Functional analysis of carpel developmental genes in California poppy (*Eschscholzia californica*)

Funktionelle Analyse von Karpel Entwicklungsgenen im Kalifornischen Mohn (*Eschscholzia californica*)

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Summary

The main objective of my study is to understand the functional evolution of novel morphologies in correlation with changes in the molecular genetic mechanisms over a period of time. One can understand the source for the existence of a wide diversity of forms by uncovering the developmental processes behind it. The plant evolutionary developmental biology (evo-devo) emerged as a branch of study that aims at unraveling the molecular and genetic mechanisms responsible for the origin and diversification of plant morphologies during the process of evolution. Flowering plants or angiosperms are the most dominating terrestrial plant ecosystems and flowers are the reproductive structures responsible for their successful adaptation. The flower comprises of four different floral organs as sepals, petals, stamens, and carpels. Variations in these organs have contributed considerably to the diversification of angiosperms. Moreover, the origin and diversification of the female reproductive organ, the carpel, was a major contributor for the evolutionary success of flowering plants. Therefore, the functional analysis of carpel developmental genes in phylogenetic informative species is one way of deciphering plant development in an evolutionary context.

The molecular mechanisms governing carpel development have been studied intensively in the core eudicot model species *Arabidopsis thaliana* (Arabidopsis) and to some extent in the monocot model plant *Oryza sativa* (Rice). However, such studies are limited in other evolutionary lineages due to lack of genetically tractable model systems. To overcome this obstacle, the basal eudicot plant, *Eschscholzia californica* (California poppy) has been established as a versatile developmental model species based on its phylogenetic position and its amenability to genetic manipulation. Hence, the molecular genetics of carpel development in California poppy helps in bridging the evolutionary gap between monocots and higher eudicots.

AGAMOUS (AG) is one of the important carpel developmental genes involved in specifying stamen and carpel identity in *A. thaliana*. In *E. californica,* there are two *AG* homologs, *EScaAG1* and *EScaAG2* which exhibit high sequence similarity at both nucleotide and protein levels. However, expression analyses through real-time qRT-PCR have shown that *EScaAG2* is being expressed stronger in the inner stamen whorls and *EScaAG1* transcripts are more abundant in the central carpels. Furthermore, down regulation of *EScaAG1* through Virus-induced gene silencing (VIGS) resulted in the homeotic conversion of outer, peripheral whorls of stamens into

petals and VIGS-*EScaAG2* led to the homeotic transformation of inner and central whorls of stamens into petals. Additionally, functional analysis of both *EScaAG* genes through VIGS has resulted in the homeotic conversion of carpels into petal-like structures. According to the ABCE model of floral organ specification, petal identity requires the presence of the floral homeotic B function genes. The results of the present study have shown that the expression of a subset of B function genes extends into the central fourth whorl when the C function is reduced. This suggests a phenomenon of B function gene regulation by the floral homeotic C function gene *EScaAG2*, a new functional domain of C class genes that has not been uncovered in any other model species.

In the second project, *Agrobacterium tumefaciens* mediated stable genetic transformation was attempted for *E. californica* with a special emphasis on establishing a reproducible transgenic regeneration system. As a new source of explant tissue that was used as a starter culture, unripe seeds were selected and the protocol was optimized to produce embryogenic calli with efficient somatic embryogenesis and subsequent plant regeneration. The unripe seeds collected during a timeframe of 22-24 days after anthesis (DAA) proved to be suitable to induce callus production. Furthermore, the addition of sucrose in all the tissue culture growing media enhanced the efficiency of subsequent somatic embryogenesis, plantlet regeneration and root induction from the unripe seed sources.

Zusammenfassung

Eines der wichtigsten Themen der biologischen Forschung ist es, die Entstehung und Entwicklung neuer Morphologien zu verstehen, die auch durch Änderungen der zu Grunde liegenden genetischen Netzwerke über evolutionäre Zeiträume entstehen. Diese Entwicklungsprozesse aufzudecken ist ein Weg, sich der Vielfalt biologischer Formen wissenschaftlich zu nähern. Der Forschungszweig der evolutionären Entwicklungsbiologie (Evo-Devo) versucht dabei, die molekularen und genetischen Mechanismen zu entschlüsseln, die für die Entstehung und Diversifizierung der Pflanzenmorphologie während des Prozesses der Evolution verantwortlich sind.

Blütenpflanzen oder Angiospermen dominieren heute die Landökosysteme. Ihre Blüten sind reproduktive Strukturen und bestehen im Allgemeinen aus vier verschiedenen Organen, den Kelchblättern, Blütenblättern, Staubblättern und Fruchtblättern. Variationen in den Blütenstrukturen haben selbst viel zur Diversifizierung der Angiospermen beigetragen. Auch war die Entstehung und die Diversifizierung der Fruchtblätter und des daraus entstehenden Fruchtknotens ein wichtiger Faktor für den evolutionären Erfolg der Blütenpflanzen. Daher ist die funktionelle Analyse von Karpell-Entwicklungsgenen in phylogenetisch informativen Spezies ein vielversprechender Forschungsansatz, um die pflanzliche Entwicklung in einem evolutionären Kontext besser zu verstehen.

Die Fruchtblatt-Entwicklung wurde intensiv in der höheren eudikotylen Modellpflanze *Arabidopsis thaliana* und dem monokotylen Getreide *Oryza sativa* untersucht. Ähnlich intensive Studien sind in solchen evolutionären Linien, die zwischen den höheren eudikotylen und den monokotylen Arten vermitteln, auf Grund fehlender Modellsysteme begrenzt. Aus diesem Grund wurde der basale eudikotyle Kalifornische Mohn, *Eschscholzia californica,* aufgrund seiner phylogenetischen Position und der Möglichkeit genetischer Manipulationen als ein vielversprechender Modelorganismus für Studien zur Karpell-Entwicklung etabliert. Diese Studien können dazu beitragen, die evolutionäre Entwicklung besser zu verstehen, die zwischen monokotylen und höheren eudikotylen Pflanzen stattgefunden hat.

AGAMOUS (*AG*) ist das C-Klasse Gen, das die Identität der Staubblätter und Karpelle in *A. thaliana* bestimmt. In *E. californica* gibt es zwei *AG* Gene, *EScaAG1* und *EScaAG2,* welche

sowohl auf der Nukleotid-, als auch auf der Proteinebene eine hohe Ähnlichkeit aufweisen. Real-time qRT-PCR Experimente zeigen, dass *EScaAG2* stärker in den Staubblättern exprimiert wird, während Transkripte von *EScaAG1* stärker in den Karpellen nachweisbar sind. Der transiente knock-down von *EScaAG1* via virus-induced gene silencing (VIGS) resultiert in der homeotischen Transformation der äußeren Staubblattwirtel in Blütenblätter, der von *EScaAG2* in der Transformation der inneren Staubblattwirtel in Blütenblätter. Die funktionelle Analyse beider *EScaAG* Gene zusammen durch VIGS führt zu homeotischer Transformation aller Staubblätter in Blütenblätter, sowie der Karpelle in Blütenblatt-ähnliche Strukturen. Die Blütenblatt Identität erfordert dabei das Vorhandensein der floralen homeotischen B-Klasse Gene. Die dargestellten Ergebnisse zeigen, dass die Expression eines Teils der B Funktion in den zentralen Wirtel hinein erweitert wird, wenn die Expression der C-Funktion reduziert wird. Diese Ergebnisse lassen eine funktionelle Domäne der C-Klasse Gene in der Regulation von B-Klasse Genen erkennen, die bisher noch nicht bei anderen Modellorganismen entdeckt wurde.

In einem weiteren Projekt wurde mit Hilfe der *Agrobacterium tumefaciens*-vermittelten stabilen genetischen Transformation ein weniger transienter Ansatz zur Erstellung transgener Pflanzen, mit einem Schwerpunkt auf die Optimierung des Regenerationssystems, etabliert. Hierbei wurden unreife Samen als die optimalen Explantate etabliert und dahingehend optimiert, embryogene Kalli mit effizienter somatischer Embryogenese und Regeneration zu produzieren. Die unreifen Samen, welche während eines Zeitraums von 22- 24 DAA gesammelt wurden, zeigten sich als optimal geeignet, eine ausreichende Kallus Produktion zu induzieren. Die Zugabe von Saccharose in alle Wachstumsmedien der Gewebekulturen verbesserte weiterhin die Effizienz der somatischen Embryogenese, die Plantlet-Regeneration sowie die Wurzelinduktion aus den Explantaten unreifer Samen.

1 Introduction

1.1 Evolutionary developmental biology of angiosperm flower

Angiosperms or flowering plants are the most dominating seed plants on this planet and they show wide diversity of plant morphologies. On the other hand, they exhibit a group of common characteristics and flower is one of those conspicuous features and center of focus in the evolution of angiosperms (Baum and Hileman, 2006). Flowers are the reproductive structures of angiosperms. The flower is usually made up of four floral organs sepals, petals, stamens, and carpels, which are sculpted into a compact, whorled structure. Angiosperms display immense yet aesthetic floral diversity with these four floral organs either by showing alterations in the arrangement of floral organs, number, colour, size or symmetry. Moreover, the wide floral diversity was found to be rapid and present right from the evolution of angiosperms. This has fascinated the biologists to understand the gene regulatory networks (GRN) and developmental mechanisms that have evolved under different selection pressures to produce unique floral structures (Della Pina et al., 2014).

In that direction, there have been extensive studies carried out for the past two decades in several eudicot model species, mainly in the *Arabidopsis thaliana, Antirrhinum majus, Petunia hybrida,* and *Oryza sativa.* Based on these studies, the four floral organs are identified by the specification of four classes of homeotic genes, which act in a combinatorial fashion and control the development of the flower (Bowman et al., 1991; Coen and Meyerowitz, 1991). Even though the flower development was studied in detail in several eudicot model species and more specifically in *A. thaliana,* the famous saying of origin and sudden appearance of angiosperms by Charles Darwin (1879) as an 'abominable mystery' still remains as a mystery to a large extent even today (Crepet, 2000). This is partly because of two reasons: firstly, flower developmental studies were concentrated in few model species of highly evolved core eudicots and secondly, lack of fossil records to trace back the primitive structures of flower origin.

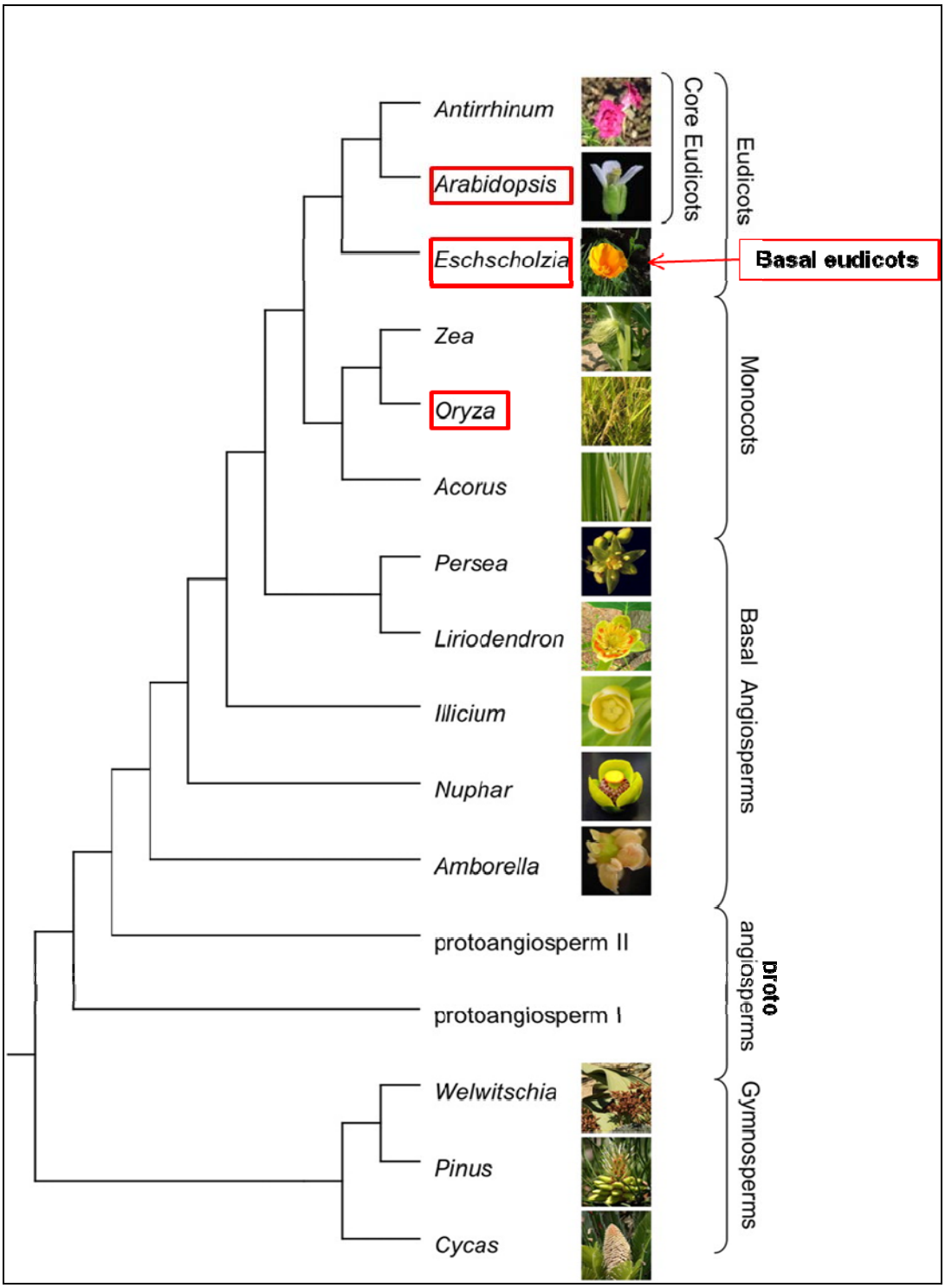
Antirrhinum
Arabidopsis
Eschscholzia
Zea
Oryza
Acorus
Persea
Liriodendron
Illicium
Nuphar
Amborella
protoangiosperm II
protoangiosperm I
Welwitschia
Pinus
Cycas
Core Eudicots
Eudicots
Basal eudicots
Monocots
Basal Angiosperms
angiosperms
Gymnosperms

Fig.1: The molecular phylogenetic tree of seed plants

The phylogenetic tree showing the relationship between the gymnosperms and other angiosperm plant lineages. The highly evolved core eudicots model species *A. thaliana,* a basal eudicot model species *E. californica* and a monocots model species *O. sativa* were highlighted in the red box. Besides that, basal angiosperms have shown as a sister lineage to all other highly evolved angiosperms and gymnosperms are the sister clade to angiosperms (Chanderbali et al., 2009).

