestion (rare cutter and frequent cutter) of genomic DNA, followed by ligation of double stranded (ds) adapters complementary to the restriction sites and finally selective PCR amplification using 3 bp extension. The amplified PCR products are separated and visualised on denaturing polyacrylamide gels through silver staining, autoradiography or fluorescence methodologies[75, 102]. AFLP assay produces 50–100 bands per assay ranging from 60 to 500 bp. Number of amplicons per AFLP assay depends on number of selective nucleotides in the AFLP primer combination, the selective base pairs extension, GC content and physical genome size and complexity[47].

The use of AFLP in sex determination of dioecious plants was introduced by Reamon-Buttner et al. (1998)[52]in *A officinalis*. Nine sex-linked AFLP markers were identified after testing upon pooled DNA of male and female plants of *A. officinalis*. Of the nine AFLP markers identified, three fragments namely E31M56, E41M50 and E33M53 were found to be tightly linked to sex locus and mapped at 0.5, 0.7 and 1.0 cM, respectively to the sex locus on L5 chromosome.

Parrish et al. (2004)[103] developed a 246 bp fragment specific to male individuals in two *Ficusfulva* populations (Table 1.5). This marker segregated in a 1:1 ratio in open-pollinated progenies from natural populations implying males to be heterogametic sex. Sequence analysis of the AFLP fragment (246 bp) showed no sequence homology to any sequence available in the database.

In *Salix viminalis* 88 selective primer combinations were screened using bulked males and female DNA samples from four families(Table 1.5)[104]. More than 1000 polymorphic fragments were obtained, of which only four co-segregated with sex. These four sex-linked markers were subsequently scored in individuals that were used for bulked sample preparation. A pair of primers that amplified the sex-linked fragments was constructed from one of the sex-linked amplified fragment length polymorphism (AFLP) fragments. In hybridization of

**Table 1.5: List of few gender specific AFLP markers in Dioecious Plants (With inputs from Milewicz and Sawicki, 2013 [90] and Herikrujam*et. al.*, 2015 [59].**

| Species name | Primer name /Restriction Enzyme | Gender specificity | References |
|---|---|---|---|
| *Asparagus officinalis* | EcoRI/MseI. Isolated the 9 sex linked AFLP markers [52].cloned and sequenced. Low copy AFLP fragments namely EM4150, EM3156 and EM3660 were chosen for designing STS primers | Male | [105] |
| *Eucommiaulmoides* | EACA/M-CTT$_{(350bp)}$ | Male | [106] |
| *Rumexacetosa* | Mse1.1/Pst1.3$_{(600,100bp)}$ | Male | [107] |
| *Salix viminalis* | Eco AA/Mse AT$_{(485bp)}$ | Female | [104] |
| | Eco AA/MseCAG$_{(192bp)}$ | Female | |
| | Eco TA/Mse CCG$_{(528bp)}$ | Female | |
| | Eco TG/Mse CAA$_{(302bp)}$ | Female | |
| *Simmondsiachinensis* | EcoGC/MseGCG$_{(525bp)}$ | Male | [108] |
| | EcoTAC/MseGCG$_{(325bp)}$ | Male | |
| | EcoTAC/MseGCG$_{(270bp)}$ | Female | |
| *Ficusfulva* | Eco-AGG/MseCA | Male | [103] |
| *Uapacakirkiana* | Eco-ACT/MseCTG | Female | [109] |

southern blot filters with the sex-linked DNA fragments, the band was present in females and absent in males.

In *R acetosa* 1085 bands were geerated using fifteen primer combinations, four of which were male specific (Table 1.5)[107]. The two male associated DNA fragments, *MADR1* (583 bp) and *MADR2* (133 bp) were isolated and sequenced. These sequences showed similarity to the known sequences from several organisms. The deciphered amino acid sequence of *MADR1*showed sequence similarity to the phosphatase genes of *A. thaliana* while Amino acid sequence of *MADR2* was found similar to retroelement sequences.

A total of 84 selective primer combinations were screened using bulked segregant analysis (BSA) for males and female plants of *Uapacakirkiana*[109]. More than 110 polymorphic markers were obtained but each of the four primer pairs (E-ACT/M-CTG, EACA/ M-CAA, E41+A/M-CTA and E-AGG/M-CTC) showed one band that was linked to sex(Table 1.5). When the four primer pairs were tested in ten individuals from different populations only one primer pair (E-ACT/MCTG) amplified a 320 bp band in female plants only.

AFLP technique has the ability to simultaneously screen of different loci distributed randomly throughout the genome.It can generate fingerprints of any small amount of DNA even of partially degraded samples regardless of its source and without any prior knowledge of DNA sequence[75, 102]. Requirement of a limited set of generic primers and a high multiplex ratio are some of the other advantages of this technique. However AFLP technique is technologically demanding and relatively costly, thus it is highly preferable to convert the AFLP fragments to a reliable and locus specific STS markers or SCAR markers which have been gainfully employed for identification of the sex.

#### 1.5.2.2.3 Inter Simple Sequence Repeat (ISSR)

Inter simple sequence repeat (ISSR) marker[76]is a DNA fingerprinting technique based on abundance and hypervariability of microsatellites (simple sequence repeats—SSR). It comprises PCR amplification of regions between adjacent, inversely oriented SSR-microsatellites. This technique utilises SSR-microsatellites as primers consisting of di-, tri-, tetraorpenta-nucleotides, usually 15–30 nucleotide long. The primers are either unanchored or

**Table 1.6: List of few gender specific ISSR markers in Dioecious Plants (With inputs from Milewicz and Sawicki, 2013 [90] and Herikrujam*et. al.*, 2015 [59].**

| Species name | Primer name | Gender specificity | References |
|---|---|---|---|
| *Calamustenuis* | ISSR 4-$(AAG)_5CC$ | Female | [110] |
| *Carica papaya* | $(GATA)_4$ | Male | [111] |
| | $(GACA)_4$ | Female and Hermaphrodite | |
| *Humuluslupulus* | K-22 –$[CA]_8GT$ | Male | [112] |
| | K10- $AC]_8YG$ | Male | |
| *Simmondsiachinensis* | UBC807-$(AG)_8T$ | Male | [48] |
| *Nyholmiellaobtusifolia* | ISSR 807 (600 bp) | Male | [113] |
| | ISSR 807 (500bp) | Female | |
| *Phoenix dactylifera* | HB9- $(GT)_6GG$ | Male | [114] |
| | HB10-$(GA)_6CC$ | Male | |
| | HB12-$(CAC)_3GC$ | Male | |
| | 814-$(CT)_8TG$ | Male | |
| | 844-A$(CT)_8AC$ | Male | |

anchored at 3' or 5' end with 1–4 degenerate bases extending into the flanking region, which increases its specificity [115]. The amplicons generated are usually 200–2000 bplong that can be separated either by agarose/EtBr staining or polyacrylamide/silver staining electrophoresis.

In *Calamustenuis* a total of 30 ISSR primers were screened on the ten male and ten female wild plants of *C. tenuis* for polymorphisms and reproducibility (Table 1.6)[110]. Of the 30 ISSR primers used to amplify the bulk DNA from male and female individuals, 23 primers gave a reproducible ISSR pattern. However, the number of amplicons varied from three to five with an average of four, and the fragment sizes ranged from 300 to 1000 bp. Of all the 30 primers tested, only one primer, ISSR4, showed sex specificity in bulk analysis. ISSR4 [5'-$(AAG)_5$CC-3'] produced a unique 600-bp fragment in female bulk DNA, and this band was absent in male bulk DNA.

In *Carica papaya* microsatellites and minisatellites were explored for their potential to serve as sex-specific markers(Table 1.6)[111].The microsatellite repeats $(GATA)_4$ and $(GAA)_6$ detected sex-specific differences in *Hin*fI or *Hae*III digests while microsatellite repeats $(TG)_{10}$, $(CAC)_5$, $(GGAT)_4$ and $(GACA)_4$ and the minisatellites PV47 and M13 failed to detect any sex-specificpatterns. The microsatellite $(GAA)_6$ revealed a male-specific difference only in Coimbtore cultivars (CO2 and CO6). However, no sex-specific polymorphism was observed in other cultivars and wild species studied. In case of $(GATA)_4$ a sex-specific band of 5 kb is seen in males of three dioecious cultivars, namely CO-2, Pusa-giant and Washington and isabsent in the corresponding female DNAs.

Twenty two inter simple sequence repeat (ISSR) primers were screened on female and male hop (*Humuluslupulus*) genotypes of Russian and European origin (Table 1.6)[112]. Two ISSR primers revealed fragments specific for male plants of hop. Based on the sequences two pairs of primers were designed. These male specific sequence tagged site (STS) markers were tested on male hop accessions of Russian origin and female hop accessions of Russian, European and American origin. A high homology of male specific hop DNA sequences to expressed sequences from EMBL plants EST database was found, most of which code cell wall glycoproteins.

In case of *Simmondsiachinensis*42 primers were analysed with a bulk sample of pooled male female DNA[48]. Only one primer, UBC-807, produced a unique ~1,200 base-pair fragment in the male DNA (Table 1.6). For the validation of this result, the primer was re-tested

with individual male and female samples from eight cultivars. A similar unique ~1,200 bp fragment was present in the male individuals of all eight cultivars and completely absent in the female individuals tested.

ISSR assay is simple, easy, quick to do and offers several advantages over RAPD and AFLP markers and thus this marker system has been adopted widely for plant genome analysis that includesassesment genetic diversity, cultivar identification, genetic fidelity, genomemapping, phylogenetics studies and population genetics, etc.[116-123]. The multilocus and dominant ISSR marker adopts higher primer annealing temperature as compared to RAPD resulting in greater band reproducibility. Sex linked ISSR markers can be linked both with coding and non-coding regions in the genome. However, involvement and mechanism of action of such sex-linked intronic sequences in the regulation of gene expression is yet to be evaluated

**1.5.2.2.4 Sequence Characterised Amplified Region (SCAR)**

Sequence characterized amplified region (SCAR) [78] marker was introduced to overcome the inherent sensitivity of RAPDs, converting into a more reliable and stable marker. SCAR markers were developed by cloning the amplified bands of RAPD and/or other multilocus markers like AFLP, then sequencing their ends and using the sequence information to design longer forward and reverse SCAR primers (22–24 nucleotides long). Such discreet bands are visualized on agarose gel supplemented with ethidium bromide or silver stained polyacrylamide gel. SCAR markers being sequence specific are not biased to minor variables in experimental condition and therefore are highly reproducible, reliable, simple to use and suitable for multiplex PCR analysis[124].The Polymorphisms of the RAPD or AFLP marker from which SCAR marker is derived are retained either as presence or absence of amplified band (dominant nature) or can appear as length polymorphisms

Gao*et. al*. in 2007 [125] identified two female specific markers in *A.officinalis* by screening 100 RAPD markers in 30 male and 30 female plants. One primer (S368) produced two markers (S368-928 and S368-1178) in female plants (Table 1.7). The female-linked S368-928 marker was sequenced and specific primers were synthesized to generate a 928 bp marker of sequence characterized amplified regions (SCAR) in female plants. $SCAR_{928}$ was able to identify homozygous *mm* female plants of *A. officinalis*.

**Table 1.7 List of few gender specific SCAR markers developed from RAPD markers in Dioecious Plants (With inputs from Milewicz and Sawicki, 2013 [90] and Herikrujam*et. al.*, 2015 [59].**

| Species name | Primer name | Gender specificity | References |
|---|---|---|---|
| *Pandanusfascicularis* | RAPD - OPD-08<br>SCAR - MSSR-01 | Male | [126] |
| *Asparagusofficinalis* | RAPD- S368<br>SCAR - S368-SCAR | Female | [125] |
| *Aucuba japonica* | RAPD- OPA 10<br>SCAR - SCAR OPA-10 | Male | [127] |
| *Calamussimplicifolius* | RAPD- S1443<br>SCAR - CsMale1 | Male | [128] |
| *Hippophaerhamnoides* | RAPD-OPA 04, OPT 06<br>SCAR – *HrX1, HrX2* | Female | [49] |
| *Momordicadioica* | RAPD- OPA-15<br>SCAR - MSSM | Male | [129] |

| | | | |
|---|---|---|---|
| *Eucommiaulmoides* | RAPD- OPF-08 | Female | [130] |
| | SCAR - MEP | | |
| *Piper longum* | RAPD- OPA-10 | Male | [124] |
| | SCAR - MPS1 | | |
| *Pistaciaatlantica* | RAPD- BC | Female | [131] |
| | SCAR - PVP | | |
| *Pistaciavera* | RAPD- OPO-08 | Female | [132] |
| | SCAR - PVF1 | | |
| *Salix viminalis* | RAPD- UBC 354 | Female | [133] |
| | SCAR - SCAR UBC 354 | | |
| *Simaroubaglauca* | RAPD- OPA-08 | Male | [134] |
| | SCAR - SCAR12 | | |

In *A. japonica* two sex-linked fragments were identified by RAPD analyses [127]. After the screening of 165 decamers on the genomic DNA of 24 male and 24 female plants, 785 fragments were obtained (Table 1.7). A 710 bp fragment from OPA10 and 1095 bpfragment from OPN 11 amplified in male plants only. The amplified fragments were cloned in pTAC1 vectors and were converted into two SCAR markers namely SCAR OPA 10 and SCAR OPN 11.

Korekar*et. al*. in 2012 [49] screened 60 RAPD primers on the DNA bulk samples from 20 male and 20 female plants of *H. rhamnoides*. Two primers, OPA-04 and OPT-06 consistently amplified female-specific (FS) fragments of 1,164 and 868 bp, respectively, which were absent in the male samples. A sequence-characterized amplified region marker *HrX1* (JQ284019) and *HrX2* (JQ284020) designed for the two fragments, continued to amplify the FS allele in 120 female plants but not in 100 male plants tested in the study.

The RAPD technique has been known for its reproducibility problems among different laboratories since it is sensitive to PCR condition variations. The SCAR markers are preferred over RAPD as these are locus specific and highly reproducible.

## 1.6 Gender Determination in Dioecious plants

Dioecious plants evolved from hermaphrodites. As a consequence hetromorphic sex chromosomes developed in dioecious plants in order to limit recombination between the different sex determining genes [39].Heteromorphic sex chromosomes in higher plants were first identified in *Rumexacetosa* and *Silenedioica* (Melandriumrubrum) by Kihara and Ono (1923) [135] and Blackburn (1923) [136]. Differentiated sex chromosomes have been established clearly in only six families, representing about eight species and two major species groups. Sex-chromosomes in flowering plants have evolved independently but have a number of features in common. The size of X and Y chromosomes is larger in comparison to autosomal chromosomes. [39].

Unisexual in dioecious plant species result from modification during development of a perfect flower by suppression of one or other organ sets. In most of the species both sets of sex organs are initiated and the inappropriate set of organs develops to some extent before abortionDespite of increasing research efforts on a number of different plant species, there is relatively little information available on the molecular basis of sex determination. In case of

seabuckthorndistinguishable sex chromosomes were reported with the males being heteromorphic [137]. Two main approaches areadopted in attempt to isolate sex determining genes from plants i.e. using homologues of genes known to be involved in flower development in hermaphroditic model plants such as *Arabidopsis* or *Antirrhinum* and using cloning strategies involving enrichment for sex chromosome sequences or enrichment for sex-linked transcripts.

In *Rumexacetosa* the expression patterns of the putative B and C function homeotic genes were shown to be strikingly different from those seen in hermaphroditic species [138]. The C function gene of *R. acetosa* showed a sex specific expression pattern. The expression of C function genes reduced in the organs which cease to develop in comparison to normal floral organs [138]. A similar situation was found in *Liquidambar styraciflua*, a monoecious tree species, where C function expression was considerably reduced in the degenerating stamens of the male flower [139]. Sex related expression of organ identity genes (CLASS B) was also recorded in *Humuluslupulus*[140].

In maize (*Zea mays*), sex determination occurs through abortion of female carpels in the tassel and arrest of male stamens in the ear. The *Tasselseed6* (*Ts6*) and *tasselseed4* (*ts4*) mutations permit carpel development in the tassel while increasing meristem branching, showing that sex determination and acquisition of meristem fate share a common pathway. Chuck *et. al.* in 2007 [140] showed that *ts4* encodes a *mir172* microRNA that targets *APETALA2* floral homeotic transcription factors. Sexual identity in maize is acquired by limiting floral growth through negative regulation of the floral homeotic pathway.

Koizomi*et. al*. in 2010 [141] showed that carpel suppression is atrigger for sexual dimorphism in *S. latifolia*. On the examination of expression patterns of *SlCLV1*, *SlSTM* (*SlSTM1* and *SlSTM2*) and *SlCUC* in young flowers of R025 and wild-type males and females it was found that the differences in expression patterns of the three genes occur at the same stage. This suggested that *SlCLV1* is also involved in carpel suppression in *S. latifolia*.

Song et. al. in 2013 [142] performed the Comparative transcriptome and physiological analysis to characterize sex-specific development of female and male flowers. Transcriptome analysis identified genes significantly differentially expressed between the sexes, including genes related to floral development, phytohormone synthesis and metabolism, and DNA methylation. Correlation analysis revealed a significant correlation between phytohormonesignaling and gene expression, identifying specific phytohormone-responsive genes and their cis-regulatory elements. Two genes related to DNA methylation,*Methyltranscferase1* (MET1) and*Decreased DNA Methylation 1* (DDM1), which are located in the sex determination region of Chromosome XIX, have differential expression between female and male flowers.

Seabuckthorn is a dioecious plant with limited genomic resources. The genome sequence of this important dioecious species is not yet available. However three transcriptome datasets are available at NCBI SRA database i.e. seed, root and leaf. Sharma et. al. (2012) selected 4700 clones from the seabuckthorn leaf cDNA library and sequenced them using sanger's method leading to the generation of 3412 high quality EST sequences, which were later submitted to dbEST, NCBI [143]. Clustering of EST's using CAP3 resulted into a total of 1665 unigenes comprising of 345 contigs and 1320 singletons. On the basis of Gene Ontology (GO) annotation 1665 unigenes were identified. Apart from EST sequencing, next generation sequencing of leaf and root tissue libraries yielded more than 90 million short reads comprising of approximately 8.5 billions nucleotides, which could be further assembled into 88,297 putative unigenes[143, 144]. 454 sequencing of sea buckthorn cDNA collections from mature seeds yielded 500,392 sequence reads, which identified 89,141 putative unigenes represented by 37,482 contigs and 51,659 singletons. Functional annotation by Gene Ontology and computational prediction of metabolic pathways indicated that primary metabolism (protein>nucleic acid>carbohydrate>lipid) and fatty acid and lipid biosynthesis pathways were highly represented categories in these seed tissues. Sea buckthorn sequences related to fatty acid biosynthesis genes in Arabidopsis were identified, and a subset of these was examined for transcript expression at four developing stages of the berry [145]. Identification of transcripts responsible for the unisexual floral development in seabuckthorn from these datasets is difficult since gender determination of dioecious plants occur in floral tissues [43] and there are chances that gender determining transcripts are not available in these transcriptomes. Thus sequences of floral regulatory genes which could act as sex determining genes identified from floral

transcriptomes of other model plant species like cucumber, *S. Latifolia,* papaya could be used to mine homologues in seabuckthorn transcriptome resources.

In case of cucumber Roche-454 massive parallel pyrosequencing technology, was used to generate a total of 353,941 high quality EST sequences with an average length of 175bp, among which 188,255 were from gynoecious flowers and 165,686 from hermaphroditic flowers. These EST sequences, together with ~5,600 high quality cucumber EST and mRNA sequences available in GenBank, were clustered and assembled into 81,401 unigenes, of which 28,452 were contigs and 52,949 were singletons.Digital expression analysis identified ~200 differentially expressed genes between flowers of WI1983G and WI1983H and provided novel insights into molecular mechanisms of plant sex determination process [146].

Papaya (*Carica papaya*) is a trioecious plant species that has male, female and hermaphrodite flowers on different plants. Although draft sequences of the papaya genome were already available, the genes for sex determination had not been identified, likely due to the complicated structure of its sex-chromosome sequences. To identify the candidate genes for sex determination, a transcriptome analysis of flower samples from male, female and hermaphrodite plants was conducted using high-throughput SuperSAGE for digital gene expression analysis Among the short sequence tags obtained from the transcripts, 312 unique tags were specifically mapped to the primitive sex chromosome (X or $Y^h$) sequences. An annotation analysis revealed that retroelementswere the most abundant sequences observed in the genes corresponding to these tags. The transcriptome analysis identified$Y^h$ chromosome-specific female determination genes, including a MADS-box gene, were identified [147].

In dioecious shrub willows Shrub Willows (*Salix suchowensis*)a large-scale transcriptome sequencing of flower buds which were separately collected from two types of sexes was performed. Totally, 1,201,931 high quality reads were obtained, with an average length of 389 bp and a total length of 467.96 Mb. The ESTs were assembled into 29,048 contigs, and 132,709 singletons. These unigenes were further functionally annotated by comparing their sequences to different proteins and functional domain databases and assigned with Gene Ontology (GO) terms. Digital expression analysis identified 806 differentially expressed genes between the male and female flower buds [148].

Transcriptome analysis of male and female flowers of *Populustomentosa* identified genes significantly differentially expressed between both the sexes, including genes related to

floral development, phytohormone synthesis and metabolism, and DNA methylation. Two genes related to DNA methylation, *METHYLTRANSFERASE1* (*MET1*) and *DECREASED DNA METHYLATION 1* (*DDM1*), which are located in the sex determination region of Chromosome XIX, have differential expression between female and male flowers. A time-course analysis revealed that*MET1* and *DDM1* expression may produce different DNA methylation levels in female and male flowers [142].

## 1.7 Research Gap

Out of the seven species of seabuckthorn, *H. rhamnoides*has been widely explored. In India two other seabuckthorn species i.e. *H. salicifolia*and *H.tibetana* should be further explored so that commercial potential of all the species of seabuckthorn could be tapped.There is only single report of RAPD based sex linked marker for *H. salicifolia*byRana*et. al.* (2009)[100]in which only 10 genotypes were used. There is no report on sex specific marker development for *H. tibetana* till date. Very few attempts have been made to elucidate gender determining mechanism in seabuckthorn. Existing genomic resources could help unravel the genetic mechanisms responsible for gender determination. In case of seabuckthorn very few genomic resources are available, which makes it tedious for identifying genes responsible for separate male and female seabuckthorn plants.

## 1.8 Hypothesis

1. There are seven species of seabuckthorn around the world. SCAR based sex linked molecular markers had been developed for *H. rhamnoides* only. SCAR markers developed for *H. rhamnoides* could be applied to other species of seabuckthorn like *H. salicifolia* and *H. tibetana*.

2. Sex determination in dioecious plants is controlled at genetic level. Differential expression of floral regulatory genes lead to gender specific organ development in male and female flowers of dioecious plants. Thus some candidate genes of floral regulatory pathways could act as sex determining genes in dioeciousseabuckthorn.