Next generation sequencing has become a great tool by providing whole genome sequence for several model species. Based on the sequencing data, well refined molecular phylogenetic trees were developed to analyse the relationships among various genes and plant lineages (Mathews and Donoghue, 1999; Nickrent et al., 2000; Qiu et al., 1999; Soltis et al., 1999; Yang and Rannala, 2012). According to modern phylogeny, gymnosperms are the extant seed bearing plants and closest relatives of angiosperms (Fig.1) (Doyle, 1998). They bear reproductive organs as cone structures, consisting of microsporophylls as male reproductive structures and megasporophylls as female reproductive structure and there is no compact whorled flower architecture (Gernandt et al., 2011). Based on these structural differences between cones and flowers, comparative genetic studies between angiosperms and gymnosperms were hindered. Furthermore, there is no much evidence to support the idea of origin of flowers from the cone structures (Bateman et al., 2006).

Subsequently, plant evolutionary developmental biology (evo-devo) has emerged as a branch of study to analyse the molecular basis of genetic mechanisms that could cause an effective phenotypic variation in floral forms during evolution. The sequencing data coupled with comparative genomics has provided a platform to understand the sequence of developmental events that can lead to the expansion of novel floral forms during evolution.

In general, the genes and their encoding proteins control the development of an organism; however, the dynamics of morphological variation is not reflected in the sequence of the gene. Based on the trait homology studies among various phylogenetically informative species, several phenomena at the level of gene regulation were revealed. One of the scenario is, large sets of developmental regulatory genes act as transcription factors (TFs) in the plant kingdom and the number of transcription factor families is conserved among the land plants. On the other hand,

the number of transcription factors per family has increased drastically from mosses (10) to angiosperms (20-25). The enlargement of TF gene families is most frequently related to genome duplication events (Carroll, 2001; Dias et al., 2003; Hsia and McGinnis, 2003). Furthermore, the plant genomes are very large and main reason being the whole genome duplications (WGD). In plants, gene and genome duplications are the major factors contributed for the evolution of novel forms. Indeed it is widely accepted that one WGD has occurred in the common ancestors of all seed plants (gymnosperms and angiosperms) and one more in the ancestors of flowering plants (Jiao et al., 2011) and an additional duplication event occurred in the basal eudicots after divergence of Ranunculales from core eudicots (Cui et al., 2006). Also, there were several independent WGDs identified in many plant lineages. This infers that several present day flowering plants (including Arabidopsis, soybean, poplar, maize) carry a diploid genome of at least six successive WGD events and they refer as paleopolyploids (Blanc and Wolfe, 2004b).

During WGD, a significant number of duplicated genes were deleted through a process known as 'fractionation' and some genes were retained non-randomly in the genome due to the action of differential selection pressures (Blanc and Wolfe, 2004a; Blanc and Wolfe, 2004b; Maere et al., 2005). The evolutionary fate of these duplicated genes through biased retention has been explained in two scenarios as ‘neo-functionalization and sub-functionalization’(Jiang et al., 2013). The duplicated gene copies or paralogous genes, which are involved in gene regulatory networks (GRN) provide robustness to the networks (GRN) and facilitate diversification at the molecular level during evolution. Usually duplicated genes never retained for longer time in the evolution and they usually lost after duplication (Force et al., 1999; Nowak et al., 1997; Wagner, 1999). However, gene copies that act as transcription factors are preferentially retained and lead to the development of new morphological forms (Liu et al., 2010). One of the good examples is the *SEPALLATA* (*SEP1-4*) genes, which are involved in various functions in different lineages and put forward a mechanism for floral diversification. *SEP* genes act redundantly to specify floral organ identity in *A. thaliana*, however, in monocots, they are involved in specifying inflorescence and floral organ identities by showing alterations in the expression pattern (Yockteng et al., 2013).

The number and expression profile of a TF for a given plant species reflect the unique characteristics of that species, as the organogenesis and developmental behaviour of a plant species depends on the differential expression pattern of TFs. (Lespinet et al., 2002). The

changes in the protein coding regions of TFs can cause modifications in their expression profile and further effects its downstream gene regulatory networks (Stern and Orgogozo, 2008). One prominent example is TCP gene that regulates floral symmetry across different species by showing alterations in the spatio-temporal expression patterns and downstream genetic interactions (Hileman, 2014). Also, mutations in the promoter regions of target genes are responsible for change in the expression patterns and bring about significant morphological diversity (Eyre-Walker, 2006; Kaufmann et al., 2005). Besides that, mutations in the *cis*-regulatory elements of developmental genes can also cause changes in the protein-protein interactions and leads to the change of expression domain either by adding a new expression domain or restricting its expression to certain locations (de Bruijn et al., 2012). The gain of expression domain is a rare case in animal systems and change in the spatio-temporal expression is a common pattern; while in plants due to gene duplications, gain of expression pattern is the main source of variation.

Even though candidate gene approach is one of the ways to study the trait homology in a number of phylogenetically informative species to reveal sources and mechanisms of morphological variation, it has its own limitations in non-model plant species that are recalcitrant to genetic modification. However, this can be conquered to some extent through virus inducing gene silencing (VIGS) (Becker et al., 2011).

1.2 Molecular genetics of flower development

1.2.1 Floral organ specification

The process of flower transition takes place in three successive phases: (i) transformation of shoot apical meristem (SAM) into inflorescence meristem (IM), (ii) conversion of inflorescence meristem to floral meristem (FM) and (iii) transformation of floral meristem into floral organs (Coen and Meyerowitz, 1991; Simpson and Dean, 2002). After a period of vegetative growth in the plant, a particular combination of endogenous and environmental signals converges and activates the expression of floral meristem identity genes *LEAFY (LFY)* and *APETALA1 (AP1)*. These genes execute the transition of small outgrowth of cells at the flanks of inflorescence meristem into a floral meristem in each individual flower by repressing the inflorescence meristem genes (Chandler, 2012; Irish and Sussex, 1990; Weigel et al., 1992). Afterwards, floral organ primordia are developed from a small group of floral organ founder cells in the uppermost

cell layer of the FM (Bossinger and Smyth, 1996). FM identity genes as a second function activate floral organ identity genes in the respective whorls.

1.2.2 ABCE model of the flower organ development

Floral organ identity genes are the master regulators that play distinct functions in flower development and were grouped into ABCE classes. Hence, the genetic basis of flower development is best explained through ABCE model of flowering (Coen and Meyerowitz, 1991; Theissen, 2001). The homeotic mutant studies in two model species *A. majus* and *A. thaliana* has formed the basis for establishment of the ABCE model of floral organ identity. The homeotic mutants with transformation of sepals into carpels in the first whorl and petals into stamens in the second whorl define the A-function (Fig.2). The mutants with homeotic conversion of petals into sepals in the second whorl and stamens into carpels in the third whorl define the B-function (Fig.2). Whereas the mutants consist of only perianth organs without any reproductive organs define the C-function (Fig.2) (Krizek and Fletcher, 2005). A- function consisting of two genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*) which specify the sepals in the first whorl, A (*AP1* & *AP2*) function combined with B class genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) specify the petals in the second whorl. While B function genes (*AP3* & *PI*) in combination with C class gene *AGAMOUS* (*AG*) specify the identity of stamens in the third whorl and C function gene (*AG*) alone specifies the carpels in the fourth whorl (Bowman et al., 1989; Coen and Meyerowitz, 1991). Thus, it has been postulated that ABC class genes act in a combinatorial fashion to specify four floral organs in four whorls. The other important feature of the ABC model is that A and C class genes act in a mutually antagonistic manner, as the C class genes oppose the expansion of A class genes into the 4th whorl and A class genes negatively regulate the expression of *AG* into the 1st whorl (Gustafson-Brown et al., 1994). Furthermore, floral organs are nothing but the modified leaves; however, the mutation in the three ABC class genes did not result in the transformation of floral organs into leaf-like structures. The four *SEPALLATA* genes (*SEP 1-4*) act redundantly as co-factors by involving in a higher order protein complex for organ specification in all the floral whorls. This has formed the basis for the extension of ABC model to ABCE model of flowering, as the *SEP* genes were grouped into E class (Lohmann and Weigel, 2002; Theissen and Saedler, 2001). The D function includes the genes required for ovule

formation and not involved in the floral organ specification (Pinyopich et al., 2003). According to the ABCDE model of flowering, class A + E genes form into a quaternary complex and specify the identity of sepals, A + B + E class genes specify the petals, B + C+ E complex specifies the stamens, C + E complex specifies the carpels, and D + E class proteins specify the ovules (Theissen, 2001). Floral homeotic genes determine not only the identity of floral organs but also regulate their differentiation (Bowman et al., 1989; Ito et al., 2007; Ó'Maoiléidigh et al., 2014; Wuest et al., 2012). The sepals are specified at the very onset of flower development (Causier et al., 2010; Kaufmann et al., 2010), and the petals are determined during intermediate stages of flower development (Wuest et al., 2012). Whereas, the stamens and carpels are specified immediately after the commencement of expression of B and C class genes. The carpels are the last organs formed in the central whorl of the flower and the FM is completely consumed in the process of gynoecium development, hence FM is determinate, unlike root meristem or shoot meristem, which are indeterminate (Fletcher, 2002).

Moreover, all the A, B, C, D and E-function genes are $MIKC^{C}$-type of MADS-box genes, with the exception of *AP2* that belongs to AP2/ERF family of transcription factors. The floral organ identity genes specify various floral organs by forming into multimeric higher order complex (Bowman et al., 1989; Coen and Meyerowitz, 1991).

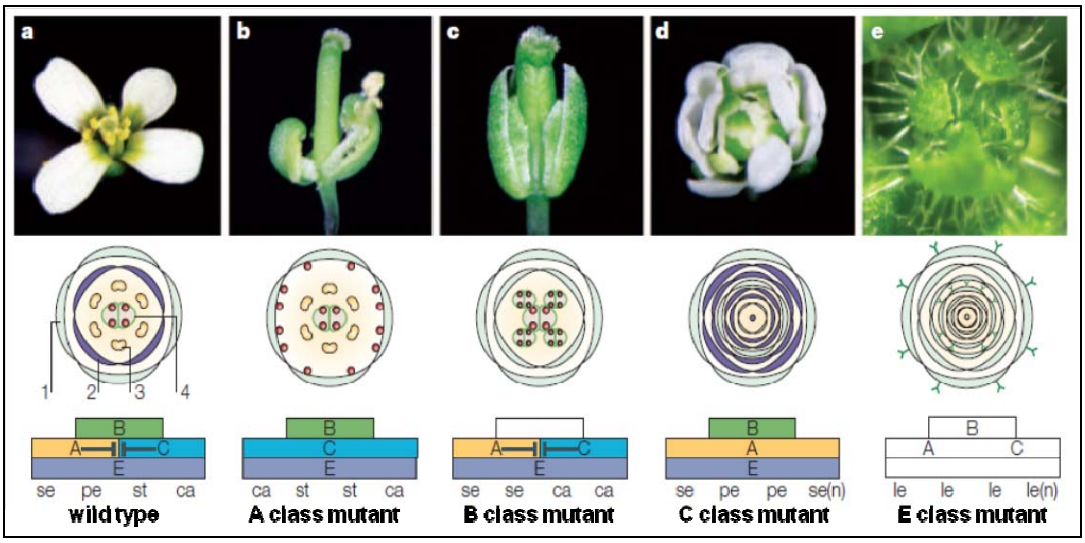

Fig.2: **Schematic representation of the ABCE model of flower development in *Arabidopsis thaliana* and homeotic transformations in the respective mutants**.

a) Wild type flower containing sepals, petals, stamens and carpels from outer to inner whorls. **b**) A class mutant (*ap2*) flower consisting of carpels in the first whorl, stamens in the second and third whorls, and carpels in the fourth whorl. **c**) B class mutant (*pi*) flower consists of sepals in the first and second whorls, carpels in the third and fourth whorls. **d**) C class mutant (*ag*) flower consists of sepals in the first whorl, petals in the second and third whorls, and reiterations of perianth organs in the interior whorls. **e**) E class mutant (*sep1 sep 2 sep3 sep4*) flower consists of whorls of leaf-like organs (Krizek and Fletcher, 2005).

1.3 MADS-box genes are the main players of flower development

The MADS-box transcription factors are widely spread throughout the eukaryotes and it is one of the best-studied gene families among the plants. They play significant roles in the morphogenesis of different plant organs and are involved in various processes extending from embryonic development, gametogenesis, root development and floral organogenesis (Smaczniak et al., 2012). The MADS box proteins are available to a lesser extent in protists, fungi, and other animals. About one to two MADS-box proteins were found to be present in algae, around 20 in mosses, and are greatly expanded to around 100 in flowering plants (Shore and Sharrocks, 1995; Theissen et al., 1996). Moreover, the expansion and diversification of MADS box genes play a crucial role in the evolution of flowering plants and many of them show conserved functions (Winter et al., 2002). The name MADS was derived from the first letters of the genes *MCM1* gene from *Saccharomyces cerevisiae*, *AGAMOUS* from *Arabidopsis thaliana*, *DEFICIENS* from *Antirrhinum majus* and *SERUM RESPONSE FACTOR* (*SRF*) from *Homo sapiens* in which this domain was first identified (Norman et al., 1988; Passmore et al., 1988; Schwarz-Sommer et al., 1990; Yanofsky et al., 1990).

There are two types of MADS-box proteins identified until now as Type I, and II, which were differentiated based on their structure, specificity, and ability to bind to the DNA. Type I MADS box proteins consist of a conserved 180 bp MADS domain and a variable region (De Bodt et al., 2003; Kofuji et al., 2003; Pařenicová et al., 2003). The Type I MADS- box genes are mainly involved in the female gametogenesis and seed development (reviewed by (Masiero et al., 2011)). Type II MADS proteins constitute a modular domain called MIKC that has been named

after its characteristic domain structure: MADS domain (M), intervening domain (I), keratin-like region (K), and C-terminal domain (C) (Theissen, 2001). All the MADS box proteins possess characteristic MADS domain at the N-terminal end which is involved in the dimerization of proteins by binding to the target DNA at the CArG-box (the consensus sequence (CC (A/T6) GG)) (Riechmann et al., 1996b). MADS box proteins never bind to CArG-box containing target genes until they form homo or heterodimers (Fig.3) (Huang et al., 1996; Riechmann et al., 1996a). Following the MADS box, a less conserved I- domain is present and which is involved in the formation of selective DNA binding dimers. Moreover, I- domain itself is very sufficient for the formation of DNA binding dimers along with MADS domain. K- domain is the second best conserved domain after MADS and is involved in the protein dimerization and C-terminal region is the most variable region of the MADS-box proteins entailed in transcriptional activation and in the formation of multimeric protein complexes (Becker and Theißen, 2003). The highly variable C-functional motif plays a significant role in determining functional specificity to the MADS box proteins (Krizek and Meyerowitz, 1996; Lamb and Irish, 2003; Riechmann et al., 1996b; Riechmann et al., 1996c).

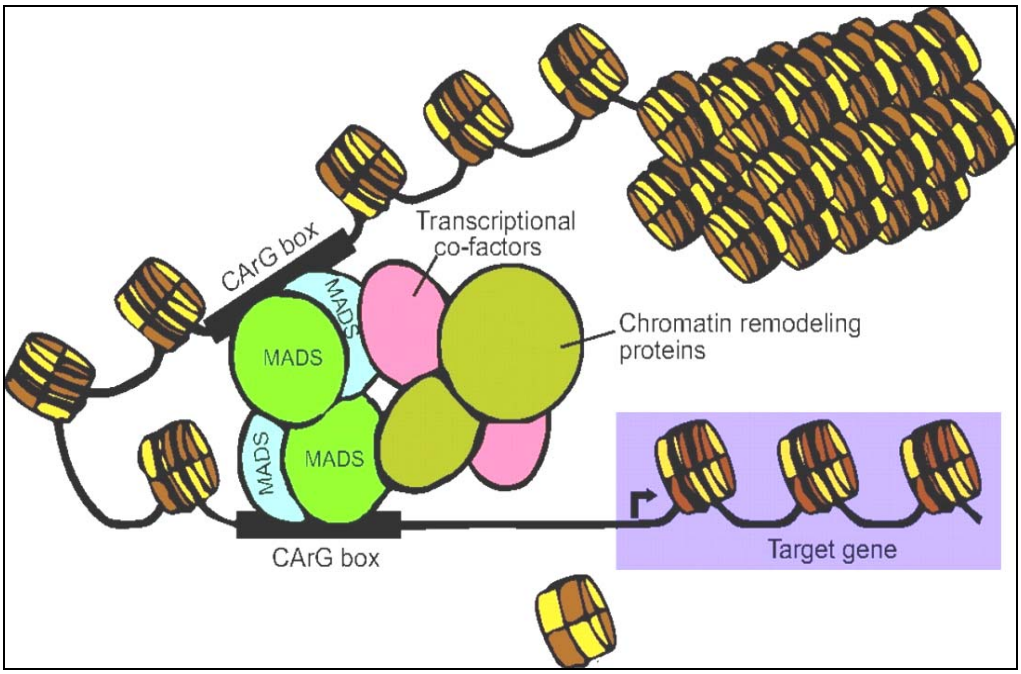

Fig.3: Schematic representation of MADS-box proteins in higher order complex formation.

As a first step, the MADS domain proteins (green and blue) form homo or hetero dimers and then they form higher order protein complex or quaternary complex as a second step. Subsequently, the complex binds to target gene at the CArG-box as a third step. Later on, MADS domain proteins recruit additional transcriptional co-factors (pink), which can mediate the transcriptional regulation and further influence the target gene specificity (Smaczniak et al., 2012).

The type II MADS-box genes exist in two forms as MIKC* and MIKC^c (Henschel et al., 2002). Genetic studies have revealed that the MIKC*-type genes control the development of male gametophytes (pollen) (Adamczyk and Fernandez, 2009; Verelst et al., 2007) and the MIKC^c genes are the master regulators of the floral organ identity (members of ABCE class genes excluding *AP2)* (Coen and Meyerowitz, 1991; Sommer et al., 1990; Theißen and Saedler, 2001). They are also involved in various other processes such as *AGL12* and *AGL17* subfamily members act in the root development (Han et al., 2008; Tapia-López et al., 2008), *OsMADS25* is involved in primary and lateral root development in rice (Yu et al., 2015). Whereas *AGL15* regulates the process of embryogenesis (Heck et al., 1995). Additionally, MADS-box transcription factors show pleotrofic functions. The *FRUITFULL* (*FUL*) gene is involved in more than one function such as carpel development and meristem identity and also involved in the regulation of fruit ripening (Airoldi and Davies, 2012; Fujisawa et al., 2013).

1.4 Origin and evolution of the carpel

The carpels are the female reproductive organs of angiosperms and are modified spore bearing leaves. Carpels often fused to form a gynoecium and enclose the ovules inside. Gynoecium is the most complex and multifunctional organ of the plant displaying high degree of morphological variability across the species. The basic structure of gynoecium consists of stigmatic tissue at the apex which facilitate pollination and pollen germination, a long style in the middle through which the pollen tube grows down towards ovules and a broad ovary at the base which encloses the placenta and ovules. The gynoecium attaches to the floral tube through a small stalk called gynophore.

The gynoecium also exhibits self-incompatibility mechanisms for the pollen and promotes out-breeding in many plant species. After pollination, the directional growth of compatible pollen tubes ensures the fertilization of ovules, which subsequently transform into seeds, and the ovary transforms into a fruit. Overall, the gynoecium protects the seeds and aids in their dispersal by employing different mechanisms in different species. Based on these advantages, the gynoecium has proven to be a major factor in the evolutionary success of angiosperms (Scutt et al., 2006) and has become a well-suited model system for the investigation of plant development during evolution.

The origin and evolution of carpels can be best understood through the comparative genetic studies in different phylogenetically informative model plant species. Based on the molecular phylogenetic data, extant gymnosperms are the closest relatives to angiosperms (Fig1). They consist of male and the female reproductive organs on separate branches or even on separate plants. The female reproductive structures called as megasporophylls are similar to carpels in angiosperms. However, megasporophylls bear the ovules naked and which is in contrast to the carpels that enclose the ovules inside and are well protected. Nevertheless, *AG* is the floral organ identity gene specifying stamen and carpel identity in angiosperms and its orthologue *CyAG* was found to be regulating the reproductive organ identity in the gymnosperms (Zhang et al., 2004). This infers that C class genes have originated before the divergence of angiosperms and gymnosperms, and its function is well conserved during evolution.

On the other hand, basal angiosperms constitute the sister group to all other angiosperm lineages and which includes three major plant groups as Amborellales, Nymphaeales, and Austrobaileyales (ANITA group) (Fig.1). The flowers of which are usually small, bisexual, and protogynous. Carpels are the simple structures (apocarpic) and are incompletely closed by substances secreted from the carpel margins. The stigma consists of multicellular protrusions and is secretory. Carpels enclose single ovules in an anatropous placentation and ovules are covered by two integuments and possess a large (crassinucellar) nucellus. The female gametophyte is four-celled/ four nucleate structure (Williams and Friedman, 2004). Double fertilization produces an embryo and a biparental diploid endosperm (Williams and Friedman, 2002).

The genetic analysis of carpel development in basal angiosperms shows that C class genes are expressed in the third and fourth floral whorls but in much broader domains and E- function genes are expressed in all the floral organs (Scutt et al., 2006). Additionally, a putative

orthologue of *CRABSCLAW* (*CRC)*, an another important gene involved in carpel development is also present in basal angiosperms and shows similar expression pattern in the carpels as in the *A. thaliana* (Fourquin et al., 2005).

In contrast, monocots are one of the major distinctive monophyletic group of angiosperms consisting of grasses, orchids and several economically important plant species and whose lineage was diverged from basal angiosperms at around 145 MYA (Scutt et al., 2006). In monocots, the flowers of grasses are highly derived and floral organ identity genes have been predominantly studied in two grass model species, rice, and maize, both belonging to Poaceae or grass family (Goto et al., 2001; Schmidt and Ambrose, 1998). Grasses contain unique flower structure that constitute stamens and carpels but lack obvious sepals and petals, instead, special structures called lemma and palea are present in the place of sepals; lodicules are present in the place of petals. The grass carpel comprises stigma, style, and an ovary with a single ovule. The transmitting track and septum are absent (Yamaguchi et al., 2004). In two grass model species, there are two C class genes due to gene duplication events and the two paralogues had shown clear subfunctionalization. Additionally, *CRC* orthologue *DROOPINGLEAF* (*DL*) in rice is involved in specifying carpel identity, floral meristem determinacy and midrib formation (Yamaguchi et al., 2004).

Besides, core eudicots are the highly evolved monophyletic group in the angiosperm lineage containing several well-studied model species such as *A. thaliana, A. majus* and *P. hybrida*. The core eudicot plants consist of highly developed, well structured gynoecium and the molecular genetics of its development is well studied in several model species, albeit most thoroughly in *A thaliana*.

1.4.1 Morphogenesis of the *Arabidopsis thaliana* carpels

The gynoecium of *A. thaliana* consists of different organs and tissues which are organized into a complex structure in order to maintain the reproductive competence (Larsson et al., 2013). The complex structure of gynoecium comprises different spatial domains with apical-basal axis consisting of stigmatic tissue with single layer of papillary cells at the apex, followed by the short solid style containing the apical portion of the transmitting tract. Following the style, a

large and broad ovary is present and attaches to the flower base through a small stalk called gynophore (Fig.4a).

Organogenesis of complex gynoecium in *A. thaliana* starts as a small dome of carpel primordium in the center of the floral meristem at stage 6 (Smyth et al., 1990). The carpel primordium cells divide and grow into a short tube like structure at stage 6 and 7, followed by the elongation of the short tube in the apical basal axis. While elongating longitudinally, the two carpels fuse congenitally and develop into a syncarpous gynoecium. Furthermore, the medial regions of the carpels grow inwards until they merge and form the medial domain. During this process, the gynoecium is differentiated into various regional domains such as apical *vs.* basal domains, medial *vs.* lateral and abaxial *vs.* adaxial (Larsson et al., 2013). The ovary walls are composed of two lateral valves, which represent the major portion of the ovary chamber (Balanza et al., 2006). Between the valves and replum, a specialized tissue called the valve margin develops (4b), which assist in releasing the seeds after fertilization (Ferrándiz, 2002). The valves and valve margins represent the lateral domains of the gynoecium. The medial domain contributes to the formation of septum, replum, placenta, ovules, transmitting tract, style, and stigma (Girin et al., 2009).