## 1.9 Objectives of the study

On the basis of the research gap ascertained through literature survey, the current study was designed to be completed through the following three objectives.

1. Validation of Sex linked SCAR markers developed for *H. rhamnoides* on *H.salicifolia* and *H.tibetana.*
2. Identification of putative sex determining genes in seabuckthorn by differential gene expression study in male and female floral buds of *H. rhamnoides.*
3. Identification of different transcription factor families playing role in sex determination in *H. rhamnoides.*

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# CHAPTER 2

# VALIDATION OF SEX LINKED SCAR MARKERS DEVELOPED FOR *HIPPOPHAE RHAMNOIDES* ON *H.SALICIFOLIA* AND *H.TIBETANA*.


## Abstract

Early sex determination of dioecious plants is essential for successful genetic improvement and commercial cultivation. Male and female seabuckthorn plants are morphologically similar and cannot be distinguished prior to 3–4 years of growth, i.e., at the time of flowering. India has three species of seabuckthorn namely *Hippophae rhamnoides*, *H. salicifolia* and *H. tibetana.* Applicability of sex linked SCAR markers *HrX1* and *HrX2* developed for *H. rhamnoides* were tested in *H. salicifolia* and *H. tibetana* using *H. rhamnoides* as control. *HrX1* produced female specific amplification in both *H. salicifolia* and *H. tibetana* while *HrX2* did not amplify except for *H. rhamnoides.* Sequence based analysis of amplified SCAR marker products *HrX1*, *HsX1* and *HtX1* has shown homology with known plant acyl CoA ligases.

## 2.1 Introduction

There are seven species of genus *Hippophae*, out of which three are found in India, namely *H. rhamnoides*, *H. salicifolia* and *H. tibetana* [1]. In contrast to *H. rhamnoides*, *H. salicifolia* and *H. tibetana* are lesser explored high value medicinal species of seabuckthorn [2]. Compared to *H. rhamnoides*, *H. salicifolia* is less thorny and contains all essential polyunsaturated fatty acids particularly omega-3 and omega-6, high quality berries with late maturation character, etc. [2].Vitamin C content in *H. salicifolia* is highest among all species of seabuckthorn [3]. In India, Himachal Pradesh has a large population of *H. salicifolia*, thus it can be exploited for berries by the local wine and juice industries and can provide raw materials for nutraceutical industry in general. Moreover *H. rhamnoides* occurs in Ladakh and Himachal Pradesh region only while *H. salicifolia* and *H. tibetana* occur in other parts of the country like Uttrakhand, Sikkim and north east regions of the country [1]. Seabuckthorn generally propagates by root suckers, softwood and hardwood cuttings and seeds [4]. Male and female seabuckthorn plants are morphologically similar and cannot be distinguished prior to 3–4 years of growth, i.e., at the time of flowering [5]. Commercial seabuckthorn plantation requires only 10% male plants for adequate pollination [6]. Thus early sex identification of seabuckthorn is useful for research as well as commercial purposes. Financial resources and valuable time can be saved if undesired male/female plants can be discarded at an early stage of research trials and commercial plantation. In *H. rhamnoides* techniques like RAPD, isozymes, SSR and SCAR have been used for sex linked marker development [7-9]. These techniques were applied on a limited population set and were not tested on other species of seabuckthorn. SCAR markers are reproducible and non-susceptible to PCR variations. There is only single report of RAPD based sex linked marker for *H. salicifolia* by Rana et al., 2009 [10]which only 10 genotypes were used. For *H. tibetana* there is no report on sex linked marker development till date. Keeping in view the limited genomic resources for *H. salicifolia* and *H. tibetana*, the applicability of two sex linked SCAR markers HrX1 and HrX2 [8] was tested on population of *H. salicifolia* and *H. tibetana*.

## 2.2 Material and Methods

### 2.2.1 Plant Material Collection

Disease-free leaf samples of *H. salicifolia* were collected from Lahaul valley, India and that of *H. tibetana* from Zanskar valley, India (Table 2.1). The leaf samples of *H. rhamnoides* collected from Indus valley, India were used as a control for validation of SCAR markers in the present study. The leaf samples were kept in ice boxes packed with cooling packs immediately after collection from field and subsequently kept at -80°C in the laboratory till further processing.

**Table 2.1:** List of plant samples collected for the current study

| **Species name** | ***H. rhamnoides*** | ***H. salicifolia*** | ***H. tibetana*** |
|---|---|---|---|
| **No. of male plants** | 12 | 100 | 50 |
| **No. of female plants** | 24 | 100 | 50 |

### 2.2.2 Genomic DNA Isolation (Doyle and Doyle 1990 [11] )

Grind thirty-day-old fresh leaf tissues from 3-5 plants in liquid nitrogen with the help of pestle and mortar. Transfer around 0.5 g of leaf powder to a 50 ml polypropylene tube. Add fifteen ml of preheated ($65^0$C) CTAB-DNA extraction buffer (100 mM Tris HCl (pH 8.0), 600nM NaCl, 10mM EDTA, 2% β-mercaptoethanol, 0.1% CTAB) and mix well. Incubate for 2 hours at $65^0$C with gentle shaking after every 10 minutes by inverting the tubes. Remove tubes from the water bath, cool for 5 minutes and add 15 ml of Chloroform: isoamyl alcohol (24:1v/v). Mix gently and centrifuge at 10,000 rpm for 10 minutes. Pour off the top aqueous layer to new 50 ml tubes, add equal vol. of chloroform: isoamyl alcohol (24:1v/v). Centrifuge at 10000 rpm for 10 minutes. Transfer the supernatant to a fresh tube and add equal volume of ice-cold isopropanol. Keep the tubes undisturbed for 15 minutes. Pellet down the DNA by centrifugation at 12,000 rpm for 5 minutes. Wash with 70% ethanol for 1-2 minutes. Keep for air-drying overnight. Dissolve in minimum amount of TE buffer. Add DNAse free RNAse (30 µg /100 µl DNA) to the dissolved DNA. Incubate for 30 min at $37^0$C. Again extract the DNA with equal volume of chloroform: isoamyl alcohol (24:1v/v). Centrifuge at 10,000 rpm for 10 minutes. Add $1/10^{th}$ volume of 3M Sodium Acetate (pH 6.8) and 2 volumes of 95%

ethanol. Pellet out the precipitated DNA and dissolve in TE buffer (10mM Tris HCL pH8.0), 1mM EDTA (pH8.0). When DNA is completely dissolved in TE, quality and concentration can be checked. To check this, run an aliquot of DNA sample on 0.8% agarose gel.

### 2.2.3 SCAR marker validation

Female specific SCAR markers, *HrX1* and *HrX2*, developed for *H. rhamnoides* were tested on the collected populations of male and female *H. salicifolia* and *H. tibetana* while *H. rhamnoides* was used as a control. DNA of 20 female plants and 20 male plants of each species was pooled. Amplification was carried out with *HrX1* and *HrX2* SCAR primers using the following PCR program; (Initial Denaturation - 94°C for 5 minutes ; 35 cycles of the following program was followed - Denaturation at 94°C for 30 seconds, Annealing at 56°C for 30 seconds, Extension at 72°C for 1 minute; Final Extension at 72°C for 10 minutes) The amplified PCR products were electrophoresed on 1.2% agarose gel at 100 volts and data was recorded. After the successful amplification in pooled DNA, SCAR markers were then tested on individual male and female plants of collected population of each species.

### 2.2.4 SCAR fragment sequencing and analysis

The amplified *HrX1* SCAR fragment of *H. salicifolia* (*HsX1*), *H. tibetana* (*HtX1*) and *H. rhamnoides* (*HrX1*) were sent for sequencing to Xcelris Labs Ltd, Ahmedabad, India. The quality of the sequences was checked by chromatogram analysis provided along with the sequence. Homology based sequence analysis of the amplified fragments was done against publically available NCBI database using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). IntronSite finder (http://dna.med.monash.edu.au/~torsten/intron_site_finder/) and Gen Scan (http://genes.mit.edu/GENSCAN.html) were used to find the introns and the coding DNA sequences (CDS) regions within amplified sequences. The homology of the CDS region within amplified fragments was determined by tBLASTx (http:// blast.ncbi.nlm.nih.gov/ Blast.cgi) analysis. Clustal W (http://ebi.ac.uk/Tools/msa/clustalw2/) was used for multiple sequence alignment among the sequences.

## 2.3 Results and Discussion

The data pertaining to testing of SCAR markers *HrX1* and *HrX2* in *H. salicifolia* and *H. tibetana* are presented in Table 2.2 Both markers worked well in *H. rhamnoides* (control) and amplified the desired 470 bp and 436 bp female specific fragment in all the 24 female plants where as no amplification was observed in the male plants. SCAR marker *HrX1* discriminated male and female plants of *H. salicifolia* and *H. tibetana* as it amplified the 445 bp fragment in 85% and 100 % female plants of these species respectively, with no amplification in the any of male plants of both the species. *HrX2* did not amplify in female or male plants of both the species (Fig. 2.1).

**Table 2.2: SCAR marker amplification in *H. rhamnoides, H. salicifolia* and *H. tibetana***

| Seabuckthorn Species | SCAR marker | Total no. of female plants | No. of female plants in which marker amplified | Total no. of male plants | No. of male plants in which marker amplified |
|---|---|---|---|---|---|
| *Hippophae rhamnoides* | *HrX1* | 24 | 24 | 12 | 0 |
| | *HrX2* | 24 | 24 | 12 | 0 |
| *Hippophae salicifolia* | *HrX1* | 100 | 85 | 100 | 0 |
| | *HrX2* | 100 | 0 | 100 | 0 |
| *Hippophae tibetana* | *HrX1* | 50 | 50 | 50 | 0 |
| | *HrX2* | 50 | 0 | 50 | 0 |

The quest for finding sex linked markers in *H. rhamnoides* is continued from past 15 years. However, very few attempts have been made to develop sex linked markers for *H. salicifolia* and *H. tibetana*, which are two important species of the genus. There is a single report on attempt to develop a sex-linked RAPD markers for *H. salicifolia* [10] in a small population of 10 genotypes and no attempt have been made on *H. tibetana* till date. So the present work is an attempt to fill this void. The female specificity of the *HrX1* and *HrX2* SCAR marker had been tested in larger population sets of both the species.

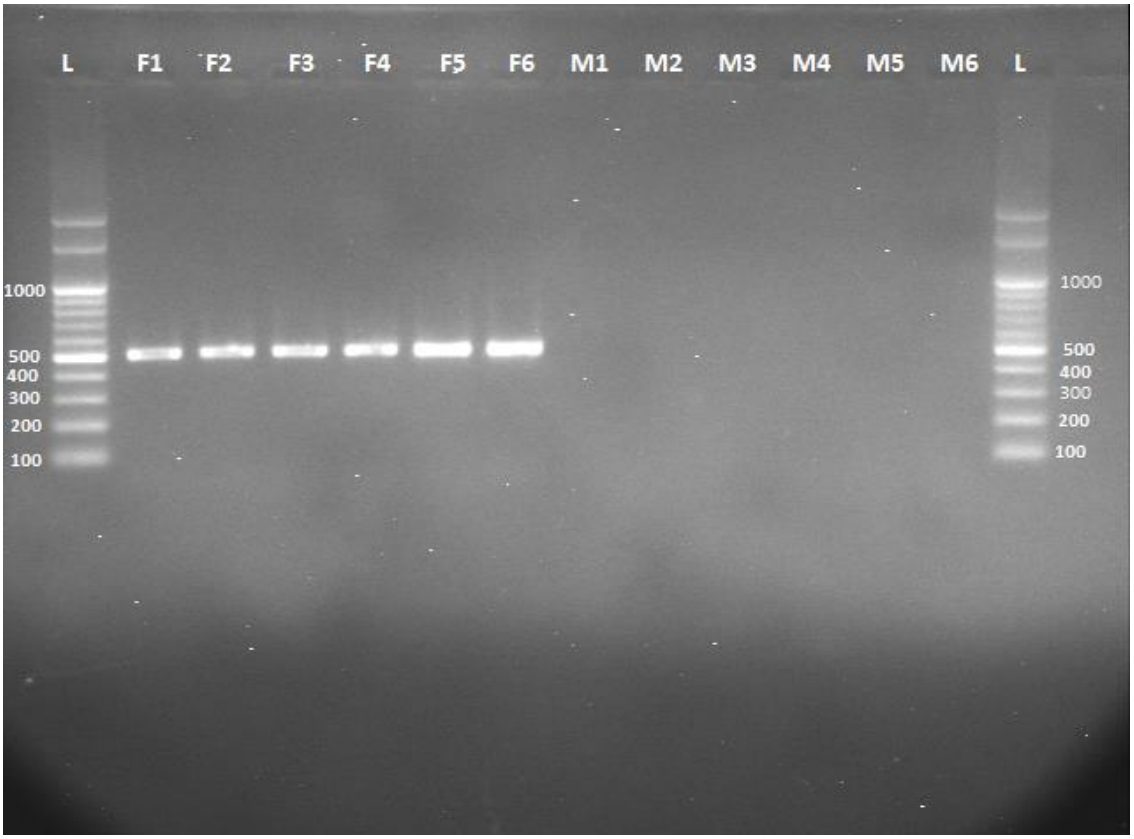


**Fig 2.1: Application of *HrX1* SCAR marker in female and male plant samples of *H. rhamnoides, H. salicifolia and H. tibetana.* L = 100bp ladder, (F1-F2) *H. salicifolia* female plants, (F3-F4) *H. tibetana* female plants, ( F5-F6) *H. rhamnoides* female plants, (M1-M2) *H. salicifolia* male plants, (M3-M4) *H. tibetana* male plants, ( M5-M6) *H. rhamnoides* male plants, L = 100bp ladder.**

The applicability of this single marker in all the three species has circumvented the need for de-novo development of sex linked markers in *H. salicifolia* and *H. tibetana*, thus saving both the time and resources.

### 2.3.1 DNA sequence analysis

The amplified *HrX1* SCAR fragment from *H. salicifolia* (*HsX1*) and *H. tibetana* (*HtX1*) were sequenced and the sequences were submitted in NCBI Genbank database (Genbank accession number KF234568 and KF359498 respectively). BLASTn analysis of *HsX1* & *HtX1* sequence revealed its sequence similarity with plant lipid biosynthetic genes as shown in Table 2.3. *HsX1* has shown similarity to *Vitis vinefera* acyl CoA synthatase, *Ricinus communis* AMP dependent ligase while *HtX1* gave similarity hits with *Cicer arietinum* acyl-activating enzyme 6-like mRNA, *Vitis vinifera* acyl-CoA synthetase. *HrX1* amplified from *H. rhamnoides* showed similarity with *Vitis vinefera* acyl CoA synthatase and *Cicer arietinum*

**Table 2.3: BLASTn analysis of *HsX1, HtX1and HrX1* with NCBI database.**

| **BLASTn analysis of *HsX1*** | | | |
|---|---|---|---|
| BLAST hits | Query Cvg. | Identity | e-value |
| *Vitis vinefera* acyl CoA synthatase | 84 % | 68 % | 8e-26 |
| *Solanum lycopersicum* acyl-activating enzyme 6-like | 61 % | 73 % | 1e-22 |
| *Vitis vinifera* medium-chain-fatty-acid--CoA ligase | 84 % | 67 % | 6e-21 |
| *Cucumis sativus* acyl-activating enzyme 5-like | 37 % | 74 % | 9e-19 |
| *Arabidopsis thaliana* acyl-activating enzyme 6 | 30% | 76% | 4e-17 |
| **BLASTn analysis of *HtX1*** | | | |
| BLAST hits | Query Cvg. | Identity | e-value |
| *Cicer arietinum* acyl-activating enzyme 6 | 30 % | 72 % | 6e-09 |
| *Vitis vinifera* acyl-CoA synthetase | 21 % | 77 % | 2e-08 |
| *Cucumis sativus* acyl-activating enzyme 5 | 12 % | 80 % | 0.005 |
| *Ricinus communis* AMP dependent ligase | 22 % | 73% | 1e-05 |
| Vitis vinifera contig VV78X019176.3, shotgun sequence | 21 % | 76 % | 8e-07 |
| **BLASTn analysis of *HrX1*** | | | |
| BLAST hits | Query Cvg. | Identity | e-value |
| *Cicer arietinum* acyl-activating enzyme 6 | 30 % | 73 % | 1e-11 |
| *Vitis vinifera* acyl-CoA synthetase | 21 % | 79 % | 4e-11 |
| *Vitis vinifera* medium-chain-fatty-acid--CoA ligase | 19 % | 75 % | 4e-05 |
| *Populus trichocarpa* acyl:coa ligase acetate-coa synthetase | 14 % | 81 % | 4e-05 |
| *Cucumis sativus* probable acyl-activating enzyme 5 | 12 % | 82 % | 1e-04 |

**Table 2.4: Prediction of CDS region and peptides using Genscan server.**

| Sequence Name | Prediction of Peptide and CDS | Length of Peptide (aa) | Length of CDS (bp) |
|---|---|---|---|
| ***HsX1*** | Yes | 72 | 216 |
| ***HtX1*** | No | NA | NA |
| ***HrX1*** | No | NA | NA |
| **JQ284019** | Yes | 180 | 540 |
| **JQ284020** | Yes | 235 | 705 |

**Table 2.5: tBLASTx analysis of *HsX1*, JQ284019 and JQ284020 predicted CDS.**

| **tBLASTx analysis of *HsX1*** | | | |
|---|---|---|---|
| BLAST hits | Score | Query Cvg. | E-value |
| *Vitis vinifera* acyl-CoA synthetase | 374 | 96 % | 1e-30 |
| *Arabidopsis thaliana* acyl-activating enzyme-6 | 211 | 93 % | 3e-30 |
| *Vitis vinifera* medium-chain-fatty-acid--CoA ligase | 264 | 93 % | 1e-29 |
| *Glycine max* medium-chain-fatty-acid--CoA ligase | 206 | 93 % | 2e-29 |
| *Cannabis sativa* acyl-activating enzyme 5 | 251 | 93 % | 6e-29 |
| **tBLASTx analysis of JQ284019** | | | |
| BLAST hits | Score | Query Cvg. | E-value |
| *Pyrenophora triticirepentis* -BFP oxidoreductase | 1030 | 98 % | 2e-76 |
| *Pyrenophora teres f. teres* 0-1 hypothetical protein | 922 | 98 % | 3e-76 |
| *Penicillium marneffei* dihydrodiol dehydrogenase, | 469 | 91 % | 6e-49 |
| *Neurospora crassa* OR74A hypothetical protein | 387 | 82 % | 6e-40 |
| *Talaromyces stipitatus* dihydrodiol dehydrogenase | 357 | 72 % | 1e-39 |
| **tBLASTx analysis of JQ284020** | | | |
| BLAST hits | Score | Query Cvg. | E-value |
| *Schizophyllum commune* glycoside hydrolase | 596 | 84 % | 4e-81 |
| *Phaeosphaeria nodorum* hypothetical protein | 728 | 79 % | 2e-76 |
| *Aspergillus oryzae* BNR/Asp-box repeat protein | 486 | 78 % | 3e-72 |
| *Fusarium oxysporum* ARA1 gene | 533 | 75 % | 4e-70 |
| *Penicillium chrysogenum* abnx gene | 493 | 78 % | 6e-65 |

**Table 2.6: BLASTp analysis of *HsX1*, JQ284019 and JQ284020 predicted peptides.**

| BLAST hits | Query Cvg. | Identity | E-value |
|---|---|---|---|
| **BLASTp analysis of *HsX1*** | | | |
| **Putative conserved domain - AFD_Class_I superfamily** | | | |
| *Vitis vinifera* Acyl-CoA synthetase | 94 % | 70 % | 1e-29 |
| *Arabidopsis thaliana* Acyl-activating enzyme 6 | 94 % | 67 % | 2e-29 |
| *Theobroma cacao* Acyl activating enzyme 5 | 94 % | 67 % | 2e-28 |
| *Cannabis sativa* Acyl-activating enzyme 5 | 94 % | 69 % | 3e-28 |
| *Cucumis sativus* Acyl-activating enzyme 5 | 94 % | 70 % | 4e-28 |
| **BLASTp analysis of JQ284019** | | | |
| **Putative conserved domain - GFO_IDH_MocA superfamily** | | | |
| *Pyrenophora teres* hypothetical protein PTT_14440 | 99 % | 79 % | 2e-103 |
| *Fomitopsis pinicola* NAD-binding protein | 99 % | 66 % | 7e-80 |
| *Macrophomina phaseolina* Oxidoreductase | 99 % | 70 % | 2e-78 |
| *Neofusicoccum parvum* dimeric dihydrodiol protein | 99 % | 70 % | 1e-77 |
| Talaromyces marneffei dihydrodiol dehydrogenase | 98 % | 62 % | 3e-72 |
| **BLASTp analysis of JQ284020** | | | |
| **Putative conserved Domain - No domains detected** | | | |
| *Pseudocercospora fijiensis* glycoside hydrolase | 99 % | 65 % | 2e-100 |
| *Dothistroma septosporum* glycoside hydrolase | 90 % | 68 % | 1e-99 |
| Pseudozyma flocculosa hypothetical protein | 87 % | 62 % | 2e-84 |
| *Pseudozyma hubeiensis* glycoside hydrolase | 98 % | 55 % | 4e-80 |
| *Punctularia strigosozonata* BNR/Asp-box repeat | 85 % | 68 % | 1e-91 |

acyl-activating enzyme 6. Low query coverage and high e-values in some BLASTn hits indicated that amplified sequences match with very short portion of the similarity hit sequences. Since the SCAR markers were amplified from the genomic DNA, presence of intronic region may be the reason for such an outcome.

To increase the specificity of the sequence analysis, putative peptides and conserved DNA sequences (CDS) were identified in *HsX1*, *HrX1* and *HtX1* using Genscan server (Table 2.4). Genscan predicted 72 amino acid long peptide and 216 bp long CDS region in *HsX1* while no putative peptides were identified in *HtX1* and *HrX1*. Korekar *et. al.* (2012) [8] analysed SCAR marker sequences JQ284019 and JQ284020 using BLASTn server but the sequences did not match with any known plant genes. Putative peptides and CDS region were also identified in JQ284019 and JQ284020. The putative CDS region from JQ284019 and JQ284020 were 540 bp and 705 bp long while putative peptides were 180 and 135 amino acid long respectively (Table 2.4).