The adaxial-abaxial axes are illustrated as internal and external surfaces of the ovary with reference to the main stem (Fig.4b). The adaxial axis of the medial domain carries ovules, transmitting tract, placenta, and a false septum. The septum divides the ovary into two locules, encloses the basal part of transmitting tract and the placenta (4b). Furthermore, the adaxial surface of the fused carpels at medial position shows meristamatic activity termed as carpel marginal meristem (CMM) and each gynoecium contains two CMMs (Azhakanandam et al., 2008; Scofield et al., 2007). CMM is responsible for producing placenta, ovules, transmitting tract, style, and stigma (Wynn et al., 2011). In contrast, the abaxial axis of the medial domain encloses the replum, which is the abaxial surface of the septum (Fig 4b).

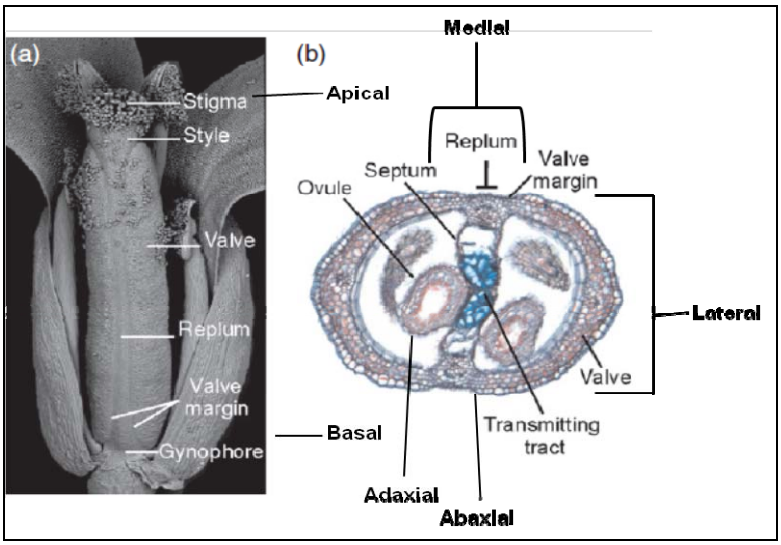

Fig.4: Depiction of *Arabidopsis thaliana* flower at anthesis stage.

a) Longitudinal view of gynoecium with apical- basal axis patterning

b) Cross section of gynoecium showing marginal tissues (Larsson et al., 2013).

1.4.2 Molecular genetics of carpel development in *A. thaliana*

In flowering plants, the core purpose of gynoecium is successful reproduction of enclosed ovules and dispersal of seeds after fertilization. A proper initiation of carpel primordia and correct patterning of the gynoecium are a prerequisite for accomplishing those functions. Moreover, the initiation and differentiation of gynoecium development is a complex process under strict genetic control. The complex gene regulatory networks ensure the proper identification of carpel primordia and differentiation of mature gynoecium along the apical-basal, medio-lateral, and abaxial-adaxial domains (Balanza et al., 2006). Although there are several genes involved in the domain specific organogenesis of the gynoecium, the contribution of each gene is limited. Indeed, many of the genes show redundant functions and at the same time, some genes function in overlapping domains. Nevertheless, their collective activity is critical for the development of competent gynoecium (Alvarez and Smyth, 1999; Azhakanandam et al., 2008; Nahar et al., 2012).

For the simplicity of understanding, the genes and genetic interactions of gynoecium development are illustrated based on the chronological sequence of events as the specification of carpel identity, the spatial distribution of regional domains followed by specialization of cells and tissues to establish a mature gynoecium.

1.4.2.1 Initiation of carpel primordia

In *A. thaliana,* FM identity genes *LFY* and *AP1* are being expressed uniformly in the young floral primordia and activate different floral-organ identity genes in distinct whorls. It has been shown that *LFY* induces the *AP1* expression and *AP1* is expressed throughout the floral primordia shortly after the *LFY* activation (Lohmann and Weigel, 2002). Later on *LFY* together with meristem identity gene *WUSCHEL* (*WUS*) activates the *AG* in the inner two whorls by directly binding to its second intron (Lenhard et al., 2001; Lohmann et al., 2001). However, the expression of *AG* is restricted to only central primordium with the contribution of *WUSCHEL* (*WUS*) (Lohmann et al., 2001). The gene, *PERIANTHEA (PAN)* regulates the expression of *AG* in a whorl specific pattern (Das et al., 2009). *LFY, PAN,* and *WUS* bind directly to the *AG* at its second intron and activate the *AG* expression in the fourth whorl (Das et al., 2009; Lenhard et al., 2001; Weigel and Nilsson, 1995). Moreover, *LFY* and *PAN* are functionally similar in the activation of *AG.* In contrast, *SEUSS* (*SEU*) encodes a transcription adaptor protein and interacts physically with various MADS-box proteins *AP1, SEP3, AGAMOUS-LIKE (AGL24),* and *SHORT VEGETATIVEPHASE* (*SVP). SEU* binds to the MADS-box protein complex through a bridge protein called *LEUNIG (LUG)* and accomplishes the repression of *AG* expression in the 1st and 2nd whorls (Sridhar et al., 2004; Sridhar et al., 2006).

1.4.2.2 Specification of carpel organ identity

AG is the C class gene involved in specifying stamen and carpel identity and is also involved in meristem termination (Bowman et al., 1991; Bowman et al., 1989). The flowers of the strong *ag-1* mutant show complete homeotic conversions of stamens into petals, carpels into sepals and recurrence of these perianth organs in an irregular phyllotaxy and it is termed as floral meristem indeterminacy (Bowman et al., 1989). Floral meristem determinacy (FMD) appears to be a

crucial step for correct patterning of the gynoecium, as the development of medial tissues are impaired in indeterminate flowers (Zúñiga-Mayo et al., 2012). *WUS* maintains stem cell fate in the FM and down-regulation of *WUS* is required to maintain the FMD (Mayer et al., 1998; Schoof et al., 2000). The floral meristem identity gene *LFY* interacts with *WUS* and activates the *AG* in the central whorls during initial stages of flower development and in turn after carpel primordia initiation (at stage 6) *AG* inactivates the *WUS* and terminates the floral meristem activity. In this pathway, *AG* interacts with a zinc finger protein called *KNUCKLES (KNU)* to repress *WUS*. Additionally, *CRABS CLAW (CRC), REBELOTE (RBL), SQUINT (SQN), ULTRAPETALA (ULT)* and *PERIANTHIA (PAN)* are involved in the regulatory loop formed by *AG, KNU* and *WUS* and control the floral meristem termination (Alvarez and Smyth, 1999; Carles et al., 2004; Das et al., 2009; Lenhard et al., 2001; Liu et al., 2011; Lohmann et al., 2001; Maier et al., 2009; Prunet et al., 2008; Sun et al., 2009).

Besides, *SHOOT MERISTEMLESS* (*STM*) is an another key factor responsible for the development and maintenance of meristems, as the loss of *stm* function causes the premature differentiation of meristematic cells. The BELL family member *REPLUMLESS (RPL)* [also called as *PENNYWISE (PNY), BELL RINGER (BLR), VAAMANA (VAN)*, *LARSON (LSN),* and *POUNDFOOLISH (PNF)]* interact with *STM* and promote the carpel formation through the positive regulation of *AG* (Bao et al., 2004; Bhatt et al., 2004; Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Yu et al., 2009). They function in parallel with *LFY* and *WUS* in the carpel development (Arnaud and Pautot, 2014). In addition to that, E class genes (*SEP 1-4*) act in combination with *AG* to specify the carpel identity in the 4th whorl (Ditta et al., 2004; Pelaz et al., 2000).

1.4.2.3 Differentiation of gynoecium

Once carpel identity is specified, the gynoecium development is initiated by activating different genetic pathways. The differentiation of gynoecium occurs on three axes of polarity: apical-basal, abaxial-adaxial and medio-lateral patterns (Larsson et al., 2013).

Apical-basal domain:

The interplay between hormones and transcription factors forms an integrative network that brings about the initiation of carpel primordia, gynoecium differentiation, ovule primordia initiation, and fruit development. The apical domain of gynoecium is represented by the style and stigma, which are regulated by several transcription factors such as *CRABS CLAW (CRC), SPATULA (SPT), ETTIN (ETT), AINTEGUMENTA (ANT), JAGGED (JAG), LEUNIG (LUG), SEUSS (SEU),* and *STYLISH1 (STY1).* These proteins determine the proper fusion of carpels and aid in fertilization (Alvarez and Smyth, 1999; Conner and Liu, 2000; Franks et al., 2002; Kuusk et al., 2002; Liu et al., 2000; Ohno et al., 2004). *SPT* shows defects in the development of the most carpel-specific tissues. Loss of *spt* function causes impaired development of apical tissues of the gynoecium and results in the improper fusion of carpels at the apex. The development of transmitting tract, style, and stigma were defective and leads to reduced frequency of fertilization and low seed production (Foreman et al., 2011; Girin et al., 2009).

Additionally, multiple lines of evidence strongly indicated the role of auxin for apical to the basal patterning of the gynoecium (Hawkins and Liu, 2014). Once the flux of polar auxin gradient establishes in the carpel primordia to determine the apical-basal patterning, the key gene regulatory network activates to determine the ontogenesis of different regional domains. Auxin synthesizes at different regions and at various time periods and transports to the targeted regions in order to ensure the robust auxin maxima. *STYLISH* (*STY1)* activates transcription of the auxin biosynthesis gene *YUCCA* (*YUC4)* in the apical part of the developing gynoecia. Hence, in *sty1 sty2* double mutant, the auxin levels are reduced and show a phenotype of split style at the apex (Kuusk et al., 2002).

Furthermore*, AUXIN RESPONSE FACTORS* (*ARFs*) perform the auxin signaling function. *ETTIN* (*ETT)* encodes an auxin response factor and specifies the abaxial fate of gynoecium (Nemhauser et al., 2000; Sessions et al., 1997). Mutant phenotype of *ett* shows diminished or lack of carpel valve tissues and extended style, stigma, and gynophores (Hawkins and Liu, 2014). Additionally, *KANADI* (*KAN*) genes are also involved in the abaxial fate of the gynoecium along with *ETT*.

Auxin transports from one cell to another in a chemiosmotic pattern. *PIN-FORMED (PIN)* and *PINOID (PID)* are the genes involved in the auxin transportation. Mutants of the *pin* and *pid*

show valveless gynoecium topped with stigmatic tissue. The weaker *pin* or *pid* mutants demonstrated that polar auxin transport is critical for gynoecium morphogenesis (Hawkins and Liu, 2014).

Abaxial-lateral domain:

In addition to the early specification of carpel identity, *AG* is also required for correct patterning of specific carpel tissues. In *ag* single mutants, the carpels are occupied by sepal-like structures due to antagonistic behavior between A and C class genes. However, in *ap2ag* double mutant with lacking A class function, carpel-associated structures were developed without valve tissues. It suggests that some more genes are involved in patterning the gynoecium and they act in an *AG* independent pathway (Liljegren et al., 2000). In that direction, *CRABSCLAW (CRC)* is one of the candidate gene involved in maintaining abaxial- adaxial patterning of gynoecium as *crc* mutants show a shorter and wider gynoecium with partially unfused carpel valves at the apex (Bowman and Smyth, 1999). Additionally, *SHATTERPROOF 1/2 (SHP)* genes specify the identity of valve margins and function in parallel to *AG* (Liljegren et al., 2000). Furthermore, *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*) promote valve margins along with *SHP1/2* (Rajani and Sundaresan, 2001), whereas *JAGGED* (*JAG), FILAMENTOUS (FIL)* and *FRUITFUL* (*FUL*) genes specify the valve identity (Dinneny et al., 2004).

On the other hand, though *REPLUMLESS* (*RPL)* is required to promote the replum identity, it does not control replum development (Roeder et al., 2003). *RPL* does the replum identity by restricting the valve margin factors *SHP1/2*, *IND* and *ALC* to the valve margins and *JAG, FIL, FUL* and *YAB3* to the valve tissues. Additionally, *BREVIPEDICELLUS* (*BP)* promote replum formation along with *RPL.* In turn, *ASSYMETRICLEAVES 1/2 (AS)* genes restrict the *BP* to the replum region. In summary, an antagonistic interaction between lateral factors like *JAG, FIL, AS1/2,* and medial factors such as *BP* and *RPL* together promote the formation of the valve and valve margin (Arnaud and Pautot, 2014).

Concurrently, *AP2* plays an important role in maintaining the growth of the replum and adjacent valve margins by repressing the action of *BP, RPL* to the replum, and *SHP 1/2, IND* to the valve margins. In addition to them, *WUSCHEL* like Homeobox 13 (*WOX 13)* shows its expression in the replum (Romera-Branchat et al., 2013).

Furthermore, the transcription factors *FIL, YABBY3 (YAB3),* and *JAG* activate *SHP* genes which function in parallel to the *AG* pathway. In addition to that, *AG* and *SHP* directly or indirectly activate *SPATULA* (*SPT)* and *CRC* (González-Reig et al., 2012).

Medio-Adaxial domain:

The adaxial surface of the gynoecium exhibits meristematic activity and which is known as central marginal meristem (CMM). *SEU* is involved in the formation of ovules from the central marginal meristem (CMM). *SEU* works with *AINTEGUMENTA (ANT*) in a redundant manner and regulates downstream genes in the formation of ovules as *seu/ant* mutants show complete loss of ovules (Azhakanandam et al., 2008). The multimeric complex formed by *LUG, SEU*, *ANT,* and *FIL* transcription factors regulate the development of medial domain of the gynoecium replum, septum, placenta, style, and stigma (Azhakanandam et al., 2008; Sridhar et al., 2004).
Besides that, *CLAVATA (CLV)* gene promotes the differentiation of cells at the periphery of meristematic zone in the shoot meristems, floral meristems, and CMM of gynoecium and restricts the differentiation of meristematic cells from the central zone . The central zone of meristem is called "meristem promoting activity" (MPA), which is more *STM* predominant, whereas peripheral zone is more *CLV* predominant. *CLV, CORYNE (CRN),* and *BARLEY ANY MERISTEM (BAM)* work in a pathway and promote the meristem maintenance in the shoot meristem, FM, and in the CMM of the gynoecium (Clark et al., 1996; Durbak and Tax, 2011).
Furthermore,

1.5 Carpel identity as specified by C-class MADS-box genes

The detailed study of C- class gene *AG* in various phylogenetically informative landmark plant species help us to understand the differential molecular mechanisms and genetic interactions responsible for the diversification of carpels during evolution. The function of *AG* was first characterized in the model plant, *Arabidopsis thaliana* by using the overexpression and knockout expression approaches. The Arabidopsis flower consists of four sepals in the outer whorl, four petals in the second whorl, six stamens in the third whorl and two carpels fused into a syncarpous gynoecium in the central whorl. whereas *ag* mutant flower shows four sepals in the outer whorl, four petals in the second whorl, six petals instead of six stamens in the third whorl and an

additional flower in the 4th whorl in the place of central gynoecium (Bowman et al., 1989). The additional flower encloses 70 floral organs of sepals and petals which are produced in a recurrent manner and it is termed as floral meristem indeterminacy (Yanofsky et al., 1990).

AG like gene clade has been found in all the seed bearing plants, including gymnosperms suggesting that the gene has been evolved around 300-400 MYA and its function is well conserved during evolution in determining the male and female reproductive organ identity.

However, owing to independent gene and genome duplication events in various lineages, the copy numbers of the *AG* gene in various plant species is varying. The consequences of gene duplication events lead to subfunctionalization or neofunctionalization. Even though the principal function of floral homeotic genes are comparable to core eudicot model species *A. thaliana*, the genetic and developmental mechanisms regulating those roles is varying between paralogous genes in different species.

The whole genome duplication at the base of angiosperm lineage led to the formation of ovule-specific D class genes (*SEEDSTICK*) and reproductive organ specifying C class genes (*AG* clade) (Kramer et al., 2004). The C class gene clade (*AG*) is responsible for the formation of carpels in angiosperms and origin and evolution of which is an important milestone in the evolutionary history of flowering plants (Becker and Theißen, 2003). The *AG* clade is further undergone recent duplication event and gave rise to *euAG* and *PLENA* clades. The *euAG* clade includes *AG* and *PLENA* clade consisting *SHATTERPROOF 1* and *2* genes (*SHP 1/2*) of Arabidopsis. The detailed study of *AG* gene in Arabidopsis, Antirrhinum, Maize, and Rice has shown its crucial roles in reproductive organ development and meristem determinacy but regulate those functions in a different pattern in different organisms. In *A. thaliana*, the single *AG* is playing both the roles of reproductive organ development and meristem determinacy (Bowman et al., 1991) . Whereas in Antirrhinum, there are two *AG* orthologous genes *PLENA* and *FARINELLI*; *PLENA* (*PLE*) belongs to *PLENA* clade responsible for carpel identity; whereas *FARINELLI (FAR)* belongs to *euAG* clade and is responsible for stamen identity and both genes together specify the floral meristem determinacy (Davies et al., 1999; Schwarz-Sommer et al., 1990). While in *Z. mays,* a monocot species consists of two *AG* orthologous genes, *ZAG1* and *ZMM2,* which have also undergone clear sub-functionalization. However, the pattern is quite different from that of *A. majus*. *ZAG1* determines the meristem determinacy and *ZMM2* is responsible for stamen and carpel identity (Mena et al., 1996). In *O. sativa*, the two *AG*

orthologues *OSMADS3* and *OSMADS58* have also undergone clear sub-functionalization; *OSMADS3* plays a major role in the stamen identity and *OSMADS58* is involved in carpel identity and meristem determinacy (Yamaguchi et al., 2006).

Moreover, *AG* subfamily members were functionally characterized in few basal eudicot model plants. In Opium poppy (*Papaver somniferum*), there is a single *AG* lineage gene identified and which produces two alternative transcripts, *PapsAG-1*and *PapsAG-2.* These two proteins play distinct roles in stamen and carpel identity and FMD along with a degree of functional redundancy (Hands et al., 2011). Whereas in *Thalictrum thalictrodes* there were two *AG* lineage genes identified as *ThAG1* and *ThAG2* and *ThAG1* specify the function of *AG* in *A. thaliana* while *ThAG2* specifies ovule identity (Galimba and Di Stilio, 2015).

Molecular basis of carpel development was majorly studied in core eudicots and to some extent in grass species of monocots and these kinds of studies are highly limited outside the model core eudicots and monocots. Hence, in order to understand the evolution of gynoecium, the molecular basis of genetic mechanisms underlying its development needs to be analyzed in more phylogenetically informative species outside the core eudicots.

1.6 California poppy *(Eschscholzia californica*) is a versatile model species for evolutionary developmental genetics

1.6.1 Unique morphogenetic characteristics of California poppy

California poppy *(Eschscholzia californica)* belongs to basal eudicots, family Papaveraceae (order Ranunculales). The basal eudicots stand in between the monocots and higher eudicots in the phylogenetic tree. Hence, it is more interesting to have a model species from basal eudicots in order to bridge an evolutionary gap between the monocots and higher eudicots (Zahn et al., 2006).

California poppy is an annual to perennial herb and is native to the west coast of North America (Cook, 1962). It is a small plant with a generation period of three months. It produces a large number of fruits with about 80 to 100 seeds per fruit. In addition to that, the cultivation of California poppy is very easy with less effort throughout the year. Owing to its bigger flower size, collection and analysis of floral parts for molecular genetic studies is easy and convenient. Furthermore, shoot and floral morphogenesis was well studied in the wild-type plants and which

assists in comparative studies of mutants plants more easier (Becker et al., 2005). Virus-Induced Gene Silencing (VIGS) system is also well established as a reverse genetic tool in *E. californica* (Wege et al., 2007). Further, California poppy is amenable to stable transformation to produce stable mutants with less generation time.

Besides that, *E.californica* has a small genome size of about six times bigger than that of *A. thaliana* (1100Mbp) and Floral Genome Project (FGP) has selected *E. californica* as a model organism to study the floral diversity among basal eudicots. Additionally, a large number of (about 6000) Expressed Sequence Tags (ESTs) of floral buds are available (Carlson et al., 2006). Several homologous genes that play a role in flower development, cell and tissue differentiation and secondary metabolism are available as large data sets (Carlson et al., 2006). Bacterial Artificial Chromosome (BAC) library resource is accessible now and large numbers of floral mutants are available along with the sequence information (Lange, 2010). Also, high throughput technologies like oligonucleotide microarray chip were compiled specifically for floral transcriptome of California poppy (Zahn et al., 2010).

Furthermore, California poppy is a good source of alkaloids. The biochemical studies of petals and stamens have revealed that the principle carotenoid contents are esters of xanthophylls and eschscholtzxanthin. California poppy has ample amount of medicinal properties. Sanguinarine, a basic benzophenanthridine alkaloid found in the roots acts as an antimicrobial agent. Hence, California poppy is a competent model species for alkaloid biosynthesis as well as in the latex biochemistry (Park and Facchini, 2000). In view of possession of several unique characteristics, California poppy was selected as a versatile model species for evolutionary developmental genetic studies.

1.6.2 Morphogenesis of *E.californica* gynoecium

California poppy flower consists of two green sepals in the outer whorl, four orange colored petals in the second whorl, 18-34 small orange colored stamens in the third whorl which are arranged in 4-5 whorls and two leaf-like carpels fused to form a solid syncarpous gynoecium in the central whorl. The gynoecium consists of a broad ovary at the base, a short style in the middle and a stigma at the top in the form of four long protrusions covered with papillae. The

ovary is superior in position and consists of a single locule with parietal placentation and ovules are being attached in two rows (Becker et al., 2005).

The presence of a floral tube around the ovary is a characteristic feature of California poppy. The apical-basal patterning of gynoecium in *E. californica* is similar to that of *A. thaliana* gynoecium. In the transverse section, the ovary consists of two valves on the lateral-abaxial position and two valve margins or carpel margins in the medial-abaxial axis (Fig.5).The replum develops between two valve margins in the medial-abaxial axis and placenta develops on the adaxial side. The two placentae grow inwards into the ovary locule and carry the ovules. In the case of *A. thaliana,* the gynoecium consists of two locules separated by a septum and a transmitting tract, whereas in *E. californica* the gynoecium consists of a single locule without a false septum and transmitting tract and hence, the pollen tube grows throughout the placenta (Becker et al., 2005).

The morphogenesis of gynoecium starts at stage 5 of the flower development (Becker et al., 2005). The carpel primordium protruded as a small arch of bulged floral meristem differentiated from the stamen primordia. At stage 6, the two carpel primordia grow longitudinally while the edge of carpel walls fuse at the medial position with developing placenta inwards. This is followed by the initiation of ovule primordia on the placental regions along with the radial growth of gynoecium at stage 7. Subsequently, each carpel constitutes 10 domains of five longitudinal, three medial and two lateral ridges at stage 8 (Becker et al., 2005). By stage 9-10, embryo sac is developed and which is followed by the formation of complete differentiation of gynoecium, which is receptive for fertilization by the end of stage 11. After fertilization, the gynoecium transforms into a fruit and encloses the seeds. The fruits elongate in size and reach to maturity at stage 12. Afterward, they become dry at stage 13. The dry fruits or capsules start to dehisce in the longitudinal direction from base to the apex while retaining both valves attached to the style at stage 14 (Cook, 1962).

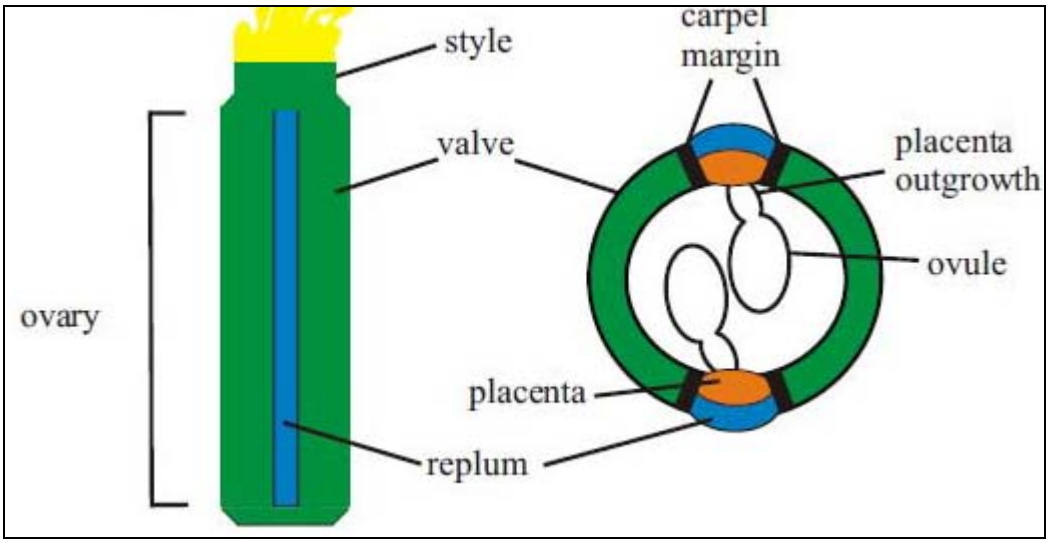

Fig. 5: **Schematic representation of *E.californica* gynoecium**

On the left side is the longitudinal view in apical-basal axis; On the right side, the transverse section of gynoecium was depicted with medio-lateral and abaxial-adaxial domains (Orashakova, 2011).

The molecular genetic studies of carpel development in *E.californica* was started with *CRC* orthologous gene *EcCRC* (Orashakova et al., 2009). *CRC* is an important gene involved in carpel development (Bowman and Smyth, 1999). Moreover, *CRC* exhibits diversified functions in different species and the developmental mechanisms responsible for showing such functional diversity are important for understanding the evolution of gynoecium. *CRC* plays an essential role in the longitudinal growth of the gynoecium, abaxial-adaxial patterning and is responsible for nectary development in Arabidopsis (Bowman and Smyth, 1999). In contrast, *CRC* orthologue *DROOPINGLEAF (DL)* in rice confers carpel identity, floral meristem determinacy, and leaf midrib formation (Yamaguchi et al., 2004). While in *E.californica*, *EcCRC* is expressed and function in the abaxial identity of the carpels and aids in the growth of tissues that develop from the carpel margins (Orashakova et al., 2009). Additionally, it is responsible for the ovule initiation and placenta formation unlike the *CRC* of *A. thaliana* and also involved in the floral meristem determinacy (Orashakova et al., 2009).