The identity of the predicted CDS regions and putative peptide was confirmed by tBLASTx and BLASTp server respectively (Table 2.5 and Table 2.6). tBALSTx analysis of *HsX1* predicted CDS showed its similarity with known plant genes such as *Vitis vinifera* Acyl CoA synthatase, *Arabidopsis thaliana* acyl-activating enzyme-6, *Glycine max* medium-chain-fatty-acid--CoA ligase, etc. In case of JQ284019 and JQ284020 tBLASTx analysis found its similarity with fungal genes such as *Pyrenophora triticirepentis* Pt-1C-BFP oxidoreductase, *Penicillium marneffei* dimeric dihydrodiol dehydrogenase, *Schizophyllum commune* glycoside hydrolase family protein, *Aspergillus oryzae* BNR/Asp-box repeat domain protein, etc. Putative peptides predicted by GENSCAN server were analysed by BLASTp server and similar results were obtained. Identification of putative peptides and CDS regions increased query coverage of similarity hits predicted by BLASTn server. Lower e-value further reinforces the significance of the tBLASTx and BLASTp hits. Thus Genscan analysis confirms the assumption that intronic regions were interfering with BLASTn analysis and further supports the association of plant lipid biosynthesis genes with sex specific SCAR marker.

In dioceous plants genetic determination of sex is linked to the X-Y chromosome system [12]. Recent studies in plants like *Silene latifolia*, *Arabidosis thaliana*, Cucumis sativus and Actinidia chinensis identified many sex determining loci and genes. The Acyl

CoA synthatase has been found to play a significant role in viable pollens in *Arabidosis thaliana* [13]. Plants harbouring mutant Acyl CoA synthatase produced non-viable pollens with a weak wall which were unable to fertilize the ovule.

The weak pollen wall was attributed to role of acyl CoA synthatase in sporopollenin biosynthetic pathway [13], which is a major constituent of rigid outer walls of the pollen. Most of other significant sequence hits in the BLASTn, tBLASTx and BLASTp analysis were ligases, which share family and E.C. no 6.2.2.3 with that of acyl CoA synthetase. As such these results indicate that the role of acyl CoA synthetase gene needs to be investigated further in relation to its influence on sex determination in the genus *Hippophae*. Genscan analysis of SCAR marker sequences submitted by Korekar *et. al.* (2012) [8] had identified domains related to fungal oxidireductases and repeat domains. None of the above sequences relate with known plant genes. Thus this is first report linking sex specific markers with known plant genes and thus warrants further investigation to provide insight in elucidation of sex determination pathways in seabuckthorn.

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# CHAPTER 3

# IDENTIFICATION OF PUTATIVE SEX DETERMINING GENES IN SEABUCKTHORN BY DIFFERENTIAL GENE EXPRESSION STUDY IN MALE AND FEMALE FLORAL BUDS OF *H. RHAMNOIDES.*


## 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.

## 3.1 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, neutraceutical 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 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 mersitem 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-kown 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 *SEPTALA 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.

## 3.2 Material and Methods

### 3.2.1 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 (Fig. 3.1). 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. 3.1. 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 µg of total RNA using Verso cDNA Kit (Thermo Scientific). The quality of cDNA was tested by amplifying 26S gene fragment using 26S primers 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.

### 3.2.2 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 3.1). 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 (Appendix I). The sequence data was manually curated and redundant sequences of the same species were discarded. Quality trimmed and

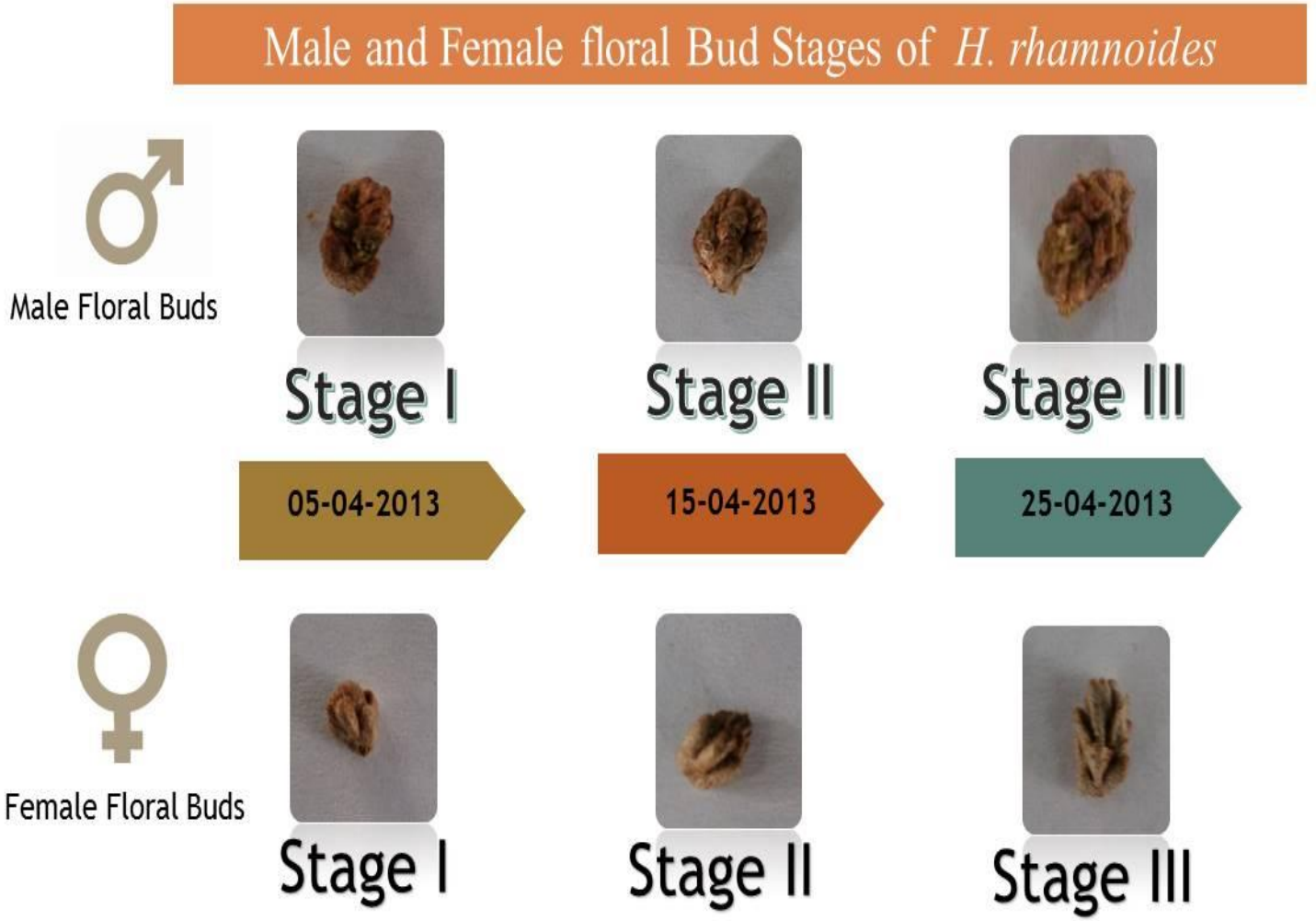


**Fig. 3.1 Temporal male and female floral bud development stages in seabuckthorn.**

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 syntenic relationship with known GISD (Table 3.2,Appendix II) BLASTN reports were analysed manually 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 3.2). To further confirm the identity of the seabuckthorn sequences, domains and repeats were identified within the GISD sequences. Nucleotide sequences of putative seabuckthorn GISD were translated to amino acid coding sequences (Appendix III) 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 seabuckthorn, protein sequences of known GISD characterized in model plant species were downloaded from NCBI Genbank database (Appendix IV). The alignment of the sequences was done with the help of CLUSTALX [39] and the final tree was constructed using MEGA 6 (Molecular Evolutionary Genetics Analysis 6.0) software [40].

### 3.2.3 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 (Table 3.3). qRT-PCR was performed with duplicate amplifications using SYBR-green-based detection system (IQ™ SYBR® Green Supermix (Biorad) in the Biorad CFX96™ Real-Time PCR Detection System). The reactions contained 100 ng cDNA template and 0.5 µM of primers in total volume of 13 µl. 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 expression data normalisation. The data presented in the figures and tables are based on the average 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 (Fig. 3.13) 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).

**Table 3.1 List of potential genes involved in sex determination in seabuckthorn**

| S. No. | Gene Name | Function in flower development | References |
|---|---|---|---|
| 1 | *APETALA1* (*AP1*) / *SQUAMOSA* (*SQUA*) | Promotes sepal Differentiation, Supresses 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 | *CLAVATA1* (*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 *EF1* 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 | *NUBBIN* (*NUB*) | Define 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 boundries within young flowers | [62, 63] |
| 21 | *SPOROCYTELESS* (*SPL*) | It is required for the initiation of sporogenesis in male and female organs of the plants. | [64] |
| 22 | *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) | Integrates vernalization and gibberellin signals in *Arabidopsis* | [65] |
| 23 | *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] |
| 24 | *SUPERMAN* (*SUP*) | It is involved in controlling cell proliferation in stamen and carpel primordia and in ovules in flower development. | [67-71] |
| 25 | *TERMINAL FLOWER 1* (*TFL 1*) | It is putative regulator gene involved in the control of flowering time and floral architecture | [72, 73] |
| 26 | *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] |

| | | | |
|---|---|---|---|
| 27 | *YABBY* (*YAB*) | Floral organ polarity | [54] |
| 28 | *SEPTALATA* (*SEP*)<br>(*SEP1, SEP2, SEP3, SEP4*) | MADS box CLASS E genes. Role in ovule formation, required to specify petals, stamens and carpels | [45] |
| 29 | *FLOWERING LOCUS C* (*FLC*) | Delays flowering in plants. Represses FLOWERING TIME (FT) gene in the absence of low temperature/ vernalization treatment. | [58] |
| 30 | *FLOWERING LOCUS D* (*FLD*) | It encodes a plant homolouge 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*) | Far red light absorbing receptor gene which senses daylight changes to promote flowering. *Arabidopsis thalianaPHYA*-null mutantplants are insensitive to floral induction by day-length extensions or night-break 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. | [77-80] |
| 35 | *PHYTOCHROME B* (*PHYB*) | Far red light absorbing receptor gene. Itinhibits flowering in *Arabidopsis*. Loss of *PHYB* accelerates flowering under both long- and short-day conditions. | [81] |
| 36 | *SHORT INTEGUMENTS* (*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 he shape of organs originating from the shoot apical meristem. | [86] |
| 41 | *DEFFECTIVE IN ANTHER DEHISCENCE 1* (*DAD1*) | It encodes 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] |

## 3.3 Results and Discussion

### 3.3.1 Morphological differences between temporal development stages of male and female floral buds of Seabuckthorn.

#### 3.3.1.1 Male Flowers

In MST I, the floral buds were brown in colour. At this stage differentiation of floral organs (stamens) was not observed after vertical dissection of the flower. The temporal developmental stage MST II (Fig. 3.3) had larger size in comparison to MST I. The colour of the bud at this stage is brown. Floral sex organs i.e. stamens (anther + filament) were observed at this stage. The colour of the anthers is dark green. In case of MST III (Fig. 3.3) the size of the buds further increased. The buds appeared brown in colour. The size of the male reproductive organs i.e. stamens (anther + filament) was larger in contrast to MST II. Moreover the colour of the anthers changed from dark green to yellow.

#### 3.3.1.2 Female Flowers

The floral development stage I (FST I) of the female flowers was dark green in colour. At this temporal developmental stage no clear differentiation of floral organs was observed after vertical dissection of the female flower. At 2$^{nd}$ development stage the colour of the floral bud remained unchanged i.e.dark green in colour. The size of the bud increased in comparison to FST I. Leaf bracts were observed at this stage. Still no clear differentiation of floral organs was observed after vertical dissection of the female flower at FST II. In case of FST III the size of the bud increased as compared to the predecessor. At FST III (Fig. 3.2) clear female reproductive organs i.e. ovary was observed. Also the size of the leaf bract increased in comparison to FST II.

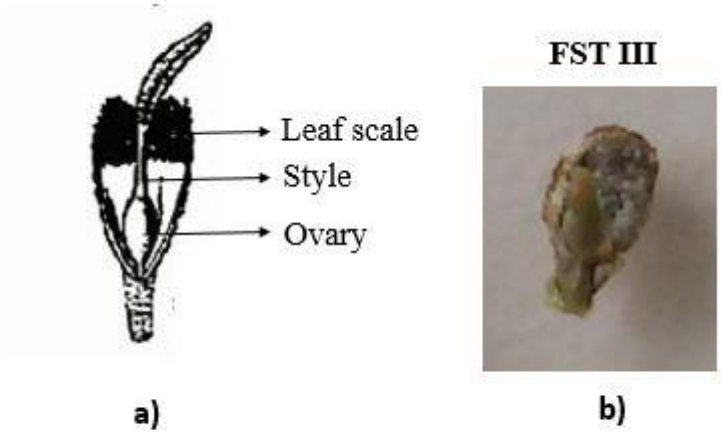


**Fig. 3.2 Vertical section of the female flower bud. a) Sketch of the female Seabuckthorn flower showing ovary, style and leaf scale. b) Vertical section of the female floral stage III (FST III)**

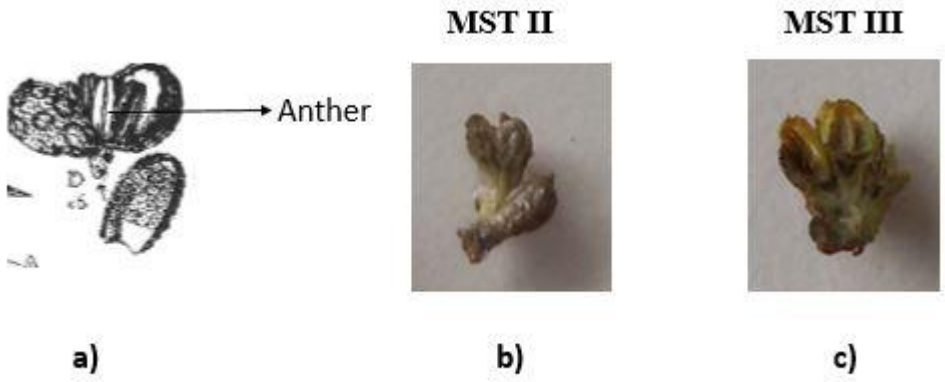


**Fig. 3.3 Vertical section of the male flower bud. a) Sketch of the male Seabuckthorn flower showing anther. b) Vertical section of the male floral stage II (MST II). c) Vertical section of the male floral stage III (MST III).**

### 3.3.2 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 floral regulatory pathways (Table 3.1) 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 3.2). Arabidopsis genes for which homologous 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 *DAD1*. 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 species or the other (Table 3.2). 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 3.2). 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 3.2). 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. 3.4) 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 plant. 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. acetosa* and *A. chinensis*, genes involved in these flowering pathways could be potential candidates of sex determination in seabuckthorn.

**Table 3.2 List of potential Seabuckthorn GISD 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 | XP_008233274.1 |
| | | | | *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 | XP_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/Crypto chrome, 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 | XP_002282465.1 |
| | | | | *A.thaliana* | 61 % | 0.0 | NP_566709.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 15 | *HrFRILK* | 84388 | Frgadia protein family | *V. vinifera*^ | 77 % | 0.0 | XP_002266233.1 |
| | | | | *A.thaliana* | 69 % | 0.0 | NP_566709.1 |
| 16 | *HrGI* | 30943 | N.D. | *P. mume*^ | 83 % | 0.0 | XP_008237480.1 |
| | | | | *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 | XP_002278263.1 |
| | | | | *A.thaliana* | - | - | - |
| 18 | *HrSI* | 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* | - | - | - |
| 20 | *HrNEF1* | 18354 | N.D. | *Theobroma cacao*^ | 84 % | 9e-87 | XP_007043754.1 |
| | | | | *A.thaliana* | 72 % | 1e-76 | NP_196843.1 |
| 21 | *HrSOC1* | 8883 | MADS box, K-box domain | *V. vinifera*^ | 71 % | 1e-98 | ABF56527.1 |
| | | | | *A.thaliana* | - | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 22 | *HrYAB5a* | 21143 | YABBY protein superfamily, HMG domain | *P. trichocarpa*^ | 78 % | 4e-73 | XP_002308074.1 |
| | | | | *A.thaliana* | 70 % | 1e-56 | NP_850080.1 |
| 23 | *HrYAB5b* | 70948 | YABBY protein superfamily, HMG domain | *P. mume*^ | 81 % | 5e-107 | XP_008242786.1 |
| | | | | *A.thaliana* | 72 % | 1e-77 | NP_850080.1 |
| 24 | *HrYAB4* | 7257 | YABBY protein superfamily, HMG domain | *G. max*^ | 82 % | 3e-117 | XP_003549900.1 |
| | | | | *A.thaliana* | 59 % | 2e-72 | NP_566037.1 |
| 25 | *HrSEP3* | 15336 | MADS box, K-box domain | *Shorea beccariana* | 84 % | 1e-142 | BAN89460.1 |
| | | | | *A.thaliana* | - | - | - |
| 26 | *HrACC* | 8293 | PPD transferase, Amino transferase CLASS I/II domain | *M. notabilis*^ | 80 % | 0.0 | EXB37292.1 |
| | | | | *A.thaliana* | - | - | - |
| 27 | *HrETR1* | 23688 | Signal transduction histidine kinase, GAF domain | *P. domestica*^ | 86 % | 0.0 | CAI64505.1 |
| | | | | *A.thaliana* | 80 % | 0.0 | NP_176808.3 |
| 28 | *HrERS* | 11717 | Signal transduction | *T. cacao*^ | 82 % | 0.0 | XP_007051012.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | histidine kinase, GAF domain | *A.thaliana* | - | - | - |
| 29 | *HrX1*[#] | 27099 | AMP-dependent synthatase / ligase, AMP-binding enzyme C-terminal domain | *T. cacao*^ | 77 % | 0.0 | XP_007034413.1 |
| | | | | *A.thaliana* | 69 % | 0.0 | NP_197138.1 |

[*]Contigs were obtained from the assembled unigenes of leaf and root transcriptome of *H. rhamnoides*. Sequences of unigenes could be downloaded from 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


**Fig. 3.4 Phylogenetic tree of the potential GISD in Seabuckthorn (*H. rhamnoides*) based on the amino acid sequence alignment.**

### 3.3.3 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 (Table 3.4). 21 GISD were analysed for differential expression among temporally corresponding male and female flower developmental stages (FDS). The CT values of 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, (Table 3.5) details of which are given below.

#### 3.3.3.1 Floral meristem identity genes

As data presented in Table 3.5 and Fig. 3.5 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 withthe 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.

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. 3.5) showed female specific expression while *HrAP2* (Fig. 3.5), expressed particularly in male flowers (Fig.3.5). *AP2* plays an important role for sex determination in maize [35]. It suppresses carpel in tassel of male flowers by targeting *TASSELSEED 4* (*TSL4*). Similarly expression of another floral meristem identity gene *HrCLV1* (Fig. 3.5) 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.

**Table 3.3 List of primers used in qRT PCR analysis of putative GISD.**

| S.No. | Primer Name | Primer Sequence | Length (bp) |
|---|---|---|---|
| **1** | HSX1CNTG1 F | CGTCGTTCCTGAGGTGTAGT | 20 |
| **2** | HSX1CNTG1 R | GCTCTCCCTCTTTCCTCCTC | 20 |
| 3 | HSX1CNTG2 F | TCACCATCGGATCCCATTCA | 20 |
| 4 | HSX1CNTG2 R | TTGAAGCGCTCTCCCTCTTT | 20 |
| 5 | HSX1CNTG3 F | TTCATCACCATCGGATCCCA | 20 |
| 6 | HSX1CNTG3 R | CTATACTCCTCCGCCACAGC | 20 |
| 7 | HSX1CNTG4 F | TCGGAATGGAGAAGTCGACA | 20 |
| 8 | HSX1CNTG4 R | TTCTTGAAGCGCTCTCCCTC | 20 |
| 9 | RTFLD F | GTCAAAACACTCCCGCCTAA | 20 |
| 10 | RTFLD R | GCTTGGTGTGGTTGTGATTG | 20 |
| 11 | RTAGL15 F | TGCCTCTCTTTGCCAGAACT | 20 |
| 12 | RTAGL15 R | GTTCTCGAAGCGAAGGAATG | 20 |
| 13 | RTCONS F | ACATACTGGCCCGAATTGAG | 20 |
| 14 | RTCONS R | CGACAACGCCAACTCTAACA | 20 |
| 15 | RTCONSB F | CGGCATCGCTTTGTACTTCT | 20 |
| 16 | RTCONSB R | TCCCGTTCTTCACTGGATTC | 20 |
| 17 | RTCONS2 F | GAGCGTGTCTGGGTATGTGA | 20 |
| 18 | RTCONS2 R | ACGAACCCATCTTCAGCATC | 20 |
| 19 | RTCRY2 F | AGTTTCAAGGTGGTGGCTGA | 20 |
| 20 | RTCRY2 R | TGCACAGAAATGCCTAGCTC | 20 |
| 21 | RTCRY1 F | GGAGAGTCGAGCAGAAGTGC | 20 |
| 22 | RTCRY1 R | CTGTCGTGGAATCTTCAGCA | 20 |
| 23 | RTCRY1B F | CCGAATGGATACACCATCCT | 20 |
| 24 | RTCRY1B R | GTGGGAAGGCAATGACAGTT | 20 |
| 25 | RTEF1 F | GGCTCAGGCAAAGAAAGTTG | 20 |
| 26 | RTEF1 R | TCTCGTCAACCTCCATCTGA | 20 |
| 27 | RTFILF F | GTTCGATGTGGTCACTGCAC | 20 |

| | | | |
|---|---|---|---|
| 28 | RTFILF R | GCAAAGTCATGGAGGCTTGT | 20 |
| 29 | RTFRIG F | CACTTGTGGTTGATCCGTTG | 20 |
| 30 | RTFRIG R | CTGTTTCCTCCAAGCAGACC | 20 |
| 31 | RTFRIGLK F | GGTCGAACAGAGCAAAGAGG | 20 |
| 32 | RTFRIGLK R | CTAATGGCAACTGGGCTCTC | 20 |
| 33 | RTFRUITFL F | ATGGAGGTGATCCTTGAACG | 20 |
| 34 | RTFRUITFL R | CCAGTTCGTCTCCCTTGAAA | 20 |
| 35 | RTGIGAN F | GATGGGCTGTTGCTAATGGT | 20 |
| 36 | RTGIGAN F | TGTGTGGCACTTGGAGTAGC | 20 |
| 37 | RTPHYB F | GGTTCCTTCCACCAACAGAA | 20 |
| 38 | RTPHYB R | TGATGCAGCCTCTATGCTTG | 20 |
| 39 | RTSHTINTG F | CCATGCCTTCAGAAAGGGTA | 20 |
| 40 | RTSHTINTG R | CCTGCCTCAACTCTTCCTTG | 20 |
| 41 | RTTRMFLR F | TGGCCTTGGAATCTCATAGC | 20 |
| 42 | RTTRMFLR R | CTTCCACAGTCACCACCAAA | 20 |
| 43 | RTTRMFLRLK F | AAGGTCGAGCCACTGACTGT | 20 |
| 44 | RTTRMFLRLK F | TTCGGAAAAGAGGTGGTGAG | 20 |
| 45 | RTAPT2 F | CTGCTTCAATCTCGGTGTCA | 20 |
| 46 | RTAPT2 R | CGCCGACAAAGTACAGGATT | 20 |
| 47 | RTAPT1 F | ATGGGCCTGTATCTGAAACG | 20 |
| 48 | RTAPT1 R | CCAGTTCGTCTCCCTTGAAA | 20 |
| 49 | RTCLV1 F | GGTCGCATTCCAGAGTTCAT | 20 |
| 50 | RTCLV1 R | AAATCACGAGGCACAAGTCC | 20 |
| 51 | RTNEF1 F | GAGCTATCGTTGTGGGCTTC | 20 |
| 52 | RTNEF1 R | GCCAACACAGCACTAGCAAC | 20 |
| 53 | RTSOC1 F | CAAAACTTGCAGCACCTGAA | 20 |
| 54 | RTSOC1 R | GCTAGGGCCATTTCCTTTTC | 20 |
| 55 | RTYABBY1 F | CCTTTGGATGAAGAGCCTGA | 20 |
| 56 | RTYABBY1 R | TTGCGGTTAGTGTACCATGC | 20 |

| | | | |
|---|---|---|---|
| 57 | RTYABBY2 F | AAGGCTTCCCTGTGGCTAAT | 20 |
| 58 | RTYABBY2 R | TGACTCAGCCTCTTCATCCA | 20 |
| 59 | RTYABBY3 F | ACCTAACCCATCACCGAACA | 20 |
| 60 | RTYABBY3 R | CGTTGGATCTCGTCCTTGAT | 20 |
| 61 | RTSEP3 F | TTGTGATGCAGAGGTTGCTC | 20 |
| 62 | RTSEP3 R | GGCTAGCAAACTCCAATGCT | 20 |
| 63 | RTACS1F | GTTTGGCTGATCCTGGTGAT | 20 |
| 64 | RTACS1R | TCCCTAGTGGGTTTGATGGA | 20 |
| 65 | RTETR1 F | GTCCACTGCCACCAGAATTT | 20 |
| 66 | RTETR1 R | GTTCTCAAAAGAGGGCACCA | 20 |
| 67 | RTGAPDH F | AGGCCATCAAGGAGGAATCT | 20 |
| 68 | RTGAPDH R | AACTGTAGCCCCATTCGTTG | 20 |
| 69 | RTERS F | GGAGGAATGTGCCTTATGGA | 20 |
| 70 | RTERS R | ACCCGAACAGCAACAACTTC | 20 |