2 Objectives

The present study has two aims:

The floral homeotic MADS-box gene *AGAMOUS (AG)* in Arabidopsis confers stamen and carpel identity and regulates floral meristem determinacy. The *ag* mutants show complete homeotic conversions of stamens into petals and carpels into sepals as well as indeterminacy of the floral meristem. Functional characterization of *AG* in several core eudicot species and in monocot species such as rice and maize suggest a conserved function of *AG* homologs in angiosperms. However, due to gene and genome duplication events, the *AG* orthologues exhibit different developmental mechanisms at various levels of gene regulation in various plant lineages.

At this point, functional analysis of *AG* orthologues in *E.californica*, a basal eudicot species could unravel some of the mechanisms involved in the development of carpels in basal eudicots during evolution. The two *EScaAG* paralogues of *E. californica* are highly similar at both nucleotide and protein sequences but are maintained in the evolution without any functional constraints. Therefore, virus-induced gene silencing (VIGS) was employed for functional characterization of *EScaAG* genes in *E. californica*.

Secondly, the stable transformation is still a critical tool for functional characterization of genes. The next objective of this study is to establish an efficient and less laborious *Agrobacterium* - mediated stable transformation of *E. californica* using unripe seeds as a new explant source. To develop a stable transformation methodology, an explant source that is amenable to transformation needs to be established. The unripe seeds were optimized in order to induce an embryogenic callus through less laborious methodology as a preliminary step and further, the tissue culture process was optimized to produce efficient regeneration of plantlets. Subsequently, transformation efficiency of unripe seeds was analyzed by using the constitutive expression of *GUS* gene and knock-down RNAi-*EcCRC* gene construct.

3 Materials

3.1 Plant material used

California poppy (*E. californica* Aurantiaca 'Orange King') seeds were obtained from B&T world seeds SARL., Paguignan, France.

3.2 Bacterial strains used

Chemically competent and electrocompetent *Escherichia coli* strain *DH5α* was used for cloning vectors. Electrocompetent *Agrobacterium tumefaciens* strain GV3101 was used for plant transformation.

3.3 Vectors used

Plasmid vector	**Bacterial selection**	**Plant selection**	**Purpose**	**Source**
pART7	Ampicillin (100 µg/mL)	-	Shuttle vector for overexpression construct	Glycerol stock
pMLBART	Spectinomycin (150 µg/mL)	Basta (10 µg/mL)	Binary vector for overexpression construct	Glycerol stock
pTRV1/pTRV2	Kanamycin (50 µg/mL)	Kanamycin (50 µg/mL)	VIGS	Glycerol stock
pHELLSGATE12	Spectinomycin (150 µg/mL)	Kanamycin (50 µg/mL)	Binary vector for ihRNAi	CSIRO, Plant Industry, Canberra, Australia.
pENTR/D-Topo	Kanamycin (50 µg/mL)	-	Shuttle vector for ihRANi	Invitrogen Life technologies GmbH, Frankfurt, Germany.
pCX35S:GUS	Kanamycin (50 µg/mL)	Hygromycin (10 µg/mL)	Binary vector for overexpression	Glycerol stock

3.4 Nucleic acid manipulation

3.4.1 PCR

The PCR reaction mixture consists of 5 µl of 10x PCR buffer, 1 µl of 10 µM dNTP mix, 3 µl of 25 mM $MgCl_2$, 0.5 µl of *Taq* DNA polymerase (5 µ/µl), 1.5 µl of forward primer (10mM), 1.5 µl of reverse primer (10mM), 50-100 ng of template DNA and finally made up the volume to 50 µl with nuclease-free water.

3.4.2 Restriction digestion

Restriction enzymes from NEB were used in all the digestion reactions. The total 10 µl reaction mixture consists of 1 µl of Buffer 4, 1 unit of restriction enzyme, 1 µg of plasmid DNA and ddH_20 up to 10 µl.

3.4.3 Ligation

The 20 µl of ligation reaction mixture was prepared by adding 1 unit of T4 DNA ligase, 2 µl of ligase buffer, 1 µg of digested plasmid DNA, 300 ng of similarly cut DNA fragment and ddH_20 up to 20 µl.

3.5 Nucleic acid analysis

3.5.1 Gel electrophoresis

For preparing the gel and documentation of the same, 1% of agarose powder (Sigma-Aldrich, Hamburg, Germany), 1x TAE buffer, 0.05% of DNA STAIN-G and 1x DNA loading dye were used.

3.5.2 Isolation of RNA

RNA was isolated by RNeasy Micro Kit (Qiagen, Hilden, Germany).

3.5.3 cDNA synthesis:

For preparing cDNA, 1 µg of total RNA, 1 µl of 50 µM oligo(dT) primer, 1 µl of annealing buffer, 2 µl of superscript III (200 U/µl) and RNase/DNase-free water up to 8 µl were used.

3.5.4 RT-PCR reaction mixture

For setting up the RT-PCR, 1 µl of 1:10 dil cDNA, 5 µl of 10x PCR buffer, 1 µl of 10 mM dNTP mix (0.2 mM each), 3 µl of 25 mM MgCl2, 0.5 µl of *Taq* DNA polymerase (5 µ/µl), 1.5 µl of forward primer, 1.5 µl of reverse primer, 35.5 µl of nuclease free ddH_2O were used.

3.5.5 qRT-PCR

For Syber Green master mix, 5 µl of 1:50 dilution cDNA, 10 µl SyBr, 5 µl of primers (400 to 600 nM) and ddH_2O upto 20 µl were used

For probe master mix 5 µl of 1:50 dilution cDNA, 100 nM UPL probe, 200 nM of primers and ddH_2O up to 20 µl were used.

3.5.6 Sequencing

For sequencing reaction, 0.5 µl of Big dye, 2. µl of the 5x buffer, 1.0 µl of gene specific primer (5mM), 1 µl of template DNA (200ng) and 5.5 µl of DNase-free dH20 were used.

3.6 Phenotypic analysis

3.6.1 SEM

For this purpose, FAE solution (2% Formaldehyde, 70% Ethanol, 5% Acetic acid), 100% Methanol, 100% Ethanol, liquid CO_2 and 2% Glutaraldehyde was used as a fixative.

3.6.2 Histology

FAE solution (3 % Formaldehyde, 5 % Acetic acid, 60 % Ethanol p.a., Tween-20), Ethanol and Rotihistol at various concentrations, paraplast, Safranin O, alcoholic fast green were used.

3.6.3 Growth media

3.6.3.1 Bacteria growth media (1 L)

LB medium is used for growing Bacteria, 1% (w/v) peptone, 0.5% (w/v) yeast extracts, 1% (w/v) NaCl, 1.5% (w/v) of agar were used.

3.6.3.2 Plant growth and tissue culture media

a) Plant sterilization

2% Sodium hypochlorite

Tween-20 or Triton X-100

b) B5 liquid media (1L)

3.16 g of B5 salts

20 g sucrose

c) Callus induction media with selection (CIM) (1L)

3.16 g B5 salts

30 g sucrose

8.4 g phytoagar

2 mg NAA

0.1 mg BAP

300 mg Timentin

d) Somatic embryo induction media (SEIM)(1L)

3.16 g B5 salts

30 g sucrose

8.4 g Phytoagar

1 mg NAA

0.5 mg BAP

300 mg Timentin

e) Plant regeneration media (PRM) (1L)

3.16 g B5 salts

30 g Sucrose

5 g Gelrite

3.7 Genomic DNA isolation by CTAB method

Liquid nitrogen, 2x CTAB buffer, 20 ml Chloroform, 5 ml Isopropanol (100%), 1 ml sterile TE buffer, 3 ul RNase (10 mg/ml), and Phenol: Chloroform in 1:1 ratio, 3 M Sodium Acetate, and 70% Ethanol were used.

3.8 Southern blotting

a. **Depurination buffer**: 250 mM HCl
b. **Denaturation buffer**: 0.5 M NaOH, 1.5 M NaCl
c. **Neutralization buffer**: 0.5 M Tris-HCl, 1.5 M NaCl
d. **20X SSC**: 3 M NaCl, 0.3 M Sodium citrate
e. **Maleic acid buffer**: 0.1 M Maleic acid, 0.15 M NaCl
f. **Hybridisation buffer**: 5x SSC, 5x Denhardt's solution, 0,5% SDS
g. **Low Stringent Buffer**: 2x SSC, 0,1% SDS
h. **High Stringent Buffer**: 0,1x SSC, 0,1% SDS
i. **Blocking solution** (Roche)
j. **Blocking reagent**: Maleic acid solution (Autoclaved), 0.5 % blocking solution (Roche)
k. **Washing buffer** B: 0.3% Tween-20 in Maleic acid buffer
l. **Detection buffer**: 100 mM Tris-Base, pH 9.5, 100 mM NaCl
m. **Stripping buffer**: 0.2 M NaOH, 0.1% SDS
n. **Antibody solution**: 0.05% Anti-Digoxigenin-AP (Roche, Mannheim, Germany) in blocking solution

3.9 Transient GUS assay

The GUS staining solutions were prepared using the following materials**:**

a) Wash buffer (10 ml) containing 0.342 ml of 1 M Na_2HPO_4, 158 ml of1 M NaH_2PO_4, 0.600 ml of 50 mM $K_3Fe(CN)_6$, 0.600 ml of 50 mM $K_4Fe(CN)_6$, 8.300 ml of MQ H_2O.

b) Stain solution (10 ml) consists of 0.342 ml of 1 M Na_2HPO_4, 0.158 ml of 1 M NaH_2PO_4,0.600 ml 50 mM $K_3Fe(CN)_6$, 0.600 ml of 50 mM $K_4Fe(CN)_6$, 0.209 ml of 2 mM X-Gluc, 8.091 ml MQ H_2O.

3.10 VIGS infiltration buffer

10 mM MgCl2, 10 mM Acetosyringone and 0.1 mM MES.

3.11 Other buffers used

a) **2% CTAB** (w/v) consists of 100mM Tris (pH=8.0), 20 mM EDTA (pH=8.0), 1.4 M NaCl, 1% PVP (Polyvinyl pyrrolidone)

b) **SOC media** consists of 2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose

c) **TE-buffer** consists of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA pH 8.0

d) **50X TAE** consists of 40 mM Tris-acetate (pH 7.5), 1mM EDTA

e) **Bacterial plasmid DNA extraction (STET)** buffer containing 8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris pH 8.0

f) **10X MOPS buffer** consisting of 0.2 M (N-morpholino) propanesulfonic acid (MOPS) and sodium acetate

g) **RNA denaturation buffer** consisting of 10ml of 100% deionized formamide, 3.5ml 40% formaldehyde, 1.5ml of 10 x MOPS buffer.

h) **RNA loading buffer** consisting of 25% (w/v) Ficoll type 400, 0.1 M EDTA solution (pH 8.0), 25% (w/v) bromophenol blue.

3.12 Enzymes used

Name	**Purchased from**
Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
T4 DNA Ligase	New England Biolabs, Frankfurt am Main, Germany
Restriction enzymes	New England Biolabs, Frankfurt am Main, Germany
Proteinase K	Invitrogen, Darmstadt, Germany
LR clonase	Invitrogen, Darmstadt, Germany
DNase I	Roche, Mannheim, Germany
SYBR Green mix	Roche, Mannheim, Germany
RNaseA	New England Biolabs, Frankfurt am Main, Germany

4 Methods

4.1 Preparation of plant material

A commercial variety of California poppy, *E.californica var Aurintiaca* was used as the base plant material for the current study. The seeds of which were sown in jiffy pots containing 1:3 ratios of peat: soil and incubated for 3 days at 4°C. Later the pots were shifted to the greenhouse, with maintaining controlled conditions of 19 hours photo period.

4.2 Plasmid vector construction

4.2.1 Construction of VIGS-based vectors

The single gene construct pTRV2-*EScaAG1* was made by amplifying a 395 bp fragment of *EScaAG1* from the *EScaAG1* ORF by using the primers VIGSEcAG1A to add a *BamH*I restriction site at the 5' end of the PCR product and EcAG1VIGS to add an *Xho*I restriction site at the 3' end. The resultant PCR fragment was digested with *BamH*I, *Xho*I and cloned into a similarly cut pTRV2 vector (Ratcliff et al., 2001) to provide the pTRV2-*EScaAG1* plasmid.
Simultaneously, a 477 bp fragment of *EScaAG2* was amplified from the *EScaAG2* ORF by using the primers VIGSEcAG2A to add a *BamH*I restriction site at the 5' end of the PCR product and EcAG2VIGS to add an *Xho*I restriction site to the 3' end. The resultant PCR fragment was subjected to digestion with *BamH*I, *Xho*I and cloned into a similarly cut pTRV2 vector to provide the pTRV2-*EScaAG2* plasmid.
The double construct pTRV2-*EScaAG1/AG2* was made by amplifying a 190 bp fragment of *EScaAG1* from the *EScaAG1* coding region by using the primers XbaVIGSEcAG1Bfw to add an *XbaI* restriction site to the 5' end of the PCR product and EcAG1VIGSXhorev to add an *Xho*I restriction site to the 3' end and resultant PCR product was digested with XhoI, XbaI. At the same time, a 214 bp fragment of *EScaAG2* was amplified from the *EScaAG2* coding region by using the primers EcoVIGSEcAG2Afw to add an *EcoRI* restriction site at the 5' end and EcAG2VIGSXbarev to add an *XbaI* restriction site at the 3' end. The resultant PCR fragment was digested with *EcoR*I, *Xba*I. Subsequently, ligation was performed with the resultant *EScaAG1*, *EScaAG2* fragments with the *EcoRI* and *XhoI* cut pTRV2 vector to provide the pTRV2-

EScaAG1/AG2 plasmid. The inserted fragments in the single and double gene constructs were confirmed by using restriction digestion and sequencing tools.