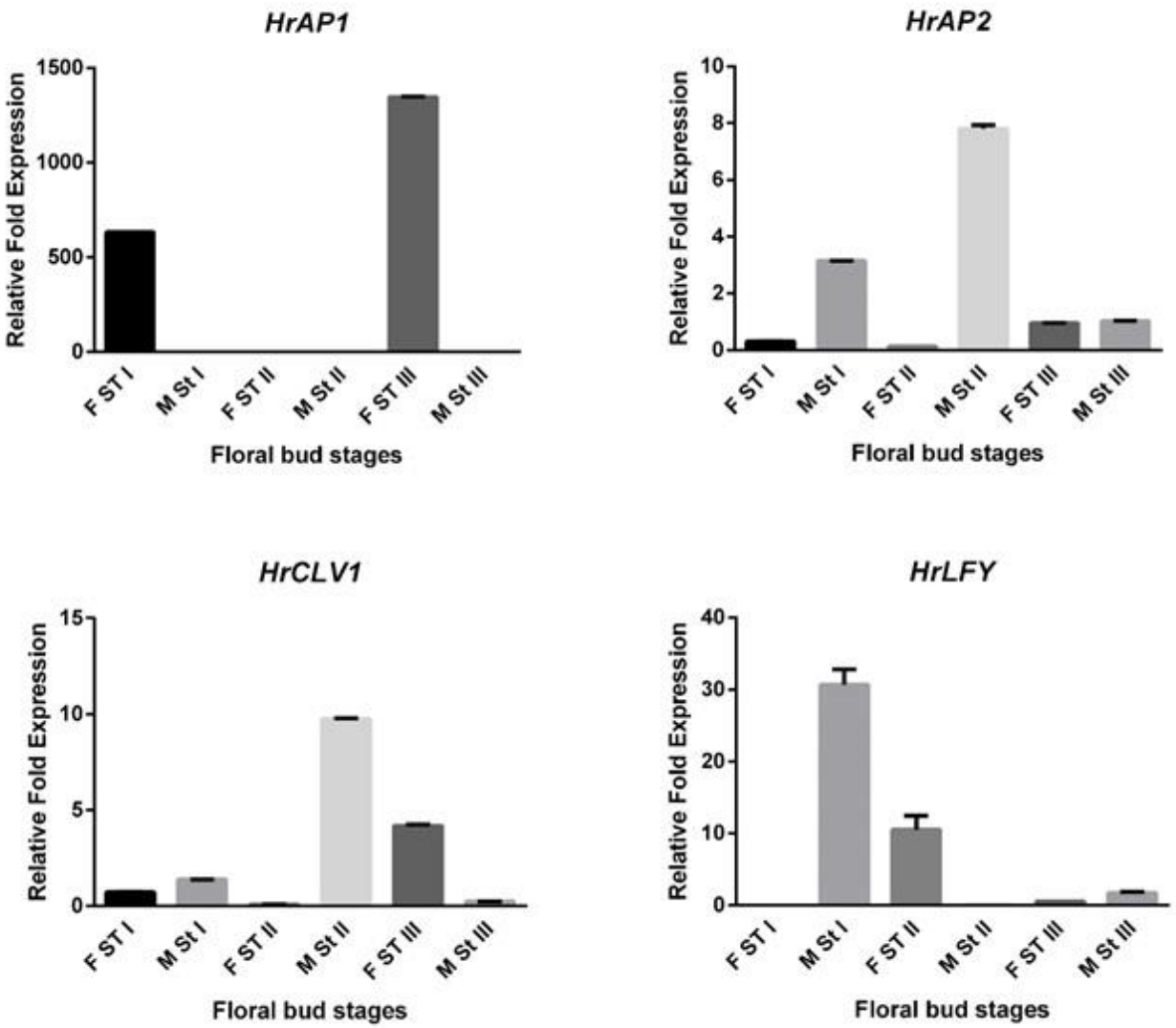


**Fig. 3.5 Relative expression of Putative seabuckthorn floral meristem identity genes.**

### 3.3.3.2 Floral organ identity genes

Among floral organ identity genes the expression of floral organ polarity gene *HrFIL* (Fig. 3.6) was higher in all FDS of female flowers. The differential expression was notably wider in FST III vs MST III (53.88 fold). On the contrary the expression of *HrYAB5* and *HrSEP3* (Fig. 3.6) was higher in all male FDS with highest differential expression of 250 fold and 1000 fold were recorded in MST II respectively. Higher expression of *SEP3* homologue was also observed in male flowers of *Asparagus officinalis* [34]. Stamen and carpel identity gene AGAMOUS (*HrAG*) (Fig. 3.6) 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* (Fig. 3.6) was flower developmental stage dependent rather than sex of flower.

### 3.3.3.3 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. 3.7). 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 3.7). The expression of *CONSTANS* (*HrCO*) responsible for flowering in long days was higher in all male FDS (Fig. 3.7) ( 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 hoologue *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. 3.7). The relative expression of genes *HrGI* and *HrEF1* was stage dependent. Thus it is concluded that expression of most of flowering time

**Table 3.4 Normalized expression values of seabuckthorn putative GISD in three temporal developmental stages of male and female flowers.** The values listed in the table are average of the fold expression values calculated by normalising with two internal reference genes i.e. 26S and GAPDH.

| Gene name | FSTI | FSTII | FSTIII | MSTI | MSTII | MSTIII |
|---|---|---|---|---|---|---|
| ***HrAP1*** | 0.632 | 0.09 | 1.347 | 0.001 | 0.107 | 0.001 |
| ***HrAP2*** | 0.219 | 0.13 | 0.168 | 0.688 | 1 | 0.172 |
| ***HrCLV1*** | 0.281 | 0.103 | 0.287 | 0.391 | 1 | 0.069 |
| ***HrX1*** | 0.281 | 0.023 | 0.129 | 0.209 | 1 | 0.199 |
| ***HrCRY1*** | 0.267 | 0.151 | 0.264 | 0.33 | 1 | 0.617 |
| ***HrCRY2*** | 1.293 | 0.919 | 0.499 | 0.01 | 0.056 | 0.026 |
| ***HrCO*** | 0.012 | 0.033 | 0.007 | 0.119 | 1 | 0.792 |
| ***HrCOLK*** | 0.138 | 0.191 | 0.117 | 0.365 | 1 | 0.252 |
| ***HrLFY*** | 0.018 | 1.346 | 0.053 | 0.579 | 0.128 | 0.095 |
| ***HrEF1*** | 0.659 | 0.277 | 1.347 | 0.29 | 0.901 | 0.179 |
| ***HrERS*** | 0.105 | 0.092 | 0.257 | 0.263 | 1 | 0.445 |
| ***HrETR1*** | 0.127 | 0.108 | 0.392 | 0.379 | 1 | 0.14 |
| ***HrFRI*** | 0.298 | 0.045 | 0.084 | 1.223 | 0.779 | 0.297 |
| ***HrFRILK*** | 0.203 | 0.108 | 0.408 | 0.443 | 1 | 0.561 |
| ***HrGI*** | 0.423 | 0.247 | 0.51 | 1.222 | 0.707 | 0.213 |
| ***HrFIL*** | 0.307 | 0.505 | 1.347 | 0.251 | 0.312 | 0.025 |
| ***HrNEF1*** | 1.293 | 0.185 | 0.135 | 0.068 | 0.441 | 0.123 |
| ***HrYAB5*** | 0.221 | 0.004 | 0.347 | 0.029 | 1 | 0.601 |
| ***HrPHYB*** | 0.436 | 0.04 | 0.069 | 0.591 | 1 | 0.515 |
| ***HrSEP3*** | 0.055 | 0.001 | 0.035 | 0.071 | 1 | 0.04 |
| ***HrAG*** | 1.293 | 0.097 | 0.084 | 0.103 | 0.615 | 0.107 |

**Table 3.5 Relative fold expression of putative GISD within temporally corresponding male and female flower development stages.** Two internal reference genes are used i.e. 26S and GAPDH.

| Genes | FST I | MST I | FST II | MST II | FST III | MST III |
|---|---|---|---|---|---|---|
| ***HrAP1*** | **632** | 0.002 | 0.84 | 1.18 | **1347** | N.E. * |
| ***HrAP2*** | 0.32 | **3.14** | 0.13 | **7.70** | 0.97 | **1.02** |
| ***HrCLV1*** | 0.72 | 1.39 | 0.10 | **9.71** | **4.15** | 0.24 |
| ***HrLFY*** | 0.031 | **32.16** | **10.51** | 0.095 | 0.56 | 1.70 |
| ***HrAG*** | **12.55** | 0.08 | 0.16 | **6.34** | 0.79 | 1.27 |
| ***HrSEP3*** | 0.77 | 1.29 | 0.001 | **1000** | 0.87 | 1.14 |
| ***HrFIL*** | 1.22 | 0.82 | 1.62 | 0.62 | **53.88** | 0.02 |
| ***HrYAB5*** | **7.62** | 0.13 | 0.004 | **250** | 0.57 | 1.73 |
| ***HrCRY1*** | 0.81 | 1.23 | 0.15 | **6.6** | 0.43 | 2.33 |
| ***HrCRY2*** | **129.3** | 0.008 | **16.41** | 0.060 | **19.19** | 0.052 |
| ***HrPHYB*** | 0.74 | 1.35 | 0.04 | **25** | 0.13 | **7.4** |
| ***HrCO*** | 0.100 | **9.91** | 0.033 | **30.30** | 0.009 | **113.14** |
| ***HrCOLK*** | 0.37 | 2.64 | 0.191 | **5.23** | 0.46 | 2.15 |
| ***HrGI*** | 0.35 | **2.8** | 0.34 | **2.8** | **2.3** | 0.41 |
| ***HrFRI*** | 0.24 | **4.1** | 0.06 | **17.31** | 0.28 | **3.5** |
| ***HrFRILK*** | 0.45 | 2.18 | 0.108 | **9.78** | 0.72 | 1.37 |
| ***HrEF1*** | 2.27 | 0.44 | 0.31 | 3.25 | **7.52** | 1.32 |
| ***HrERS1*** | 0.40 | 2.50 | 0.092 | **10.86** | 0.58 | 1.73 |
| ***HrETR1*** | 0.34 | 2.98 | 0.108 | **9.78** | 2.8 | 0.36 |
| ***HrX1*** | 1.34 | 0.74 | 0.02 | **43.47** | 0.65 | 1.54 |
| ***HrNEF1*** | **19.01** | 0.05 | 0.42 | 2.3 | 1.09 | 0.91 |

* Fold expression value less than 0.0001 are considered as Negligible Expression (N.E.).

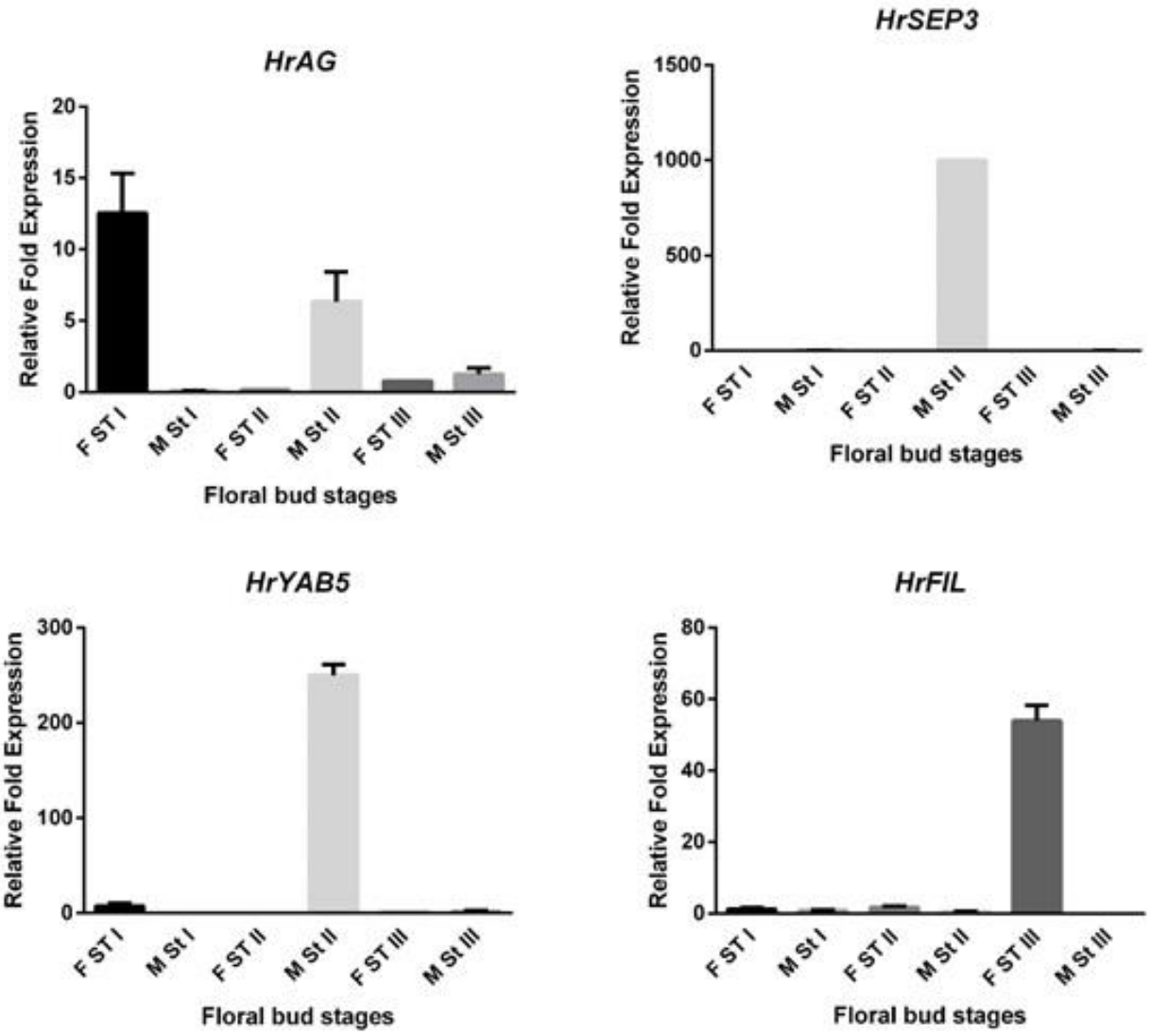


Fig. 3.6 **Relative expression of putative seabuckthorn floral organ identity genes.**

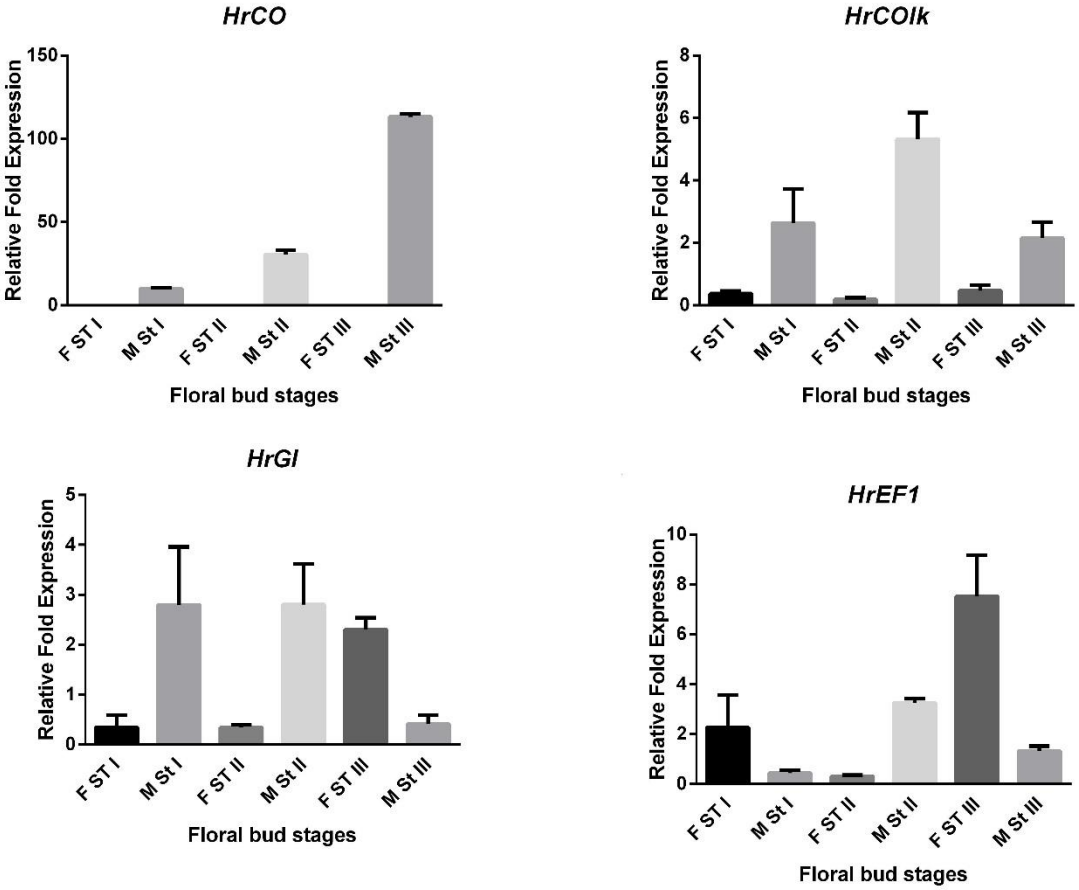


Fig. 3.7 **Relative expression of putative seabuckthorn flowering time genes.**

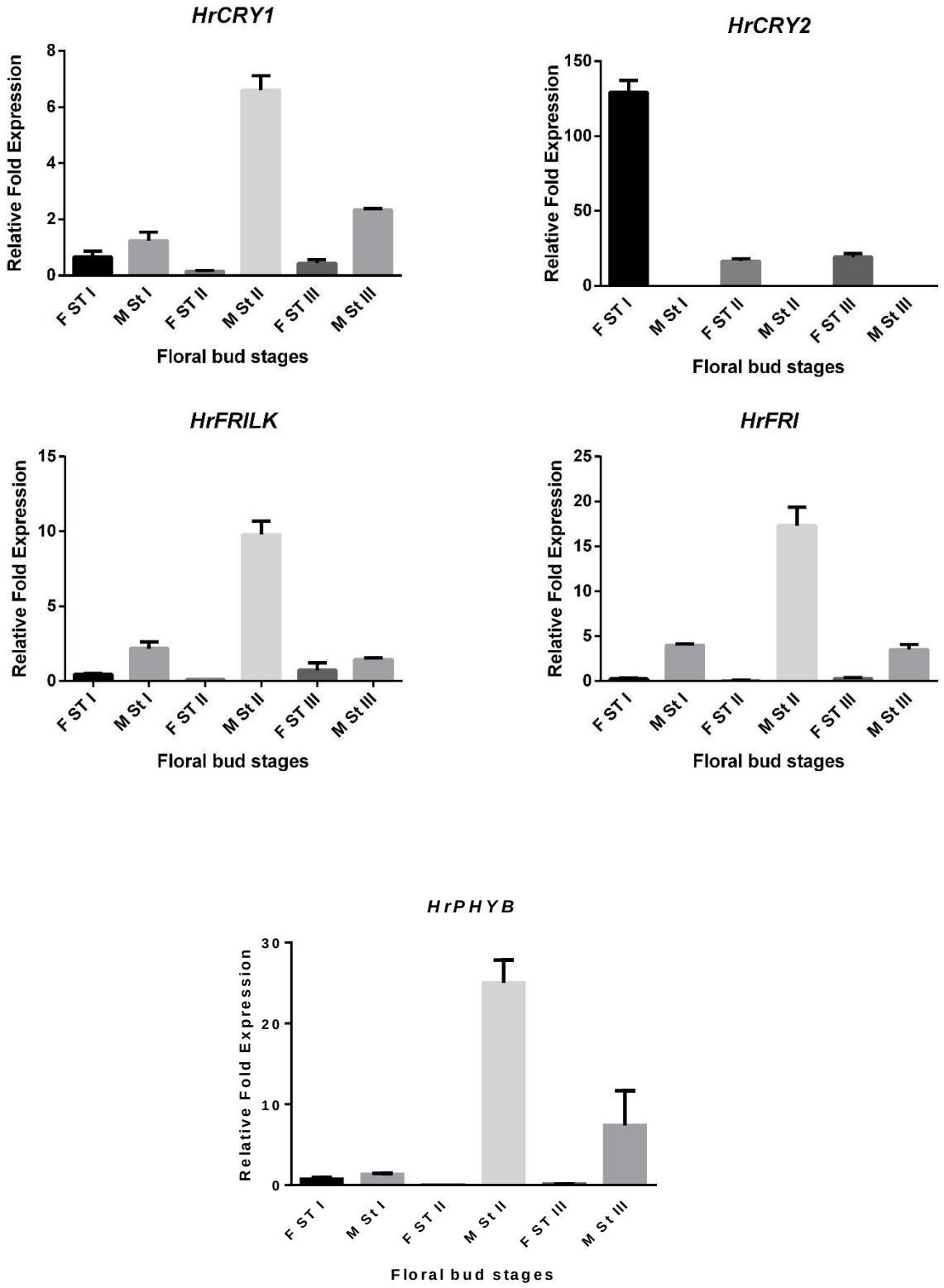


**Fig. 3.7 (Contd.) Relative expression of putative seabuckthorn flowering time genes.**

genes including *HrCRY1*, *HrPHYB* and *HrCO* was higher in all male FDS while that of *HrCRY2* was higher in all female FDS.

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 circardian pathway genes like *CO, GI* and *FT* and could alter flowering time depending upon external cues [91]. Differential 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.

#### 3.3.3.4 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. 3.8) showed 10.86 fold higher expression in MST II with respect to FST II while expression of *HrETR1* (Fig. 3.8) was recorded 9.78 fold higher in MST II with respect to FST II.

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.

#### 3.3.3.5 Pollen exine formation genes

*HrX1* is the female specific SCAR marker which was found to show high level of similarity to Acyl CoA synthatase and other related plant ligases on the basis of BLASTn and tBLASTx analysis of sequence [8]. In *A. thaliana* knocking out of acyl CoA synthatase led to production of unviable pollen, which in turn produced male sterile plants [93]. *HrX1* (Fig. 3.9) expressed 43.47 fold higher in MST II with respect to FSTII. On the contrary, expression of *HrNEF1* (Fig. 3.9) was observed to be 19.01 fold higher in FST I as compared to MST I.

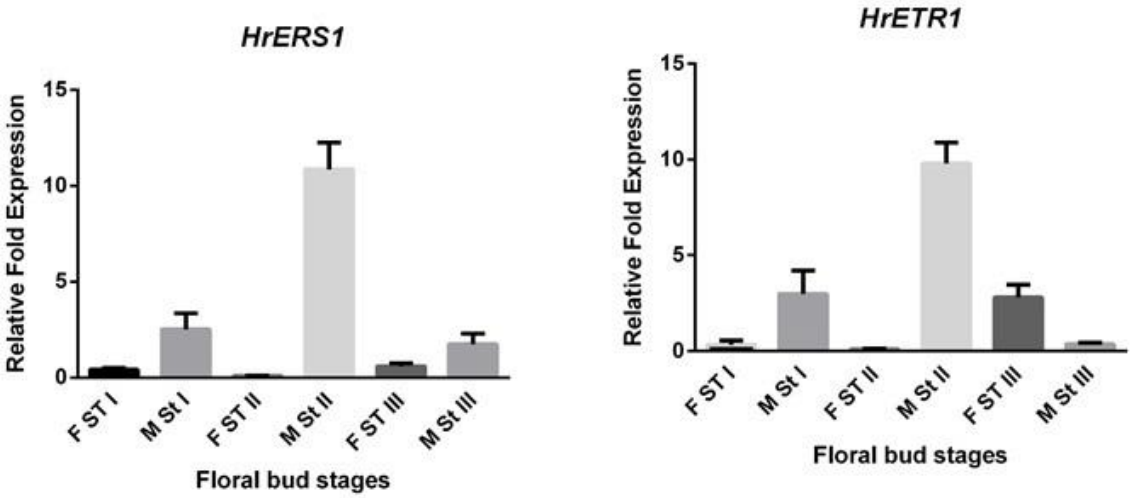


**Fig. 3.8 Relative expression of putative seabuckthorn phytohormnone ethylene genes.**

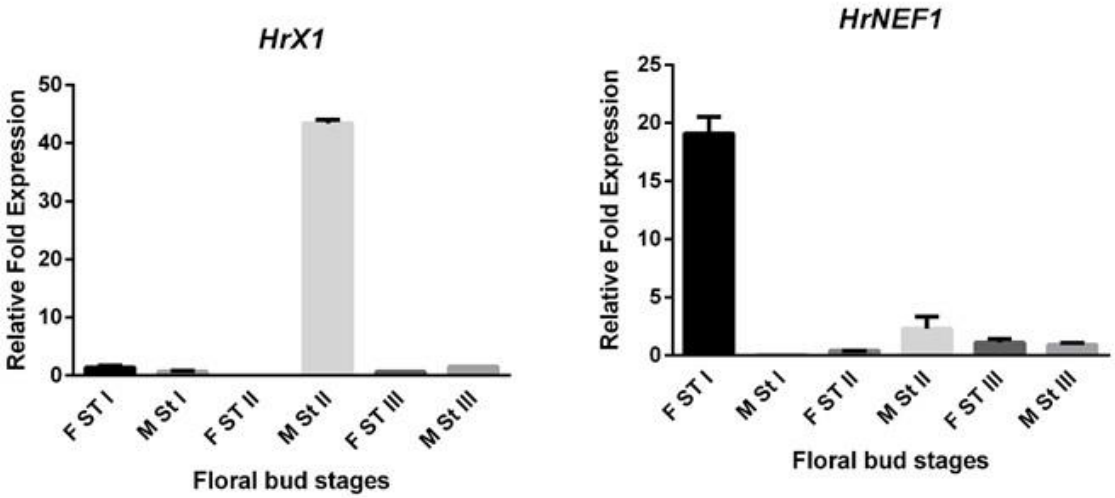


**Fig. 3.9 Relative expression of putative seabuckthorn pollen exine genes.**

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.

### 3.3.4 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. 3.10). The expression of all putative GISD except for *HrCRY2* and *HrLFY* was higher in 2$^{nd}$ developmental stage of male flowers (Fig. 3.11). 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. 3.12). Moreover, the heat map of putative GISD (Fig. 3.13) shows that male flowers have maximum GISD with higher level of expression as compared to female flowers in 2$^{nd}$ floral developmental stage.

Out of the three developmental stages, 2$^{nd}$ stage had the maximum number of genes with expression biased towards male flowers (Fig. 3.10, Fig 3.11 & Fig. 3.12). Thus stage II of male and female flowers require further investigation to justify the tilt of GISD expression towards male flowers.

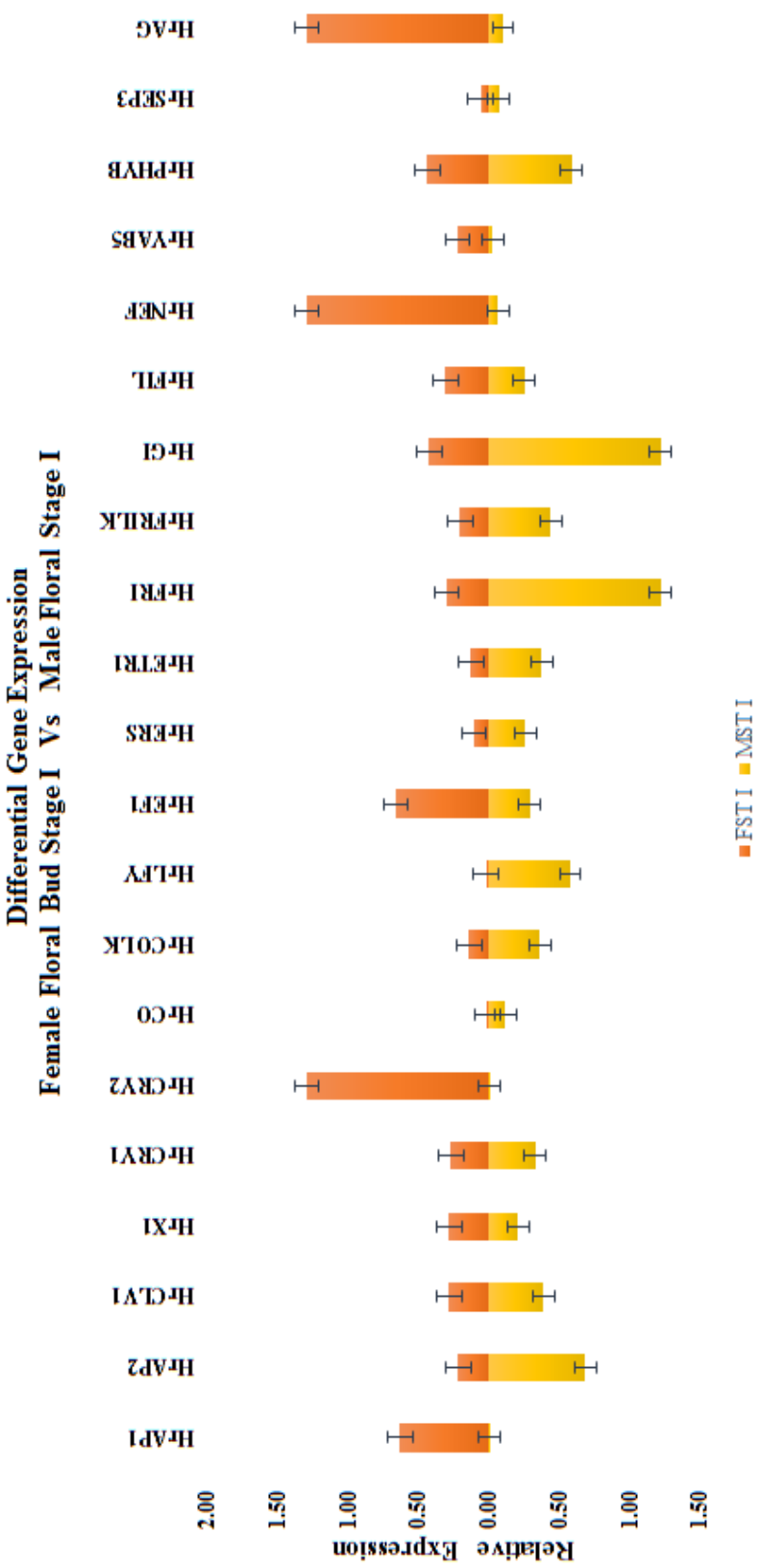


**Fig. 3.10 Comapritive expression of seabuckthorn GISD between floral Development Stages (FDS) - Male Stage I (MST I) vs Female Stage I (FST I).**

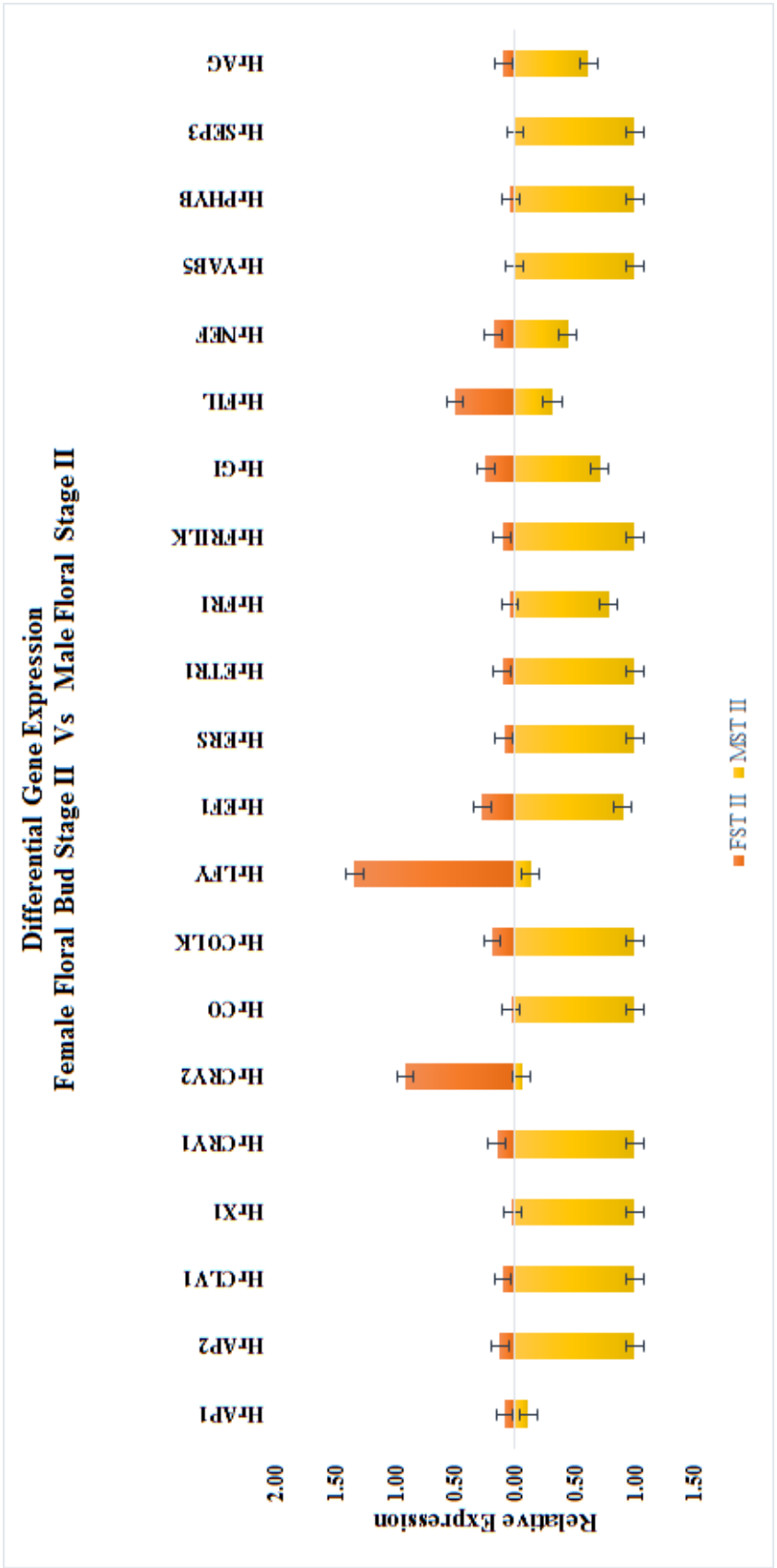


**Fig. 3.11 Comapritive expression of seabuckthorn GISD between floral Development Stages (FDS) - Male Stage II (MST II) vs Female Stage II (FST II).**

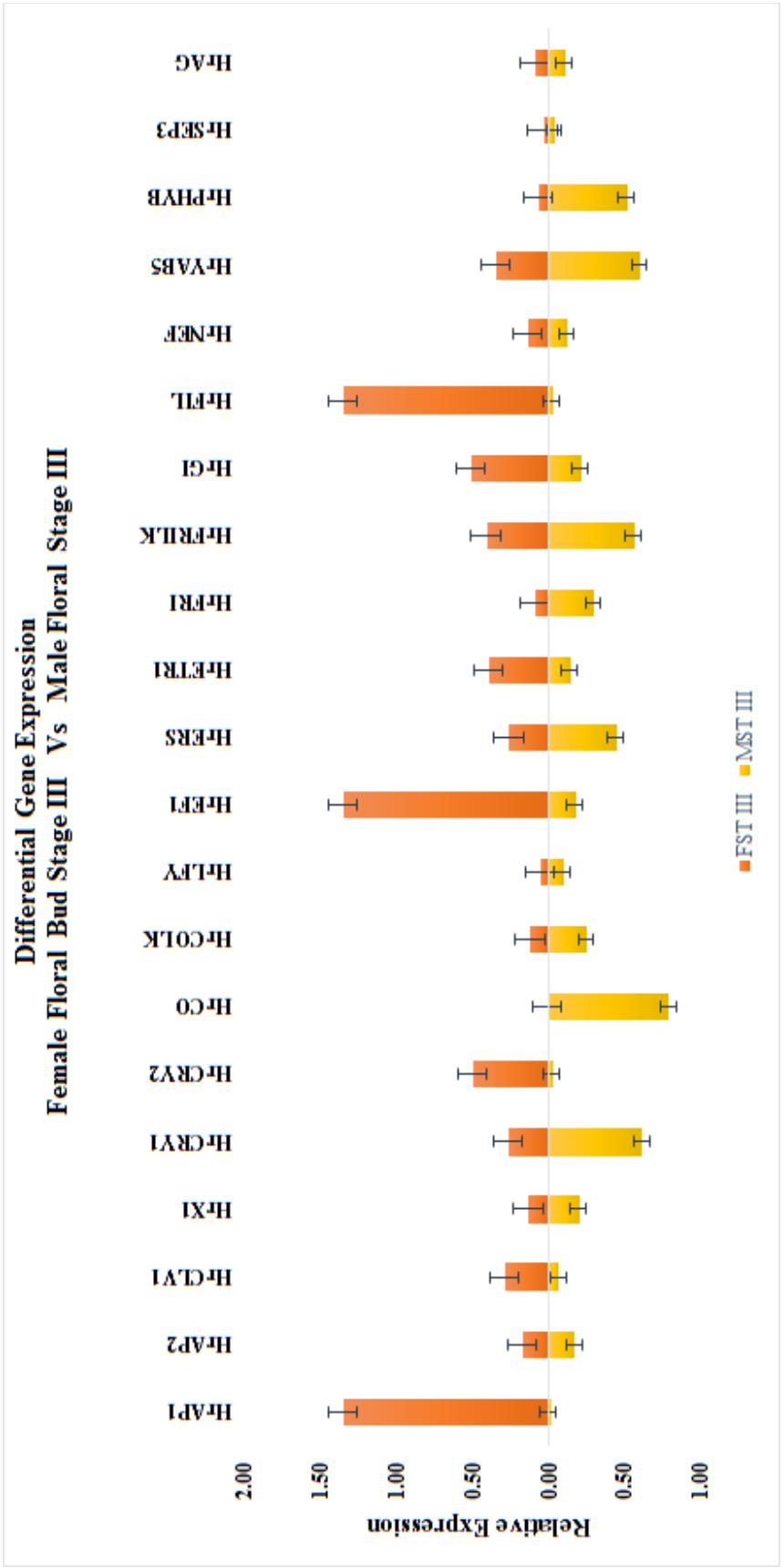


**Fig. 3.12 Comaprtitive expression of seabuckthorn GISD between floral Development Stages (FDS) - Male Stage III (MST III) vs Female Stage III (FST III).**

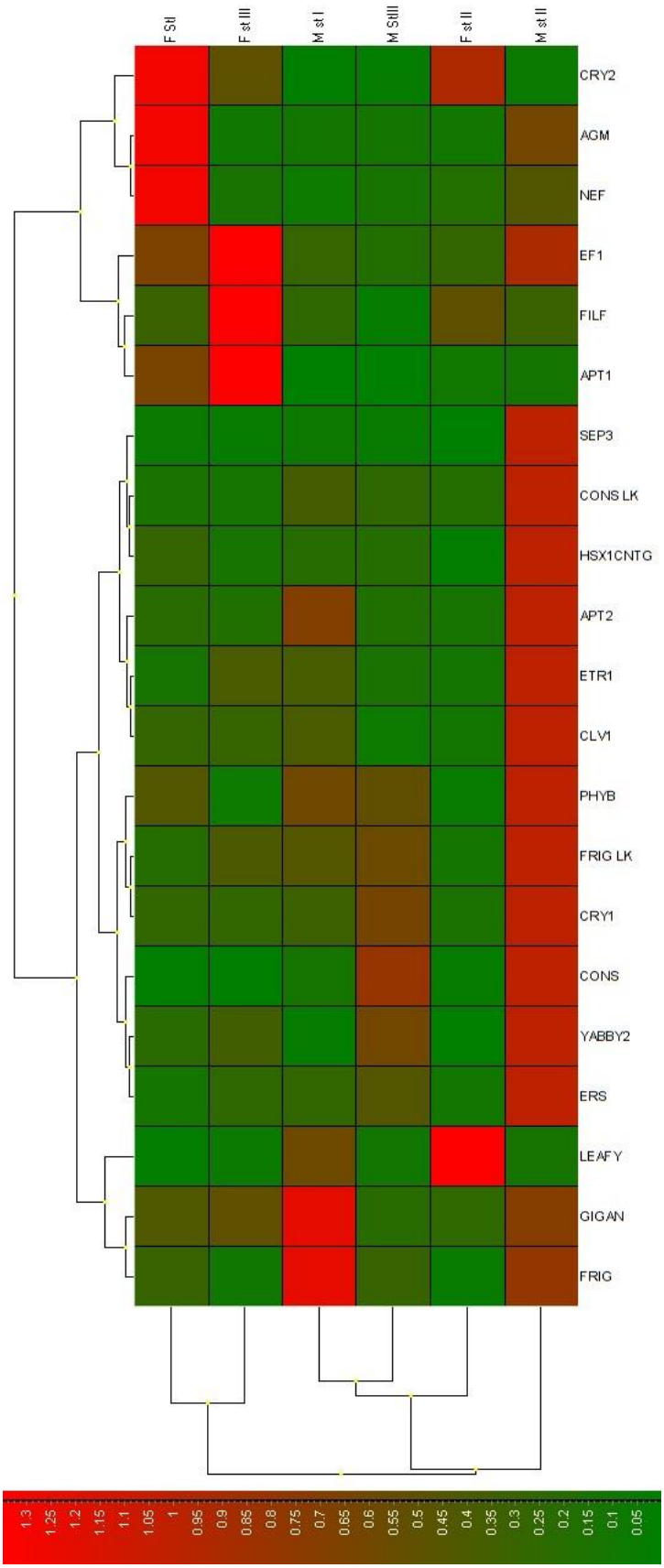


**Fig. 3.13 Heat map of relative expression of putative GISD in seabuckthorn FDS.**

## 3.3.5 Correlation of gene expression data with floral organ development in male and female floral buds of Seabuckthorn.

### 3.3.5.1 Female floral buds

The morphological observation of the female sex organs i.e. ovary at FST III indicated that the ovary might have developed in stages between FST II and FST III. The expression level of the genes *HrAP1, HrCLV1, HrFIL, HrCRY2, HrGI, HrEF1* and *HrETR1* increased in FST III with respect to FST II. However the expression level of the gene *HrLFY* decreased at FST III with respect to FST II.

### 3.3.5.2 Male Floral buds

In case of male floral buds the distinct male floral organs i.e. anthers were observed at **MST II** and it matured through **MST III**. Thus the development of the stamens started in between **MST I** and **MST II** while stamens matured through **MST II** and **MST III**. The expression of the genes ***HrAP2, HrCLV1, HrAG, HrSEP3, HrYAB5, HrCRY1, HrPHYB, HrCO, HrCOLK, HrFRI, HrFRILK, HrEF1, HrERS1, HrETR1, HrX1*** and ***HrNEF1*** increased in **MST II** with respect to **MST I.** But the expression of ***HrLFY*** decreased in **MST II** with respect to **MST I.** During the maturation of male floral bud from **MST II** to **MST III** the expression of ***HrCO*** only increased while the rest of the above mentioned genes had reduced expression levels in **MST III** with respect to **MST II**.