Consequently, the resultant positively confirmed plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 through electroporation. The transformed *Agrobacterium* colonies containing TRV1 and TRV2 plasmids were selected using gentamycin and kanamycin (50 µl/ml each). The cells were grown up to an OD550 of 0.7- 0.85, collected the pellet and dissolved in the LB liquid.

4.2.2 Construction of stable transformation based vectors

4.2.2.1 *EcCRC* overexpression construct

The whole open reading frame of *EcCRC* was used to make the overexpression construct. The pART7 plasmid was used as a shuttle vector. The *EcoR1* restriction site was introduced to the forward primer and *BamH1* restriction site was anchored to the reverse primer. The resulting PCR fragment *EcoR1-EcCRC-BamH1* was cloned into the multiple cloning site of pART7 through restriction digestion. The shuttle vector pART7 was further digested with *Not1* and the whole cassette of CaMV35S*: EcCRC* fragment was introduced into pMLBART binary vector. The construct was made available by one of my colleagues Svetlana Orashakova, was used for overexpression of *EcCRC* through *Agrobacterium*-mediated stable transformation.

4.2.2.2 *EcCRC* Knock-down expression construct

The gateway technology is one of the cloning methods that take the advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989). Topo cloning was carried out to construct the intron-hairpin cassette to knock-down the gene expression. pENTR/ D-Topo vector was used as an entry vector. Forward primer was designed by introducing a four base pair sequence CACC at the 5' end of the forward primer (EcCRCihRNAiFor) and reverse primer (EcCRCihRNAiRev) was used to amplify a 150 bp blunt-end PCR product from the *EcCRC* open reading frame. The purified PCR product was transferred into a pENTR/ D- Topo entry vector through Topo cloning reaction. After confirming the presence of *EcCRC* gene fragment through colony PCR and sequencing reaction in the entry vector, LR cloning was performed to

transfer the *EcCRC* fragment into the binary vector pHELLSGATE12 by using LR clonase enzyme. The pENTR/D-Topo-*EcCRC* vector was incubated with pHELLSGATE12 along with LR clonase enzyme for overnight at 25°C. Later the reaction was inactivated with Proteinase K for 10 min and immediately transformed into *E.coli* through freeze-thaw method. Subsequently, the two *EcCRC* fragments inserted in the opposite orientation separated by an intron in the pHELLSGATE12 vector were confirmed through colony PCR, restriction digestion, and sequencing methods.

4.3 Bacteria manipulation

4.3.1 Preparation of competent cells for *E. coli* and *Agrobacterium*

The DH5α *E. coli* cells were grown for overnight in 5 ml of LB medium at 37°C. The overnight starter culture was added to 1000 ml of fresh LB medium and incubated in a shaker until they reach to an early log phase (OD_{600} 0.2-0.4). Immediately, the culture was transferred to ice, divided the 1000 ml of culture into four parts by pouring into ice-cold centrifuge bottles, and centrifuged at 5000 rpm for 10 min at 4°C. From here onwards, the culture was maintained on the ice. The supernatant was discarded and the pellet was dissolved in ice-cold 0.1 M $CaCl_2$ at half volume of the original culture and incubated on ice for 1 hour. The cells were then centrifuged again at 5000 rpm for 10 min at 4°C and the pellet was resuspended with 0.1 M $CaCl_2$ at $1/10^{th}$ of the original volume. This was followed by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant was removed and resuspended the pellet with ice-cold sterile glycerol to a final concentration of 10% (v/v). The cells were then centrifuged again at 5000 rpm for 10 min at 4°C. Afterwards, each pellet was then resuspended by gentle swirling with 1 ml of ice-cold sterile glycerol. Later 50 µl of culture was aliquot into 0.2 ml tubes and snap freeze in the liquid nitrogen. Immediately the cells were frozen in the -80°C freezer.

The *Agrobacterium* competent cells were prepared in the same way as DH5α *E. coli* cells except that the cells were grown at 28°C instead of 37°C.

4.3.2 Transformation of *E. coli* through Electroporation

The electrocompetent *E. coli* cells were maintained at -80°C freezer in small aliquots in the tubes. The tube was taken from the freezer and kept immediately on ice for thawing. About 50 ng of a plasmid solution was added to the tube containing 50 µl of competent cells and mixed gently. Then the mixture was transferred to a pre-cooled cuvette (gap 0.2 cm) and gave a short pulse in the electroporator at 25 µF capacitor, 200 Ω (ohm) resistance and 2.5 KV field strength between 6.25 –12 kV/cm for 4 to 8 milli sec. Consequently, 1 ml of SOC medium was added to the cuvette and mixed by inversion and transferred the solution to a 1.5 ml tube. Subsequently, the tube was incubated for an hour at 37°C in a shaking incubator (200 rpm). Afterwards, 50, 100, and 200 µl of the resulting culture was spread on the LB plates (containing the appropriate antibiotics) and grown for overnight at 37°C.

4.3.3 Transformation of *E.coli* through freeze-thaw method

The cloning reaction mixture of 2 µL was added to a vial of chemically competent *E. coli* cells and mixed gently. Afterwards incubated on ice for 30 minutes and subjected to heat shock for 30 seconds at 42°C in a water bath without shaking. Immediately the vial was transferred to the ice and 250 µL of S.O.C. medium that was maintained at room temperature was added. Later, the vial was incubated at 37°C with shaking in a shaking incubator for an hour. Finally, 50 and 200 µL of bacterial culture was used to spread on the prewarmed LB plates with selective antibiotics. The plates were incubated for overnight at 37°C.

4.3.4 Transformation of *A. tumefaciens* through Electroporation

The electrocompetent *A. tumefaciens* cells were maintained at -80°C freezer in small aliquots in tubes. The tube was taken from freezer and kept immediately on the ice for thawing and about 50 ng of a plasmid solution was added to the tube containing 50 µl competent cells and mixed gently. Then the mixture was transferred to a pre-cooled cuvette (gap 0.2 cm) and gave a short pulse in the electroporator at 25 µF capacitor, 400 Ω (ohm) resistance and 2.5 KV field strength

between 6.25-12 kV/cm for 4 to 8 milli sec. Consequently, 1 ml of LB was added to the cuvette, mixed by inversion and immediately transferred to 1.5 ml tube. The tube was incubated for 1 hour at 28°C with shaking incubator (200 rpm). Afterwards, 100, 150, and 200 µl of the resulting culture was spread on the LB plates (containing the appropriate antibiotics) and grown for 2 to 3 days at 28°C (Weigel and Glazebrook, 2006).

4.3.5 *Agrobacterium* culture preparation for stable transformation

The overexpression construct pMLBART: 35S-*EcCRC*, the knockdown expression construct pHELLSGATE12:35S:ihpRNAi-*EcCRC*, pMLBART:35S-*GFP* and pCX35S*:GUS* gene constructs were transformed into *Agrobacterium* strain GV3101 through electroporation. The *Agrobacterium* strains containing those plasmids were maintained at -80°C as glycerol stocks and were later on used to infect the unripe seeds. The glycerol stocks of GV3101 carrying a binary plasmid was scratched with toothpick and infected the 5 ml of LB liquid media as a starter culture and agitated for overnight at 28°C. The next day, 1 ml of the grown starter culture was used to infect 100 ml of fresh LB media and grown for overnight (OD_{500}0.8-1.0). The culture was then transferred to a falcon tube and centrifuged at 4000 rpm for 20 min. The resulting supernatant was discarded and the pellet was resuspended in the B5 liquid medium and incubated again for two more hours (OD_{500}0.8-1.0).

4.3.6 *Agrobacterium* culture preparation for VIGS

Agroinfiltration was carried out in order to induce virus-induced gene silencing (VIGS) in *E. californica*. VIGS is a method that exploits an RNA-based antiviral defense mechanism. The plants were infected with a modified virus vector carrying a gene of interest that was targeted for silencing. *A. tumefaciens* strain GV3101 containing the vector constructs pTRV1, pTRV2-*EScaAG1*, pTRV2-*EScaAG2,* pTRV2-*EScaAG1/AG2,* and pTRV2-*E* as a negative control, and pTRV2-*EScaPDS* as a positive control were maintained as glycerol stocks at -80°C. All the six plasmids containing *Agrobacterium* cultures were grown individually for overnight at 28°C in the 4 ml LB liquid medium supplemented with 50 µg/ml kanamycin sulphate, 10 µg/ml gentamycin sulphate, and 100 µg/ml rifampicin as a starter culture. The following day, the 4 ml

starter culture was mixed with 40 ml of fresh LB media and incubated for overnight until they grow to a late exponential phase (OD_{600} 0.8-1.0). Subsequently, the cultures were centrifuged at 4000 rpm for 20 min at room temperature (RT) and the pellet was dissolved in 20 ml of infiltration buffer by vortexing thoroughly and kept at room temperature for two hours.

4.4 Plant manipulation

4.4.1 Agroinfiltration for inducing VIGS

The pTRV1 containing infiltration buffer was mixed with pTRV2-G (G=*EScaAG1, EScaAG2, EScaAG1/AG2, EcPDS* and Empty vector) containing buffer in 1:1 ratio and mixed well. The resultant suspension was injected to the shoot apical meristem of three weeks old plants. About 0.1 to 0.2 ml of suspension was injected into the shoot apical meristem by using a 2 ml syringe having a needle of 0.45 mm x 25 mm as described (Wege et al., 2007). Consequently, the infected plants were incubated for 24 hrs at 4°C in the dark and were moved to greenhouse.

4.4.2 *Agrobacterium*-mediated stable transformation

The fruits at 22 DAA were collected and sterilized by washing with 70% ethanol for 1 min, followed by rinsing in the sterile water. Later the fruits were immersed in 2% sodium hypochlorite with a drop of Tween-20 or Triton X-100 and shook for 20 min. This is followed by rinsing and washing with sterile water for 3-4 times until the remnant of the bleach was removed. Thereafter, the fruits were air dried and opened with a sterile scalpel. The seeds have been slightly wounded with a sterile scalpel while removing from the fruits in order to facilitate the better penetration of *Agrobacterium* into the seeds. Simultaneously, the wounded seeds were immersed in the B5 liquid inoculation medium, agitated for about 20 min and blot them dry on sterile filter papers. The seeds were then co-cultivated on primary callus induction medium (CIM) for two days.

After two days of co-cultivation, the seeds were scrapped out from the CIM and washed in the sterile distil water for twice and suspended in the Timentin with potassium clavulanate solution for about 20 min. Then they were blotted dry on sterile filter papers and transferred to CIM+Selection medium.

4.4.3 Callus induction and somatic embryogenesis

The seeds were transferred to fresh CIM with selection at weekly intervals. After two weeks, the seeds started forming white globulous embryogenic calli. These calli were transferred to somatic embryo induction media with selection (SEIM+Sel) (Park and Facchini, 2000). Two weeks later, the calli started forming somatic embryos, which were separated and transferred to plant regeneration medium (PRM). The developing somatic embryos were transferred to fresh PRM at 15 days interval until they develop proper root and shoot system.

Selected plantlets with healthy root systems were transferred to vermiculite and covered with polythene bag in order to maintain humidity for a week. Thereafter the plantlets were shifted to 1:3 ratio of peat: soil mixture in jiffy pots and acclimatized to greenhouse conditions.

4.5 Nucleic acid analysis

4.5.1 Plant RNA Extraction

The samples of first buds or young leaves from the wild type plants and down-regulated plants (maximum 100 mg) were collected and kept immediately in the liquid nitrogen. Then the samples were homogenised with sterilized mini pestles in 1.5 ml tubes. Thereafter the samples were processed by following the plant-RNA-OLS®Kit manufacturer's instructions (Omni Life Science, Bremen, Germany).

4.5.2 Preparation of RNA Gel

The overall quality of RNA was assessed by electrophoresis on a denaturing agarose gel. One gram of agarose was added to 72 ml of water and heated until agarose was dissolved, and then cooled down to 60°C. Subsequently, 10 ml of 10X MOPS running buffer and 18 ml of 37% Formaldehyde (12.3 M) were added. The mixture was poured immediately into a gel plate with a comb and assembled the gel by adding enough 1X MOPS running buffer to cover the gel. Afterwards, the RNA samples were prepared by adding 0.5x Formaldehyde loading dye to each RNA sample, heated to 65°C for 5 min, and loaded onto the gel. Then followed the standard

electrophoresis at 85 V for 45 min. Afterwards, the gel was viewed and analysed using a gel-doc instrument equipped with UV light and a digital camera.