## 3.4 Conclusion

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.

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# CHAPTER 4
# IDENTIFICATION OF DIFFERENT TRANSCRIPTION FACTOR FAMILIES PLAYING ROLE IN SEX DETERMINATION IN *H. RHAMNOIDES*


## Abstract

Transcription factors regulate the expression of the genes. The study was conducted to identify seabuckthorn transcription factor families involved in floral development which may have role in sex determination. Forty three transcription factor families having role in flower development were shortlisted from literature survey, and twenty two transcription factor families were identified from available seabuckthorn genomic resources. Of these, transcripts of 12 transcription factor families were found to differentially express in either male or female flower bud stages. HrTF_AP2 was significantly expressed in female flower buds only while HrTF_AUX_IAA and HrTF_SRF 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 transcription factor families under study. Information on these sex-specific transcription factor families will help in elucidating sex determination mechanism in seabuckthorn.

## 4.1 Introduction

With the completion of the *Arabidopsis thaliana* genome sequence, the information of the full complement of transcription-factor encoding genes could be utilized to identify transcription factor families in other plant species. The recent shift from a 'gene-centric' to a 'genome-centric' perspective in biology is particularly appropriate for the study of transcription factors [1]. Transcription factor (TF) genes comprise a substantial fraction of all eukaryotic genomes, and the majority can be grouped into a handful of different, often large, gene families according to the type of DNA-binding domain that they encode.

Transcription factors (TFs) are the proteins that regulate gene expression. A typical plant transcription factor with few exceptions contains a DNA-binding region, an oligomerization site, a transcription-regulation domain, and a nuclear localization signal. Most transcription factors exhibit only one type of DNA-binding and oligomerization domain, occasionally in multiple copies, but some contain two distinct types. DNA-binding regions are normally adjacent to or overlap with oligomerization sites, and their combined tertiary structure determines critical aspects of transcription factor activity. The basic amino acid residues are essential for the function of nuclear localization signals and DNA-binding domains. Multigene families encode transcription factors, with members either dispersed in the genome or clustered on the same chromosome. The expression of transcription factor genes in plants is regulated at transcriptional and post-transcriptional levels, while the activity of their protein products is modulated post-translationally [2].

The classification of transcription factors depend upon their structural features. The transcription factor families are sometimes subdivided according to the number and spacing of conserved residues in the most similar domain [2]. For example, owing to the quantity and arrangement of cysteine (C) and histidine (H) residues, the transcription factors containing zinc fingers fall into five classes: C2H2, C3H, C2C2 (GATA finger), C3HC4 (RING finger), and C2HC5 (LIM finger) [3]. Other than such classification criteria, sometimes transcription factors of the same family are classified by reference to a domain that falls outside the most conserved region. For example, homeodomain factors exist as five groups, including the homeodomain zipper [4], homeodomain finger [5], GLABRA2 [6], ELK-homeodomain [7] and twin homeodomain factors [8].

In *A. thaliana* there are 1589 transcription factor genes classified into more than 40 different families. [9]. Many of those gene families are large, with over, or close to, 100 members, and the largest of them are bHLH, AP2/ERF, and MYB-(R1)R2R3 factors. In general, transcription factor families have collectively higher expansion rates in plants than in animals.. For example, two of the largest *Arabidopsis* families, MYB and MADS, are very small in fungi and animals. The transcription factor families selected for expression analysis in floral buds of seabuckthorn are described below.

A large plant-specific family of transcription factors is the AP2/ERF group. The AP2 domain itself, however, is not plant specific, since it is found in endonucleases of different organisms. The AP2/ERF superfamily is defined by the AP2/ERF domain, which consists of about 60 to 70 amino acids and is involved in DNA binding. The AP2 family proteins contain two repeated AP2/ERF domains. AP2/ERF proteins have important functions in the transcriptional regulation of a variety of biological processes related to growth and development, as well as various responses to environmental stimuli [10, 11].

Auxin response factors (ARF) are transcription factors that regulate the expression of auxin response genes. ARFs bind with auxin response elements (AuxRE) in promoters of the genes. Most ARFs consist of an amino-terminal DNA-binding domain (DBD), a middle region that functions as an activation domain (AD) or repression domain (RD), and a carboxy-terminal dimerization domain (CTD). The ARF DBD is classified as a plant-specific B3-type, but requires additional amino-terminal and carboxy-terminal amino acids for efficient *in vitro* binding to TGTCTC AuxREs [11, 12].

Members of the ARID (AT-rich interaction domain) family of DNA-binding proteins are found in fungi and invertebrate and vertebrate metazoans. ARID-encoding genes are involved in a variety of biological processes including embryonic development, cell lineage gene regulation and cell cycle control. Although the specific role of ARID domains are yet to be elucidated, they may be involved in both positive and negative transcriptional regulation and are likely to be involved in the modification of chromatin structure [11, 12].

Aux/IAA proteins are short-lived nuclear proteins that repress expression of primary/early auxin response genes in protoplast transfection assays. Aux/IAA proteins dimerize with auxin response factor (ARF) transcriptional activators that reside on auxin-

responsive promoter elements, referred to as AuxREs. Most Aux/IAA proteins contain four conserved domains, designated domains I, II, III, and IV. Domain II play role in protein stability while domain III and IV play dimerization. A clear function for domain I had not been established. An LxLxL motif within domain I is important for conferring repression. The dominance of Aux/IAA repression domains over activation domains in ARF transcriptional activators provides a plausible explanation for the repression of auxin response genes via ARF-Aux/IAA dimerization on auxin-responsive promoters [11, 13].

The bZIP TFs are characterized by a 40- to 80-amino-acid-long conserved domain (bZIP domain) that is composed of two motifs: a basic region responsible for specific binding of the TF to its target DNA, and a leucine zipper required for TF dimerization [11, 14]. In plants, basic region/leucine zipper motif (bZIP) transcription factors regulate processes like pathogen defence, light and stress signalling, seed maturation and flower development [11, 15].

GRAS (GAI, RGA, SCR) gene family share a variable amino-terminus and a highly conserved carboxyl-terminus that posses five recognizable motifs [16]. Members of the GRAS gene family encode transcriptional regulators that have diverse functions in plant growth and development such as gibberellin signal transduction, root radial patterning, axillary meristem formation, phytochrome A signal transduction and gametogenesis [11, 17].

Homeobox transcription factor family encodes a protein domain, the homeodomain (HD), which is a conserved 60-amino acid motif present in transcription factors found in all the eukaryotic organisms. This 60-amino acid sequence folds into a characteristic three-helix structure that is able to interact with the DNA. Most HDs are able to bind DNA as monomers with high affinity, through interactions made by helix III (the so-called recognition helix) and a disordered N-terminal arm located beyond helix I. The high degree of conservation of this type of domain among diverse proteins from different kingdoms indicates that this structure is crucial to maintain the HD functionality [11, 18].

MYB factors represent a family of proteins that include the conserved MYB DNA-binding domain. Many vertebrates contain three genes related to v-Myb c-Myb, A-Myb and B-Myb and other similar genes have been identified in insects, plants, fungi and slime moulds. This domain generally comprises up to three imperfect repeats, each forming a helix-turn-helix structure of about 53 amino acids. Three regularly spaced tryptophan residues, which form a

tryptophan cluster in the three-dimensional helix-turn-helix structure, are characteristic of a MYB repeat. The repeats in c-Myb are referred to as R1, R2 and R3; and repeats from other MYB proteins are categorised according to their similarity to either R1, R2 or R3. In contrast to animals, plants contain a MYB-protein subfamily that is characterised by the R2R3-type MYB domain. MYB proteins can be classified into three subfamilies depending on the number of adjacent repeats in the MYB domain (one, two or three). [11, 19].

NAM, ATAF, and CUC (NAC) transcription factors comprise a large protein family. Proteins of this family contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal domain. NAC was originally derived from the names of three proteins, no apical meristem (NAM), ATAF1-2, and CUC2 (cup-shaped cotyledon), that contain a similar DNA-binding domain. The early reported NAC transcription factors are implicated in various aspects of plant development. NAC transcription factors play an essential role in regulating senescence, cell division, and wood formation [20].

PHD proteins are mostly found in nucleus, and their functions tend to lie in the control of chromatin or transcription. PHD fingers bind to specific nuclear protein partners, for which they apparently use their loop 2 surface. Each PHD finger has its own cognate nuclear ligand, much like RING fingers have their cognate E2 ligases. [21]

The SRF domain (MCM1, AGAMOUS, DEFICIENS, and MADS) is a conserved DNA-binding/dimerization region present in a variety of transcription factors. MADS box genes represent a large multigene family in vascular plants. In angiosperms, many of the genes of the MADS family are involved in different steps of flower development, most notably in the determination of floral meristem and organ identity. The roles that MADS box genes play, however, are not restricted to control the development of the plant reproductive structures only [22].

Plant sex determination occurs at genetic level. The role of MADS transcription factor family in plant sex determination is widely understood. However the transcription factor families other than MADS / SRF family are least explored for their role in sex determination in dioecious plants. Since the morphological variations in dioecious plants exist mostly in sex organs (flowers), transcription factor families involved in flower development could be explored for potential TF candidates for sex determination in seabuckthorn. In current study

literature survey was conducted for identification of TF families involved in floral development. After identification of TF families, the potential transcript from a family was selected for expression studies through qRT-PCR in the three temporal male and female floral development stages in seabuckthorn.

## 4.2 Material and methods

### 4.2.1Plant material, RNA extraction and cDNA synthesis

The plant material, RNA extraction and cDNA synthesis protocol are same as reported in the Material and Methods section of the Chapter 3.

### 4.2.2 Identification of Transcription factors from the genomic resources of seabuckthorn

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 The pfam ids for all the TF families were extracted from the pfam database (http://pfam.janelia.org/). Quality trimmed and filtered nucleotide sequences of seabuckthorn were retrieved from its seed [23], root and leaf [24] transcriptome (NCBI Accession No.SRX118240, SRX131619 and SRX131618 respectively) and ESTs from NCBI EST database. The unigenes from seabuckthorn leaf as well root transcriptomes were scanned against these protein family ids. For each family of transcription factor the best hit was mined out based on smallest e-value.

### 4.2.3 Expression analysis of transcription factors by qRT-PCR

The methodologies adopted for analysis of the gene expression data are same as described in Material and Methods section of the Chapter 3.

## 4.3 Results and Discussion

### 4.3.1 Identification of seabuckthorn transcription factor families

The current study was focused on 43 Transcription Factor (TF) families that were known to be involved in flower development as well plant phytohormone regulation. Out of 43 TF families 22 TF families had transcript sequences in available *H. rhamnoides* genomic resources (Table 4.1). The TF families for which transcripts were not present in the transcriptome data of seabuckthorn include Bhlh, GARP-G2, ZIM, PcG, ULT, ABI3-VP1, CCAAT-HAP2, C2C2-CO-LIKE, Trihelix, BBR-BPC, C2C2-GATA, EIL, JUMONJI, LUG, CCAAT-HAP3, GARP-ARR-B, HRT-like, LFY, NZZ, SRS, VOZ. The trancription factor families had more than one transcript in each family. Thus for testing the TF families for their potential role in unisexual flower development in seauckthorn, the transcript with the lowest e-value was chosen for expression studies on male and female floral buds and are listed in Table 4.1 .

**Table 4.1 – List of Seabuckthorn transcription factor families retrieved from available seabuckthorn resources.**

| S. no. | Transcription factor family name | PFAM ID | No. of transcripts identified | Contig* with least E-value |
|---|---|---|---|---|
| 1 | NAC | PF01849 | 18 | 9738 |
| 2 | AP2/ERF | PF00847 | 91 | 32672 |
| 3 | AUX-IAA | PF02309 | 80 | 32742 |
| 4 | Myb_DNA-binding | PF00249 | 214 | 67523 |
| 5 | Homeobox | PF00046 | 68 | 27290 |
| 6 | C3H | PF00642 | 95 | 80119 |
| 7 | TLP (tubby like protein) | PF01167 | 28 | 24353 |
| 8 | bZIP | PF00170 | 63 | 22713 |
| 9 | MADS/SRF | PF00319 | 27 | 34830 |
| 10 | WRKY | PF03106 | 146 | 71374 |
| 11 | C2C2-Dof | PF02701 | 71 | 64439 |
| 12 | C2C2-YABBY | PF04690 | 16 | 65380 |
| 13 | C2H2-zf | PF00096 | 10 | 55401 |
| 14 | GRAS | PF03514 | 92 | 7016 |
| 15 | PHD | PF00628 | 47 | 14115 |

| | | | | |
|---|---|---|---|---|
| 16 | ZF-HD dimer | PF04770 | 22 | 5691 |
| 17 | ARID | PF01388 | 9 | 31220 |
| 18 | SBP | PF03110 | 25 | 54068 |
| 19 | ARF | PF00025 | 72 | 29939 |
| 20 | SAP | PF02037 | 4 | 70287 |
| 21 | Zf-TAZ | PF02135 | 7 | 19198 |
| 22 | Tiffy | PF12171 | 38 | 18003 |

### 4.3.2 Expression analysis of transcription factors by qRT PCR

The temporal expression of the 22 transcription factors across three developmental stages of male and female flowers of *H. rhamnoides* was tested (Table 4.3). The CT values of ten transcription factors which showed values greater than 35, were not considered for further investigation. Thus remaining 12 transcription factors were analysed for differential expression among temporally corresponding male and female flower developmental stages (FDS).

#### 4.3.2.1 Ethylene-responsive element-binding factor (AP2/ERF) transcription factor family

Ethylene-responsive element-binding factor (ERF) genes constitute one of the largest transcription factor gene families in plants [25]. These transcription factors are involved in different physico-biological processes in plant systems implicated in the regulation of different developmental stages including flower development, embryo development, leaf epidermal cell identity and spikelet meristem determinacy, etc. [26-29]. In case of seabuckthorn HrTF ERF / HrTF_AP2 showed female specific expression. 163.81 fold higher expression was observed in FST I with respect to MST I (Table 4.4; Fig. 4.1). On the other hand its expression was 7.05 fold higher in FST II with respect to MST II (Table 4.4; Fig. 4.1). The results are in contrast with as reported by zhao et. al in 2006 [30] in which AP2/ ERF homologue was implicated to have role in pollen development. The down regulation of the AP2/ ERF homologue led to decreased pollen viability as well as germination activity [30]. The higher expression levels of HrTF AP2 /ERF indicates its role in female flower development in seabuckthorn.

#### 4.3.2.2 A–T Rich Interaction Domain (ARID) transcription factor family

ARID (*A*–T *R*ich Interaction Domain) transcription factors are involved in the regulation of processes implicated in the flower/fruits developmental stages. At1g76110 and At1g04880 transcription factors representing the ARID family have been characterised in *Arabidopsis thaliana*, where, they controls the expression of some candidate genes in ovule and primordia development stages [31]. ARID TF has shown higher level of expression in I$^{st}$ and

**Table 4.2 List of primers used in qRT PCR analysis of Transcription factors.**

| S.No. | Primer Name | Primer Sequence | Length (bp) |
|---|---|---|---|
| **1** | TFAP2 F | CGAGTGGTGATCAAACATGG | 20 |
| **2** | TFAP2 R | GCCGTCAGATATCCCTTTCA | 20 |
| 3 | TFARF F | AGAGCCAGTCAAGTCCCTCA | 20 |
| 4 | TFARF R | TTAGGGATGCAGTGTTGCTG | 20 |
| 5 | TFARID F | GCTGGGAAGGAACTGGTACA | 20 |
| 6 | TFARID R | TCTAAGCTGTGGAGGCAGGT | 20 |
| 7 | TFAUX_IAA F | GCCAAGGAATCAGAAAGCTG | 20 |
| 8 | TFAUX_IAA R | CTGCTAAACCACCTCCCAAG | 20 |
| 9 | TFB_ZIP F | GCCAGAGGTGACAGGAGAAG | 20 |
| 10 | TFB_ZIP R | TGCTTGACAATGGGAAATGA | 20 |
| 11 | TFGRAS F | ACGAACCCCAGTTTCTTGTG | 20 |
| 12 | TFGRAS R | GTGTTGCTGCTTACCCACCT | 20 |
| 13 | TFHOMEOBOX F | CTAAGACCGGATCGCTGAGA | 20 |
| 14 | TFHOMEOBOX R | CCCGAAATATGAGCCCTGTA | 20 |
| 15 | TFMYB F | CACCATCAAAATCAAGGGAAG | 21 |
| 16 | TFMYB R | CCGGCGTCCACTTTAATCTA | 20 |
| 17 | TFNAC F | GGGGCTGTTGATAACGAAGA | 20 |
| 18 | TFNAC R | AAGAAGACCGTTGTCGAGGA | 20 |
| 19 | TFPHD F | CTTCTTTTCAGCACCCAAGC | 20 |
| 20 | TFPHD R | TTTCGTTGAGGACGTTAGGG | 20 |
| 21 | TFSAP F | GAAACTCGTGGCAAGCTCTC | 20 |

| | | | |
|---|---|---|---|
| 22 | TFSAP R | CAATCCATGCTTCCTCAGGT | 20 |
| 23 | TFSBP F | TCCATCACAAAGCCTCCACT | 20 |
| 24 | TFSBP R | AGGTTTCATCAGCTTCCGGA | 20 |
| 25 | TFSRF_TF F | AGAGGCCCTGCTAATTTGCT | 20 |
| 26 | TFSRF_TF R | CGGTTCTTTGTGACGCTGAA | 20 |
| 27 | TFTIFFY F | AAACTGCTTGCATGGGGAAC | 20 |
| 28 | TFTIFFY R | TGTCATGTCGTGTTGAACCG | 20 |
| 29 | TFTUB F | TTCAACTAGCAAAGCAGGGC | 20 |
| 30 | TFTUB R | GTGAAAGTTTGTGGCCGTCT | 20 |
| 31 | TFWRKY F | GCTTTAGGGTCTGATGCTGC | 20 |
| 32 | TFWRKY R | ACCAAGAGACGATGTGCAGA | 20 |
| 33 | TFYABBY F | TATGGGGCACTTCGGATCTC | 20 |
| 34 | TFYABBY R | AGCGGTTAGTGTTCCTGGAA | 20 |
| 35 | TFZF-C2H2 F | GGAGTGAGTTGAGGTGGTCA | 20 |
| 36 | TFZF-C2H2 R | CTCAAGAGACACGTACGCAC | 20 |
| 37 | TFZF-CCCH F | GCACTCCACCCAGCTTATTG | 20 |
| 38 | TFZF-CCCH R | TGGTGGTTCAGGAAGTGGAA | 20 |
| 39 | TFZF-DOF F | TTGCAGAAATGACGAGGCTG | 20 |
| 40 | TFZF-DOF R | GTGCTAGCGAGAACCCAAAA | 20 |
| 41 | TFZF-HD F | GCGCATTTGAGTGAGGTAGG | 20 |
| 42 | TFZF-HD R | ACCACCACCATCATCACCAT | 20 |
| 43 | TFZF-TAZ F | CAGCACACTCACTCTCAGGA | 20 |
| 44 | TFZF-TAZ R | TCGCACATGAATAAGGCACG | 20 |

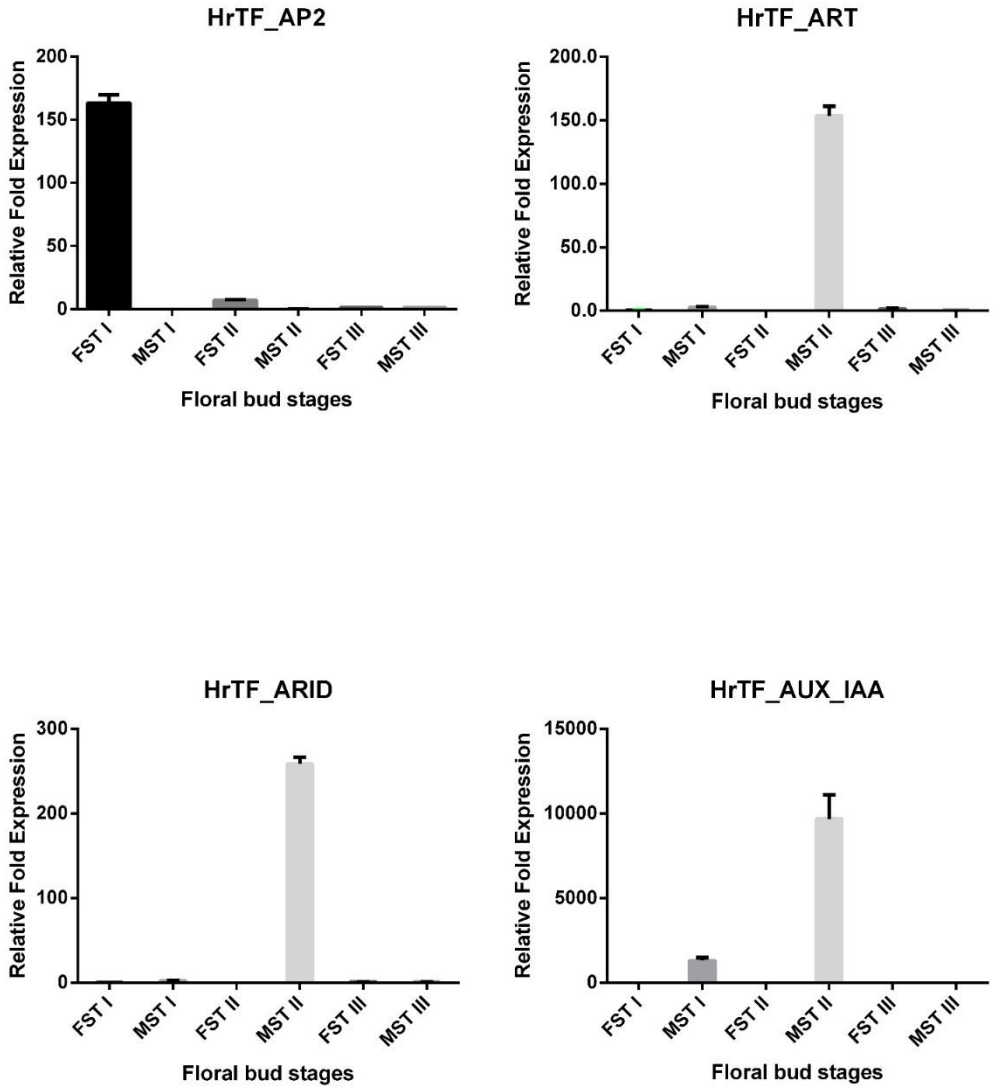


**Fig 4.1 Relative temporal expression of seabuckthorn AP2/ERF, ARF, ARID and AUX_IAA transcription factors**

II$^{nd}$ developmental stages of male flower buds (260 fold) as compared to corresponding stages of female flower buds (Table 4.4; Fig. 4.1). Thus ARID TF could have important role in development of sex organs in male flowers of seabuckthorn.

#### 4.3.2.3 Auxin Response Factor (ARF) transcription factor family

Auxin response transcription factors (ARF) are known for their role in different developmental stages of flowers and fruits in various plant species such as *Solanum lycopersicum, Arabidopsis thaliana, Nictotiana tobaccum, etc.* [32]. In seabuckthorn the highest expression of ARF transcript was observed in MST II (155 fold) with respect to FST II (table 4.4; Fig. 4.1). The expression level of ARF increases from MST I to MST II and then again decreases in MST III, indicating its role in initial development of male sex organs. In *A. thaliana* two Auxin Response Factors, ARF6 and ARF8, regulate gynoecium and stamen development in immature flowers [33]. Mutations in the miR167 (miR167 regulates the expression of ARF6 and ARF 8) target sites of ARF6 or ARF8 caused ectopic expression of these genes in domains of both ovules and anthers where miR167 was normally present. As a result, ovule integuments had arrested growth, and anthers grew abnormally and failed to release pollen [33]. Thus the HrTF_ARF transcript may have a role in development of anthers in male flowers of seabuckthorn

#### 4.3.2.4 Auxin/Indoleacetic acid (AUX/IAA) transcription factor family

A*ux/IAA*s are auxin response genes that code for nuclear localized proteins [34]. It influence apical dominance, vascular development, tropic movements, root growth, tissue and organ patterning, and flower and fruit development [35]. The expression of HrTF_AUX/IAA was biased towards the male flowers. In fact, its expression level in the first two temporal developmental stages of female flowers was negligible. In MST I the expression level of AUX/IAA transcript was 1320 fold higher in comparison to corresponding female developmental stage (Table 4.4; Fig. 4.1). The difference in the expression levels between the male and female flower buds widens further in II$^{nd}$ stage where in 9680 fold higher expression was recorded in male flower buds compared to female buds (Table 4.4; Fig. 4.1). Such an outcome indicates an important role of AUX/IAA TF family in development of seabuckthorn male flowers.

**Table 4.3 Normalised expression of transcription factors within temporally corresponding male and female flower development stages.** The values listed in the table are average of the fold expression values calculated by normalising with two internal reference genes i.e. 26S and GAPDH.

| Transcription factors | FST I | MST I | FST II | MST II | FST III | MST III |
|---|---|---|---|---|---|---|
| ***HrTF_AP2*** | 18.02 | 0.11 | 4.8 | 0.68 | 1.36 | 1.36 |
| ***HrTF_ARF*** | 0.37 | 1 | 0.03 | 4.65 | 2.14 | 1.32 |
| ***HrTF_ARID*** | 0.43 | 1 | 0.008 | 2.08 | 1.83 | 1.64 |
| ***HrTF_AUX_IAA*** | 0.0005 | 0.66 | 0.0005 | 4.84 | 0.07 | 0.36 |
| ***HrTF_BZIP*** | 4.216 | 1 | 2.43 | 4.1 | 0.943 | 2.14 |
| ***HrTF_GRAS*** | 3.892 | 0.84 | 1.73 | 4.845 | 0.95 | 1.915 |
| ***HrTF_HOMEOBOX*** | 0.613 | 0.632 | 0.011 | 4.845 | 0.817 | 1.391 |
| ***HrTF_MYB*** | 1.892 | 0.327 | 1.84 | 4.845 | 0.763 | 1.701 |
| ***HrTF_NAC*** | 0.811 | 1 | 0.666 | 1.869 | 0.95 | 1.127 |
| ***HrTF_PHD*** | 3.315 | 0.697 | 3.73 | 4.845 | 1.525 | 1.927 |
| ***HrTF_SAP*** | 0.811 | 0.611 | 0.48 | 4.845 | 0.252 | 0.794 |
| ***HrTF_SRF_TF*** | 0.307 | 0.795 | 0.88 | 4.845 | 0.207 | 1.204 |

**Table 4.4 Relative fold expression of transcription factors within temporally corresponding male and female flower development stages.** Two internal reference genes are used i.e. 26S and GAPDH.

| Transcription factors | FST I | MST I | FST II | MST II | FST III | MST III |
|---|---|---|---|---|---|---|
| ***HrTF_AP2*** | **163.81** | 0.0006 | **7.05** | 0.142 | 1 | 1 |
| ***HrTF_ARF*** | 0.37 | **2.7** | 0.006 | **155** | 1.62 | 0.616 |
| ***HrTF_ARID*** | 0.43 | **2.32** | 0.003 | **260** | 1.11 | 0.89 |
| ***HrTF_AUX_IAA*** | 0.0007 | **1320** | 0.0001 | **9680** | 0.19 | **5.14** |
| ***HrTF_BZIP*** | **4.216** | 0.237 | 0.592 | 1.68 | 0.44 | **2.269** |
| ***HrTF_GRAS*** | **4.63** | 0.215 | 0.357 | **2.801** | 0.496 | **2.016** |
| ***HrTF_HOMEOBOX*** | 0.97 | 1.031 | 0.002 | **440.454** | 0.587 | 1.703 |
| ***HrTF_MYB*** | **5.78** | 0.173 | 0.38 | **2.633** | 0.448 | **2.22** |
| ***HrTF_NAC*** | 0.811 | 1.23 | 0.356 | **2.806** | 0.842 | 1.18 |
| ***HrTF_PHD*** | **5.351** | 0.21 | 0.769 | 1.298 | 0.791 | 1.263 |
| ***HrTF_SAP*** | 1.327 | 0.753 | 0.099 | **10.094** | 0.317 | **3.151** |
| ***HrTF_SRF_TF*** | 0.386 | **2.64** | 0.181 | **5.505** | 0.172 | **5.816** |

#### 4.3.2.5 Basic region/leucine Zipper motif (bZIP) transcription factor family

Basic region /leucine Zipper motif (BZIP) transcription factors have been known for their diverse role in different metabolic process in plant systems. It is involved in controlled regulation of genes implicated in physio-biological processes including, flower development, light and stress signaling, seed maturation, pathogen defense etc. [15]. In seabuckthorn the expression of HrTF_bZIP is stage specific and does not depend upon gender. The highest expression of this TF was recorded in FST I (4.216) and MST III (2.269) (Table 4.4; Fig. 4.2). bZIP proteins play role in gender determination in case of cucumber. Differentially expressed cDNA clone 138/B10 was obtained from the monoecious flowers which had similarity to *Arabidopsis* bZIP proteins [36].

#### 4.3.2.6 Homeobox transcription factor family

HOMEOBOX TFs family have been implicated in the shoot, embryo and flower development stages through control regulation of candidate genes like *PRESSED FLOWER* (*PRS*) in *Arabidopsis* and rice [37, 38]. The expression of HOMEOBOX TF was 440 fold higher with respect to corresponding stage of female flower buds (Table 4.4; Fig. 4.2). In apple *MDH1* a Homeobox protein is a probable candidate for plant fertility control [39]. *In situ hybridization* showed that MDH1 mRNA accumulated in ovules of the preanthesis flowers of the apple. To further verify its function the *MDH1* was transformed into *Arabidopsis thaliana* under the control of the cauliflower mosaic virus 35S promoter. The transgenic *Arabidopsis* plants showed dwarfing, reduced fertility and changes in carpel and fruit (silique) shape. The size and shape of the cells in the transgenic fruit was irregular, which showed the probable role of *MDH1* in plant fertility.

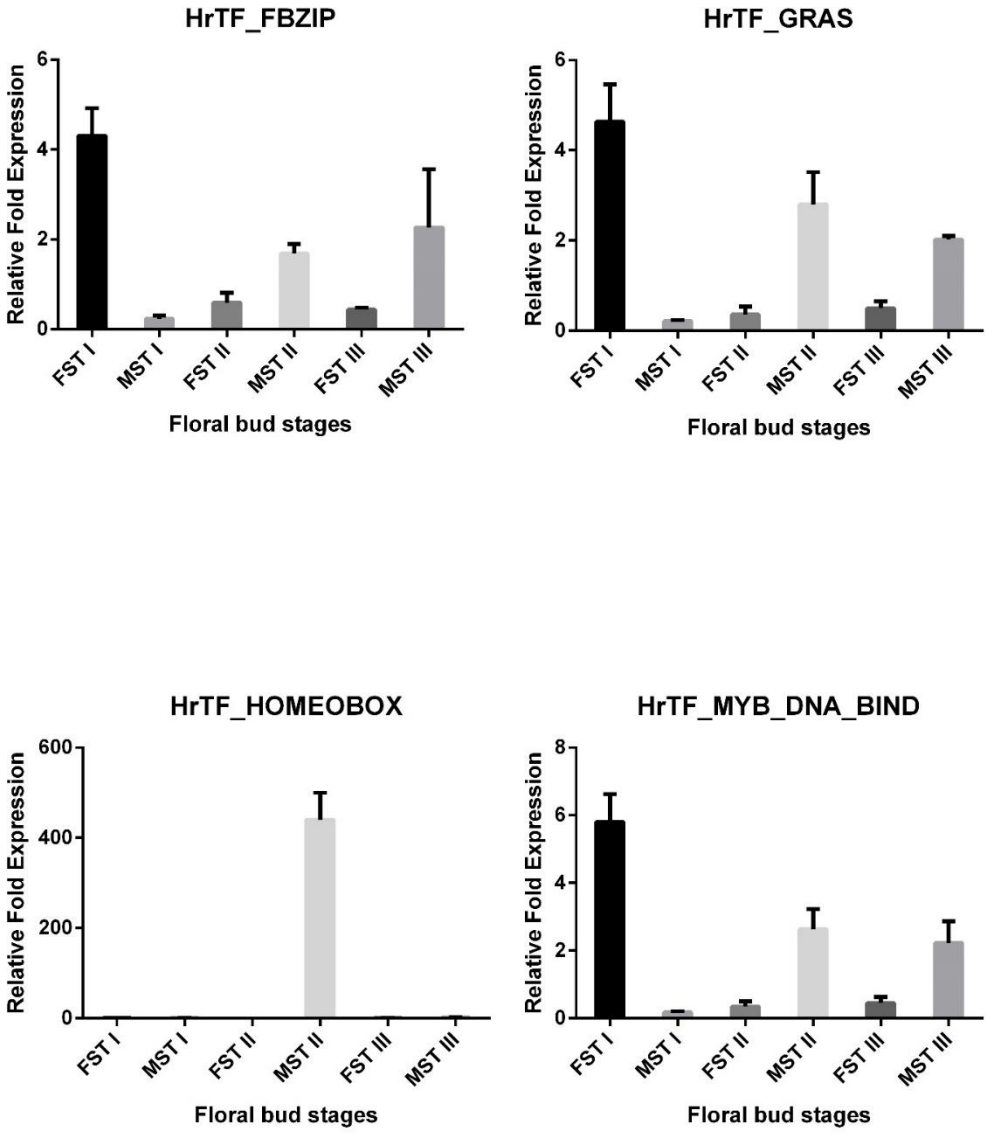


**Fig 4.2 Relative temporal expression of seabuckthorn BZIP,GRASS, HOMEOBOX and MYB_DNA_BINDING transcription factors.**

### 4.3.2.7 GIBBERELLIN-ACID INSENSITIVE [GAI] REPRESSOR OF GA1 [RGA] SCARECROW [SCR] (GRAS) transcription factor family

GRAS transcription factor family generally represented by three major proteins GAI, RGA and SCR in plant species. This family is known for its role in controlling the expression of candidate genes implicated in the fruits/flower developmental stages, signal transduction, auxin transport, stomatal development, etc. in *Arabidopsis thaliana* [40]. The expression of HrTF_GRAS was observed higher across all the developmental stages of the male flowers. MST I recorded 4.63 fold higher expression as compared to FST I while MST II recorded 2.801 fold expression with respect to FST II (Table 4.4; Fig. 4.2). Similar pattern of expression was observed in 3$^{rd}$ developmental stage as 2.016 fold expression in MST III as compared to FST III (Table 4.4; Fig. 4.2). Similar pattern of expression was observed in cucumber. The expression of *Cucumber Lateral Suppressor* (CLS) ,a putative transcription factor belonging to GRAS family, was recorded higher in male flowers and shoot tips [41].

### 4.3.2.8 MYB (myeloblastosis) transcription factor family

MYB TFs family is known for their diverse roles in plant systems, where, it controls the expression of key genes involved in the growth, flower development, phenylpropanoid biosynthetic pathway, seed dormancy, etc. [42]. As in the case of HrTF_GRAS, HrTF_MYB show male biased expression pattern. MST I showed 5.78 fold higher expression with respect to FST I (Table 4.4; Fig. 4.2). MST II and MST III recorded 2.633 and 2.220 fold expression as compared to FST II and FST III respectively (Table 4.4; Fig. 4.2). Similarly in Arabidopsis MYB TF family gene *DUO1* is responsible for controlling male gamete formation. *DUO1* is specifically expressed in the male germline, and DUO1 protein accumulates in sperm-cell nuclei. Mutations in *DUO1* produced a single larger diploid sperm cell unable to perform fertilization [43].

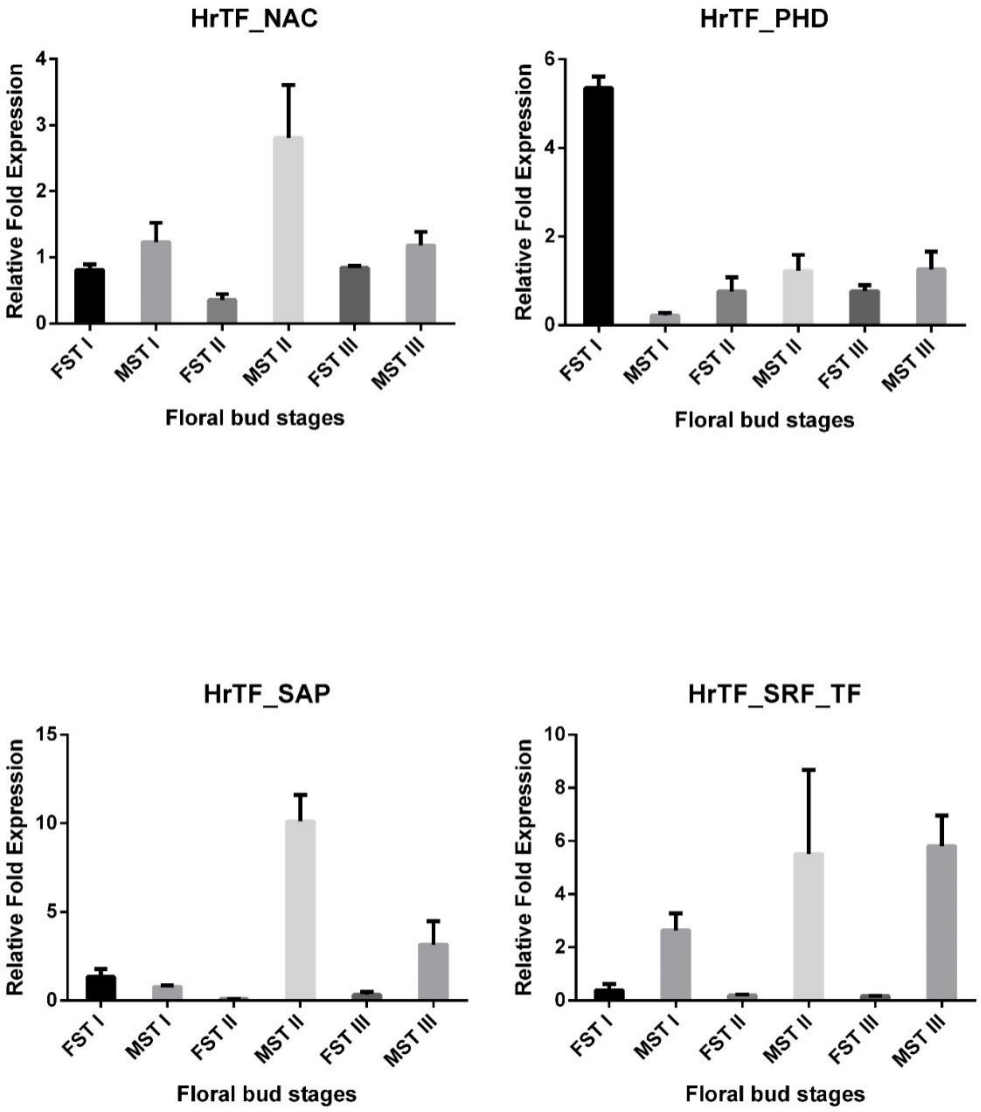


**Fig 4.3 Relative temporal expression of seabuckthorn NAC, PHD, SAP and SRF_TF transcription factors.**

#### 4.3.2.9 NAM, ATAF1/2, CUC2 (NAC) transcription factor family

The plant-specific NAC (NAM, ATAF1/2, CUC2) transcription factors play regulatory roles in diverse developmental processes and stress responses [44]. The differential expression of HrTF_NAC was detected in 2nd stage of male flowers with quantum of expression being 2.806 fold as in corresponding stage of female flower (Table 4.4; Fig. 4.3). The expression pattern of the above mentioned TF was similar in 1st and 3rd development stage in case of male and female flowers. NAC family member *SlCUC* is a strong candidate of sex determination in *S. latifolia*. Comparative *in situ* hybridization experiments between male, female, and hermaphrodite individuals reveal that *SlCUC* show a male-specific pattern of expression before any morphological difference become apparent [45].

#### 4.3.2.10 Plant Homeo Domain (PHD) transcription factor family

First developmental stage of male flowers i.e. MST I showed 5.351 fold expression of HrTF_PHD as compared to female flowers (Table 4.4; Fig. 4.3). PHD transcript exhibited stage specific expression rather than sex biased one. *MALE STERILITY1* (*MS1*) is a gene that encodes a nuclear protein with Leu zipper–like and PHD-finger motifs and has an important role in postmeiotic pollen development in *A. thaliana*. The Leu zipper–like region and the PHD motif are required for the MS1 function. Phenotypic analysis of the *ms1* mutant and the *MS1-SRDX* transgenic *Arabidopsis* indicated that *MS1* was involved in formation of pollen exine and pollen cytosolic components as well as tapetum development [46].

#### 4.3.2.11 STERILE APETALA (SAP) transcription factor family

HrTF_SAP expressed significantly in MST II (10.094**)** and MST III (3.151) with respect to their corresponding female development stages (Table 4.4; Fig. 4.3). The higher expression level of the SAP TF family in the male flowers point to their potential role in male flower development of seabuckthorn.

#### 4.3.2.12 Serum Response Factor (SRF) transcription factor family

The members of the SRF transcription factor family play an important role in flower development [47]. The expression of the HrTF_SRF was higher in all the male floral development stages. MST I, MST II and MST III recorded 2.64, 5.505 and 5.816 fold expression of HrTF_SRF against their corresponding female floral development stages (Table 4.4; Fig. 4.3). The consistent expression level of HrTF_SRF across all the male floral development stages indicate its potential role in male flower development.

### 4.3.3 Floral development stage (FDS) specific expression of transcription factors

In Floral Development Stage I of seabuckthorn higher female specific expression was observed in case of HrTF_AP2, HrTF_FBZIP, HrTF_GRAS, HrTF_MYB_DNA_BIND and HrTF_PHD while higher male specific expression was detected for HrTF_ARF, HrTF_ARID, HrTF_AUX_IAA and HrTF_SRF_TF (Fig. 4.4). Floral Development Stage II of seabuckthorn male floral buds have high gene expression of most of the TFs except HrTF_AP2, HrTF_FBZIP and HrTF_PHD. HrTF_AP2 had highest differential expression among other corresponding TFs in floral development stage II (Fig. 4.5). In Floral Development Stage III higher female specific expression of HrTF_ARF and HrTF_ARID was observed while expression of HrTF_FBZIP, HrTF_GRAS, HrTF_MYB_DNA_BIND, HrTF_AUX_IAA, HrTF_SRF_TF and HrTF_SAP was higher in male flower buds (Fig. 4.6). In all the male floral development stages HrTF_AUX_IAA and HrTF_SRF_TF had highest expression. While in case of female flowers HrTF_AP2 recorded significant expression in all floral developmental stages

Out of the three developmental stages, II$^{nd}$ stage had the maximum number of genes with expression biased towards male flowers (Fig. 4.4, Fig 4.5 & Fig. 4.6). The heat map of transcription factors (Fig. 4.7) also indicate that male flowers have maximum TFs with higher level of expression as compared to female flowers in II$^{nd}$ floral developmental stage. Thus stage II of male and female flowers require further investigation to justify the tilt of TFs expression towards male flowers.

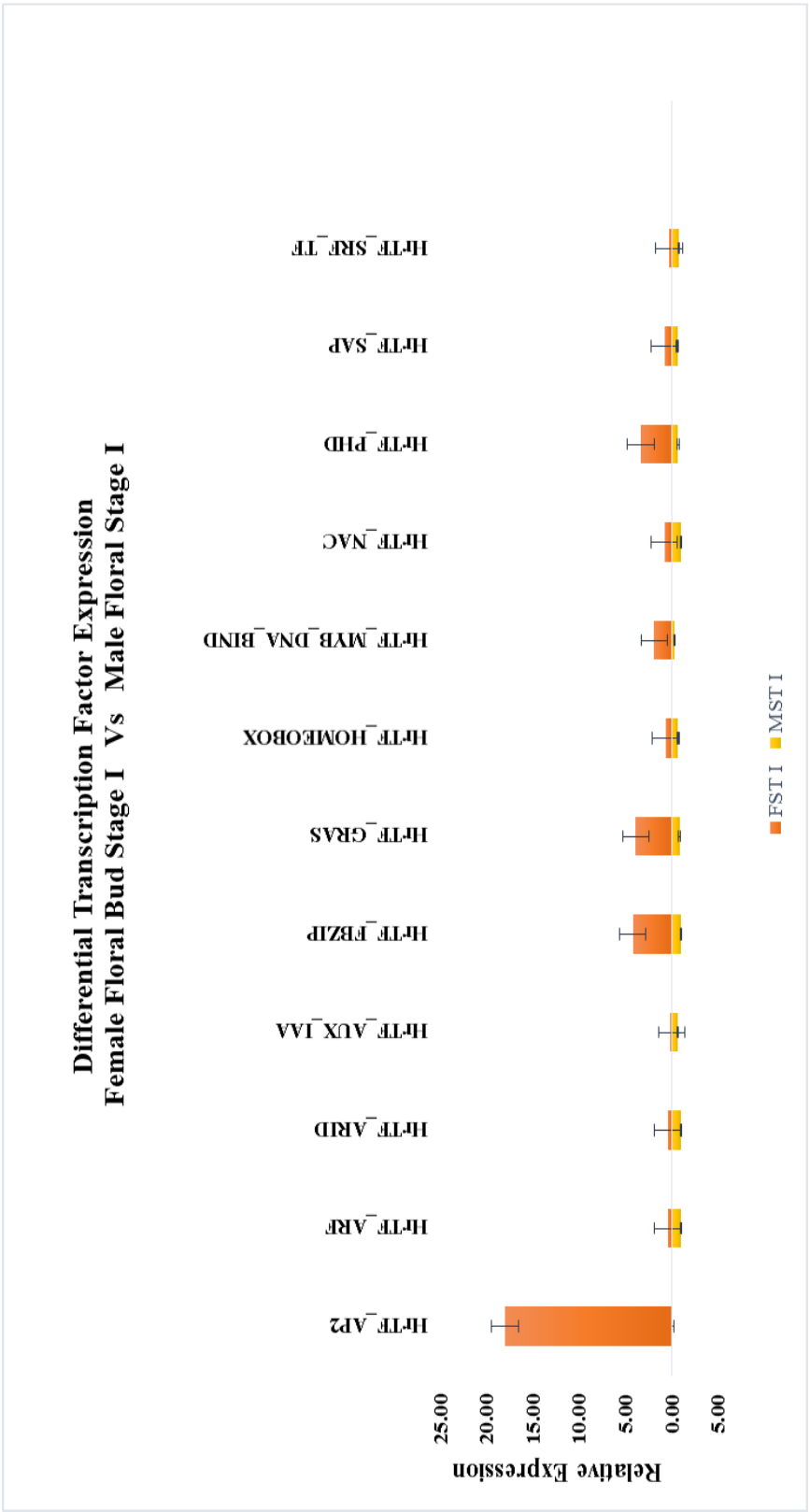


**Fig. 4.4 Comparative expression of seabuckthorn transcription factors between floral Development Stages (FDS) - Male Stage I (MST I) vs Female Stage I (FST I).**

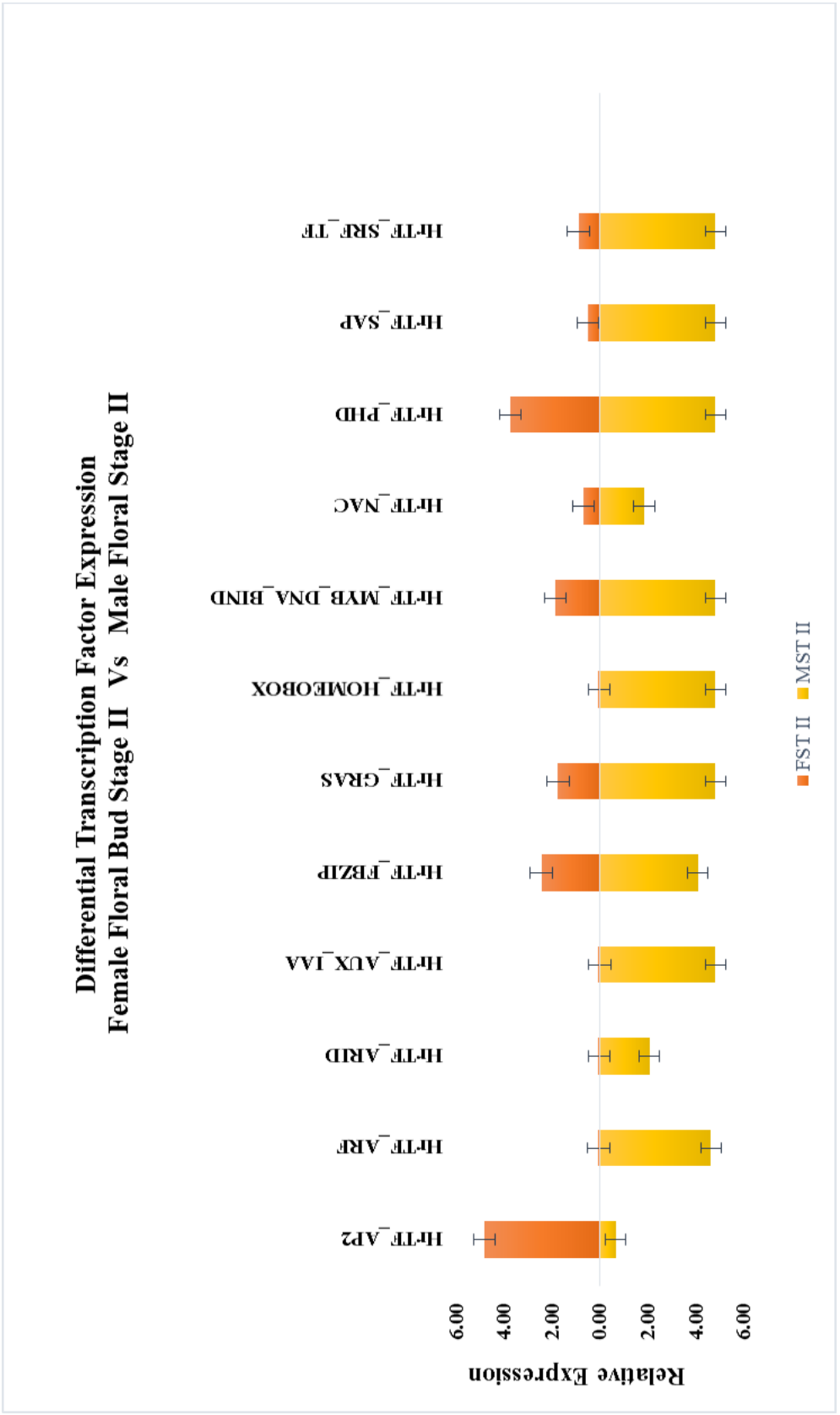


**Fig. 4.5 Comparative expression of seabuckthorn transcription factors between floral Development Stages (FDS) - Male Stage II (MST II) vs Female Stage II (FST II).**

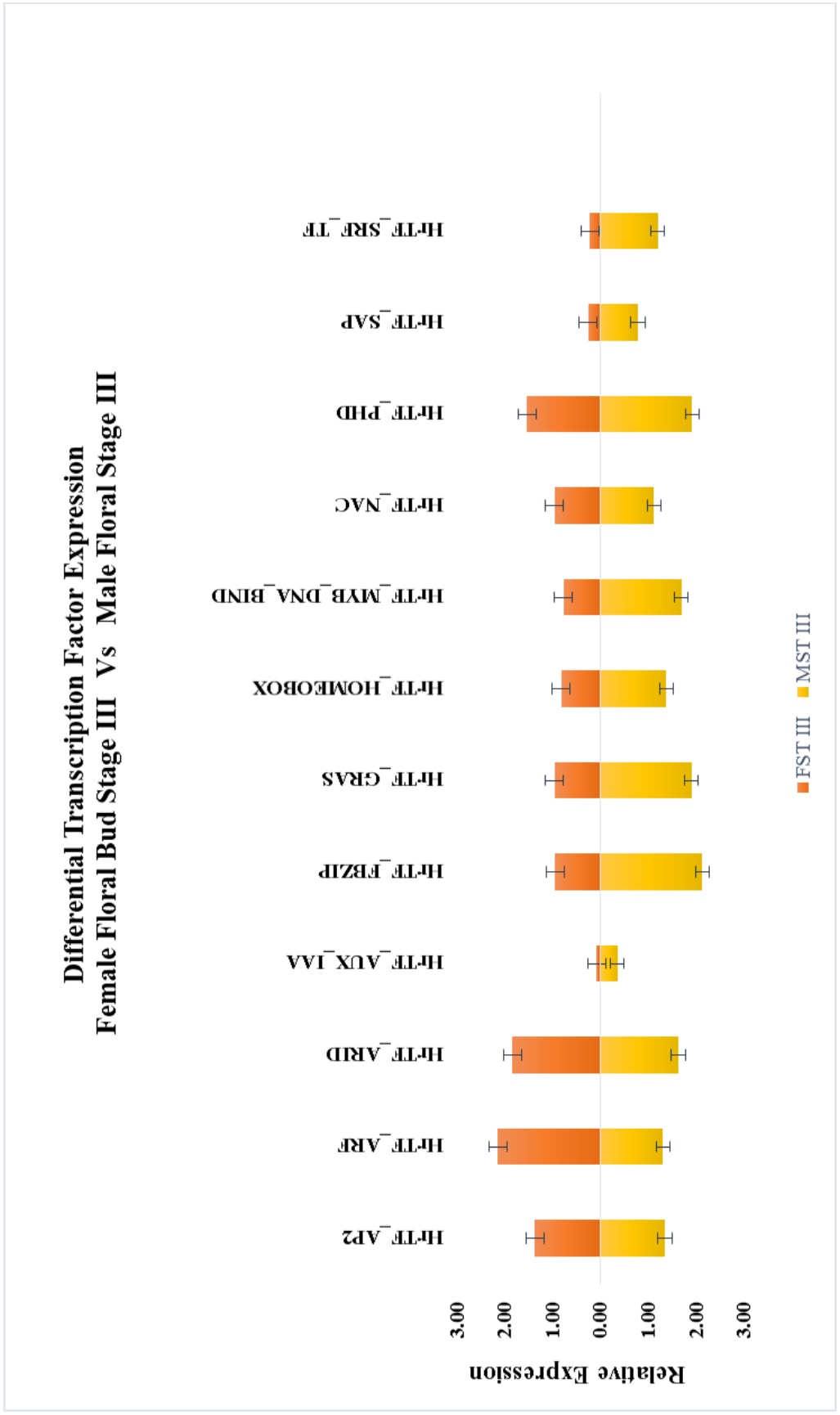


**Fig. 4.6 Comparative expression of seabuckthorn transcription factors between floral Development Stages (FDS) - Male Stage III (MST III) vs Female Stage III (FST III).**

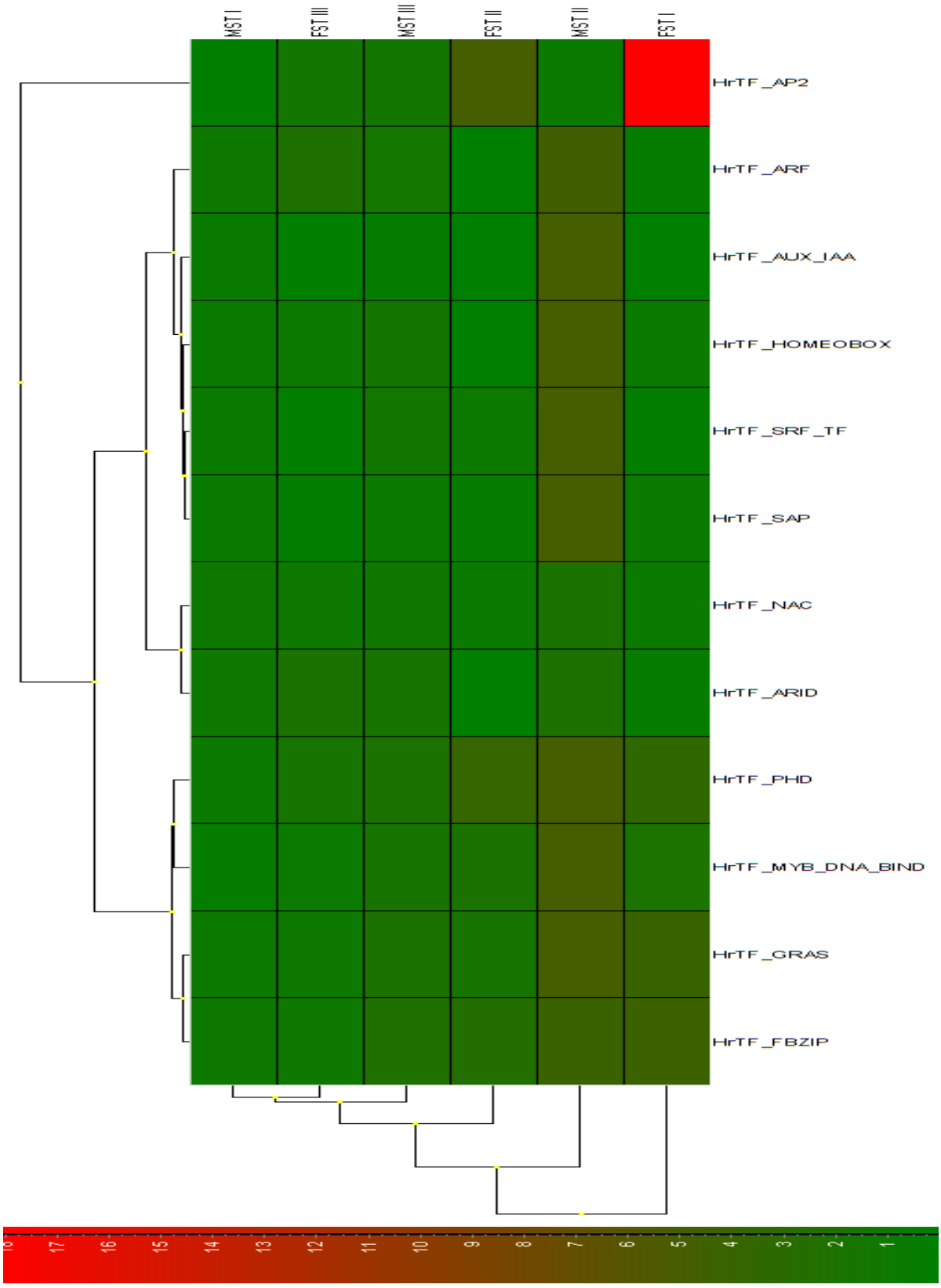


**Fig. 4.7 Heat map of relative temporal expression of transcription factors in seabuckthorn FDS.**

## 4.4 Conclusion

In conclusion, the current study showed differential expression of transcription factors in all the three floral developmental stages of both male and female flowers. The expression level of HrTF_AUX_IAA and HrTF_SRF transcription factors was observed higher in all the developmental stages of male flowers as compared to female flowers. Whereas HrTF_AP2 transcription factor significantly showed higher expression levels in the female floral developmental stages only. Further investigation is required to understand and validate the role of HrTF_AUX_IAA, HrTF_SRF and HrTF_AP2 in development of male and female flowers respectively.

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# CHAPTER 5

# SUMMARY

Seabuckthorn is a dioecious shrub which belongs to family Elaeagnaceae. The name seabuckthorn is derived from its characteristic of growing near the sea, and it possess many spines that are reminiscent of some buckthorn species of the genus *Rhamnus*[1]. The shrub has a huge potential for researchers in the field of biotechnology, neutraceutical, pharmaceutical, cosmetic and environmental sciences [2]. The female plants bears berries that are rich in vitamins, organic acids, fatty acids, flavonoids and antioxidants, thus provides various health related benefits [3].

Early sex determination of dioecious plants has commercial applications. Financial resources and valuable time can be saved if undesired male/ female plants can be discarded at an early stage of research trials and commercial plantation. Commercial seabuckthorn plantation require only 10% males for adequate pollination [4]. However, the earliest detection of male and female seabuckthorn plants is possible when flowering occurs, which is too late. This presents a serious inconvenience to plant breeders who have to retain large numbers of superfluous males for several years and leads to wastage of funds, labour, field space and valuable time in case of commercial plantations. The problem is more complicated in seabuckthorn which multiply vegetatively in the field through suckers leading to excessive male or females depending upon their initial proportion. We have noticed this problem in the Kelong area of Lahul and Spiti, India, where seabuckthorn plantation was done by the Forest department, few years back. Most of the plants turned out to be male and the entire area is presently dominated by unproductive males. Molecular marker based studies like RAPD, SSR, ISSR, SCAR etc. were conducted for past several years for gender identification in *Hippophae rhamnoides*[5-8]. To utilise the full potential of the seabuckthorn flora present in India, gender differentiating markers are required for all the three species existing in India.Thus in our current study the sex specific markers developed for *H. rhamnoides* were tested on the collected populations of male and female plants of *H. salicifolia* and *H. tibetana*. Female specific *HrX1* SCAR marker was able to distinguish female plants from male plants across the three species of seabuckthorn i.e. *H. rhamnoides*, *H. salicifolia* and *H. tibetana*[9].

Moreover it is the first sex specific SCAR marker in seabuckthorn which had shown homology with known plant genes like Acyl CoA synthatase (plant lipid biosynthetic genes).

Differences between male and female plants are mainly detected in reproductive organs, which occur through differential growth, repression or abortion of sex organs in unisexual flowers [10, 11]. The genes play role in development of the flower likemersitem identity genes, organ identity genes and flowering time genes could act as probable candidates for sex determination of dioecious plants. 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.

The genetic control of sex determination is well understood in several model plant systems like *Silene latifolia*[12-14] , *Cucumissativus*[15-17], *Salix*[18, 19], etc. Molecular and genetic studies have shown that the underlying mechanisms controlling flower development are largely conserved in distantly related dicotyledonous plant species [20]. Thus, genomic resources generated from these model plants could be used to identify the potential GISD in seabuckthorn. Different spatial and temporal development stages of flower have been used to decipher the mRNA transcripts involved in sex determination in dioecious plants like *S. latifolia*, *Rumexacetosa*, *Actinidiachinensis*, etc. [21-23]. Thus for identification of potential candidates for sex determination in seabuckthorn differential expression of known flowering genes as well as transcription factors was analysed using quantitative Real Time PCR (qRT-PCR) in three temporal Floral Development Stages (FDS) of both male and female seabuckthorn flowers.

Floral Development Stage Iof seabuckthorn recordedhigher female specific expressionfor *HrAP1*, *HrCRY2*, *HrNEF1* and *HrAG*. Whereas*HrAP2*, *HrLFY*, *HrFRI*and*HrGI*recorded higher expression levels in male flowers.At II$^{nd}$ floral development stage the expression level of most of the genes studied except *HrCRY2* and *HrLFY* was observed higher in male flowers as compared to female flowers.In Floral Development Stage III of seabuckthorn, higher female specific expression of *HrAP1*, *HrCRY2*, *HrEF1* and *HrFIL*was observed, while *HrCRY1*, *HrCO* and *HrPHYB* had male specific expression. *HrCO* showed consistent higher expression in all male floral

development stages.On the other hand *HrCRY2* recorded elevated expression levels in all the female floral development stages.

The morphological observation of the female sex organs i.e. ovary at FST III indicated that the ovary might have developed in stages between FST II and FST III. The expression level of the genes *HrAP1, HrCLV1, HrFIL, HrCRY2, HrGI, HrEF1* and *HrETR1* increased in FST III with respect to FST II. However the expression level of the gene *HrLFY* decreased at FST III with respect to FST II.In case of male floral buds the distinct male floral organs i.e. anthers were observed at MST II and which matured through MST III. Thus the development of the stamens started in between MST I and MST II while stamens matured through MST II and MST III. The expression of the genes *HrAP2, HrCLV1, HrAG, HrSEP3, HrYAB5, HrCRY1, HrPHYB, HrCO, HrCOLK, HrFRI, HrFRILK, HrEF1, HrERS1, HrETR1, HrX1* and *HrNEF1* increased in MST II with respect to MST I. But the expression of *HrLFY* decreased in MST II with respect to MST I. During the maturation of male floral bud from MST II to MST III the expression of *HrCO* only increased while the rest of the above mentioned genes had reduced expression levels in MST III with respect to MST II.

In Floral Development Stage Iof seabuckthorn higher female specific expression of Transcription factorswas observed in case of *HrTF_AP2, HrTF_FBZIP, HrTF_GRAS, HrTF_MYB_DNA_BIND* and *HrTF_PHD*while higher male specific expressionwas detected for *HrTF_ARF, HrTF_ARID, HrTF_AUX_IAA* and *HrTF_SRF_TF*. The male Floral Development Stage II recorded higher expression levels of maximum TFs under study except *HrTF_AP2, HrTF_FBZIP* and *HrTF_PHD.* In Floral Development Stage III of seabuckthorn higher female biased expression was showed by *HrTF_ARF*and*HrTF_ARID*while *HrTF_FBZIP, HrTF_GRAS, HrTF_MYB_DNA_BIND, HrTF_AUX_IAA, HrTF_SRF_TF* and *HrTF_SAP*recorded male biased expression. *HrTF_AUX_IAA* and *HrTF_SRF_TF*recorded consistent higher expression in all the male floral development stages.Out of all the TFs under study *HrTF_AP2*expressed consistently across all the female development stages.

## Conclusion

- Female specific SCAR marker ***HrX1*** is able to differentiate female plants from male plants in three species of seabuckthorn, namely, *H. rhamnoides, H. salicifolia* and *H. tibetana.* Applicability of this single marker (***HrX1***) in all the three species has circumvented the need for de-novo development of sex linked markers in *H. salicifolia* and *H. tibetana*, thus saving both the time and resources. It is the first report in seabuckthorn that sequence of sex linked marker has shown homology with known plant gene, which needs further investigation for its potential role in sex determination. Thus as per the stated hypothsis the female specific SCAR marker *HrX1* developed for *H. rhamnoides* were able differentiate gender in other two species of seabuckthorn i.e. *H. salicifolia* and *H. tibetana.*
- ***HrCO*** has shown consistent higher expression in male floral buds only. While **H*rCRY2*** was expressed throughout the development of female floral buds while its expression was very low in all the development stages of male flowers. The expression level of ***HrAP1, HrFIL, HrCRY2*** and ***HrGI*** increased only in female flowers during the development of female floral organs while the level of expression of ***HrAP2, HrAG, HrSEP3, HrYAB5, HrCRY1, HrPHYB, HrCO, HrCOLK, HrFRI, HrFRILK, HrERS1, HrX1*** and ***HrNEF1*** increased along with the development of male floral organs in male floral buds. In case of both male and female flowers the expression of ***HrLFY*** gene decreased as the sex organ development started.
- ***HrTF_AUX_IAA*** and ***HrTF_SRF_TF*** had consistent higher expression in male floral buds only. ***HrTF_AUX_IAA*** had consistent higher expression levels during the first two developmental stages of male flower development of male floral organs indicating its role in initial development of male floral organs**.** *HrTF_AP2* had consistent higher expression in female floral buds only. The expression of this TF increased in second and third stage of development.
- Out of the three development stages of male and female flowers of seabuckthorn, **MST II** recorded the highest expression levels of maximum GISD as well as TF transcripts under study.

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## Research articles under review

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**Chawla A**., Kant A., Stobdan T., Srivastava R.B. and Chauhan R.S. (2013) *Hippophae rhamnoides* SCAR marker *HrX1* genomic sequence. **Accession No. KF359497**

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# APPENDIX

**The following appendix files are present in CD-ROM attached at the back cover of the thesis**

**Appendix I** - Nucleotide sequences of the known flowering pathway genes in model plants.

**Appendix II** - Nucleotide sequences of seabuckthorn putative GISD retrieved from seabuckthorn genomic resources

**Appendix III** - Protein sequences of seabuckthorn putative GISD

**Appendix IV** - Protein sequences of the known flowering pathway genes in model plants **.**