4.5.3 Complementary DNA (cDNA) Synthesis

The RNA samples were used to synthesize the first strand cDNA using the Superscript III Kit (Invitrogen, Karlsruhe, Germany) by following manufacturer's protocol.

4.5.4 Plasmid DNA Extraction

For isolation of plasmid DNA, 2 ml of bacterial culture was grown overnight in a shaking incubator. On the next day, the grown culture was centrifuged at a maximum speed for 1 min. The resultant supernatant was discarded and resuspended the pellet with 400 µl of STET buffer and 40 µl of 10 mg/ml Lysozyme. The suspension was incubated in the boiling water for 40 seconds followed by centrifugation at a maximum speed for 5 min. The genomic DNA and other debris collected as a pellet was removed slowly using a toothpick and the plasmid DNA recovered in the supernatant was purified by adding equal volume of pre-cooled 2-propanol and centrifuged at a high speed for 5 min. Thereafter, the supernatant was removed and the pellet was washed by adding 70% ethanol followed by centrifugation at a high speed for 5 min. Consequently, the supernatant was removed and 50 µl of TE buffer supplemented with 10 mg/ml RNase A was added to the pellet and incubated at 37°C for an hour to allow the plasmid DNA to dissolve in the TE buffer. Afterwards, the concentration of the plasmid DNA was determined by using the spectrophotometer.

4.5.5 Restriction digestion of Plasmid DNA

Restriction digestion was done for construction of vectors during cloning and also for the confirmation of inserted fragment in the cloned vector. The master mix for digestion was prepared according to the recommendation of the restriction enzymes manufacturer instructions (New England Biolabs, Frankfurt am Main, Germany). The contents of the master mix were mixed thoroughly and incubated at 37°C for 1 hour, followed by inactivation in the water bath at

65°C for 20 min. Subsequently, the digested DNA was analyzed through standard gel electrophoresis.

4.5.6 Polymerase Chain Reaction (PCR)

The standard PCR was carried out either during the cloning procedure in order to amplify a gene of interest and/or for the confirmation of the cloned fragment in the plasmid vector. The standard master mix was used and the samples were mixed well and gave a short spin with a microcentrifuge. PCR program include initial denaturation for one cycle at 95°C for 5 min, followed by, 35 cycles of denaturation at 94°C for 30 sec, annealing at 52-58°C for 30 sec and extension at 72°C for 30 sec- 1 min, followed by final extension for one cycle at 72°C for a duration of 5 min was used.
Ta was used based on the formula (Ta= Tm-5°C) and usually ranged between 52-58°C. The amplification of DNA was analysed through standard gel electrophoresis.

4.5.7 Colony PCR

Colony PCR was exploited for determining the cloned fragment of DNA in plasmid constructs after cloning. Individual colonies were picked from the overnight grown bacterial plates and resuspended in 10 µl of sterile water. The resulting 2 µl suspension was used as a template for the PCR reaction and a standard PCR protocol was followed for 25 µl of total reaction mixture.

4.5.8 Agarose Gel Electrophoresis

The standard agarose gel was prepared by adding 1% agarose in 1x TAE buffer and dissolved by heating in a microwave. Then allowed it to cool down to 60°C, added 0.05% of DNA STAIN-G, followed by pouring into a gel plate and allowed it to solidify. The DNA sample was mixed with 1x DNA loading dye and loaded on the solidified agarose gel. Standard electrophoresis was carried out at 120 V for 40 min and then documented the gel using a gel-doc instrument equipped with UV light and a digital camera.

4.5.9 DNA Sequencing

The presence of gene fragment after cloning was confirmed by sequencing with gene specific primers. First PCR was performed by using a PCR program which include initial denaturation at 96°C for 1 min for one time, followed by 15 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 1 min 15 sec, followed by 5 cycles with 96°C for 10sec, 50°C for 5 sec, 60°C for 1min 30 sec, followed by 10 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 2 min. Subsequently moved the tube to 4°C and followed further sequencing reaction by adding 5 µl ddH_2O and then the samples were sent to sequencing laboratory for obtaining nucleotide sequence. The ABI files obtained from them were analysed through Bio Edit software (Platt et al., 2007).

4.5.10 Expression analysis of *EScaAG* paralogous genes through RT- PCR in VIGS down-regulated plants

Total RNA was isolated from buds of different developmental stages, floral organs at anthesis, young fruits, green and mature seeds using plant-RNA-OLS®Kit following manufacturer's instructions (Omni Life Science, Bremen, Germany). One µg of total RNA was reverse transcribed into cDNA using Superscript III Kit (Invitrogen, Karlsruhe, Germany). The amount of cDNA template to be used for each tissue was standardized by using an endogenous control of the *E. californica* EST sequence (NCBI accession: CD476630) closest to the *A. thaliana ACTIN2* gene, the primer pair actin2RTQfw and actin2RTQrev were used. Paralogue specific primer pairs spanning at least one intron were used to discriminate between *EScaAG1* and *EScaAG2*. EcAG1RTFw primer and EcAG1RTRevspan were used to amplify *EScaAG1* and EcAG2RT Fw primer and EcAG2RTRevspan were used to amplify *EScaAG2*. RT-PCR was performed for 35 PCR amplification cycles and the sizes of the amplified products were 191 bp for *Actin2*, 420 bp for *EScaAG1*, and 800 bp for *EScaAG2.*

4.5.11 Expression analysis of *EScaAG* paralogous genes through qRT-PCR in VIGS treated plants

The inherent *EScaAG1/2* genes expression was analyzed by isolating the total RNA from the wild type plant flowers of different developmental stages, floral organs at anthesis, young fruits, and leaves. Additionally, the down-regulation of *EScaAG* gene expression was measured using qRT-PCR on the first buds of untreated, empty vector (pTRV2-E), pTRV2-*EScaAG1,* pTRV2-*EScaAG2,* and pTRV2-*EScaAG1/2* treated plants.

Additionally, expansion of B class genes into the 4th whorl was also analysed through qRT-PCR in the petaloid gynoecium of VIGS treated plants using two types of samples collected viz young carpel from small buds before anthesis (1-2mm diameter) and the mature carpels of a bud close to anthesis (5-8mm diameter). One µg of total RNA was reverse transcribed into cDNA using the Superscript III Kit (Invitrogen, Karlsruhe, Germany). The reference genes selected were *E. californica ACTIN2* and *GAPDH*. The amount of cDNA and the concentration of primers were optimized and the efficiency of estimated primer pairs was found to be between 400 and 600 nM. Paralogue specific primer pairs were designed to discriminate between *EScaAG1* and *EScaAG2* by spanning at least one intron in the forward primer and extending through a 15 bp deletion part of *EScaAG1* at C-terminal end in the reverse primer. The primers were tested further for paralogue specificity using sequencing PCR. The expression of *EScaDEF1, EScaDEF2,* and *EScaGLO* was quantified using intron-spanning primers in gynoecium pools of the wild type and treated samples with the help of Light Cycler 480. For quantification of *EScaDEF1* the Universal Probe Library (UPL) probe # 132 and for *ACTIN,* UPL probe # 136 were designed by using UPL Assay Design Centre of Roche and SyBr was used for the rest of the other genes.

The qRT-PCR program consisting of heating the samples at 95°C for 5 min, followed by 45 cycles of 10 sec at 95°C, 10 sec at 60°C, and 10 sec at 72°C was employed. Melting curve analysis was performed to prevent the formation of primer dimers and unspecific PCR products.

GeNorm VBA applet was used to estimate the Cp values based on three biological replicates and further three technical replicates were used for each biological replica (Vandesompele et al., 2002) Normalization Factor (NF) and Standard Deviation (SD) were calculated based on the expression of two selected reference genes *GAPDH* and *ACTIN*.

4.5.12 Expression analysis of *EcCRC* through RT-PCR in putative stable transformants

RNA was isolated from fresh leaves of wild type, putative transformants of *EcCRC* overexpression and *EcCRC* knock-down expression plants using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). One µg of total RNA was reverse transcribed into cDNA using Superscript III kit (Invitrogen, Karlsruhe, Germany). The amount of cDNA template used for each sample was standardized by using an endogenous control of the *E. californica ACTIN*. The primer pairs actin2RTQfw and actin2RTQrev were used for amplifying the control gene *ACTIN*. Simultaneously, *EcCRC* gene specific primers EcCRCRTfor and eccrcRTQrev primers were used for 35 PCR amplification cycles to amplify a 550 bp fragment of *EcCRC* in the overexpression plants. Similarly, *EcCRC* gene specific primers forward primer (EcCRCihRNAiFor) and reverse primer (EcCRCihRNAiRev) were used for 35 cycles to amplify a 150 bp of *EcCRC* fragment from the leaf tissues of knock down expression plants.

4.5.13 Genomic DNA isolation

About one gram of young leaf tissue was collected from young plants and ground into a fine powder in the pre-cooled sterile mortar and pestle in presence of liquid nitrogen. Immediately the powder was transferred to a 50 ml Falcon tube containing 15 ml of the extraction buffer [2x CTAB buffer]. The mixture was incubated at 65°C for 20 min in a water bath with occasional gentle swirling. Then cooled the mixture to room temperature (RT), added an equal volume of chloroform and shook gently. Subsequently, the solutions in the falcon tubes were centrifuged at 5000 *g* for 10 min and collected the supernatant. Afterwards, the supernatant was extracted with an equal volume of Isopropanol and incubated for 10 min at RT and then subjected to centrifugation for 30 min at 5000 rpm at 4°C. The supernatant was discarded and added 1ml of STE buffer (sterile TE), 3 µl of RNase (10 mg/ml) and incubated for 40 min at 37°C for digesting the RNA. Afterwards, phenol: chloroform was added in the 1:1 ratio to get rid of the garbage and the supernatant was collected. Later $1/10^{th}$ volume of 3 M Sodium Acetate (NaAc) and 0.7 vol of Isopropanol were added to precipitate the DNA. Afterwards, the pellet was washed with 70% ethanol, followed by drying under vacuum and re-hydrated the precipitate in

50 µl of TE for at least an hour. The quality of genomic DNA was analysed through gel electrophoresis and quantity was measured through spectrophotometer.

4.5.14 Genotyping of putative transformants through southern blotting

Genomic DNA was extracted using Peqlab Mini Gold kit (Peqlab, Erlangen, Germany). The young and fresh leaves were collected from the wild type and putative transformants. The collected samples were frozen immediately in the liquid N2 and were homogenized using sterile pre-cooled mortar and pestle. Further processing of samples was performed according to the used kit manufacturer's instructions. The concentration of genomic DNA was measured and about 5 µg was digested with Hind111 for overnight followed by separation of digested fragments through electrophoresis in 0.7% agarose gel at 50 mA for 8 hours. Simultaneously, followed the gel washing by depurination for 15 min in 250 mM HCl, denaturation for twice for 15 min incubation time, followed by neutralisation for twice for about 15 min and finally followed the equilibration for 25 min in the 20x SSC.

Subsequently, the DNA fragments from the gel were transferred to a Hybond N+ membrane by using the capillary blotting System. The DNA probe for *BAR* gene was prepared by DIG labeling PCR and the pMLBART vector was used as a positive control. Afterwards, the membrane was washed in 2x SSC and then dried for 2 hours in the oven at 80°C. This is followed by overnight hybridization with DIG-labeled probe and performed the stringent washing of the membrane. Subsequently, the detection of signal was carried out using a LAS- 3000 mini luminescent image analyzer.

4.6 Phenotypic analysis

4.6.1 Scanning Electronic Microscopic examination of gynoecium

SEM was performed for detecting the surface structure of phenotypic flowers. Gynoecia and petals of the wild type flower as a positive control and gynoecia of *EScaAG1* and *EScaAG2* VIGS treated plants at anthesis stage were collected. The samples were processed in a Methanol-Ethanol based method followed by critical point drying (CPD). Further, the samples were gold

coated and observed under SEM for the changes in the cellular structure of gynoecium tissue of VIGS-*EScaAG* treated plants.

4.6.2 Histological sectioning of buds

It has been deployed for detailed microscopic examination of cells and tissues of phenotypic flowers and fruits. The first buds at around 1.6 to 2.5 mm diameter were collected from the wildtype, pTRV2-E, pTRV2-*EScaAG1*, pTRV2-*EScaAG2,* and pTRV2- *EScaAG1/EScaAG2* infected plants. These fresh buds were fixed first in the FAE solution and then embedded firmly in the Paraplast Plus (Tyco Healthcare, http://www.tyco.com). Afterwards, the enclosed bud tissues were sectioned with microtome as 7 mm thickness and were stained with Safranin and Fast Green for 24 hours and counterstained with alcoholic Fast-Green (Chroma, http://www.chroma.com) solutions for 3 min as described (Orashakova et al., 2009).

4.6.3 Transient GUS assay

The calli samples were transferred to a microfuge tube containing the GUS staining solution. The tubes were left open to remove any trapped air and were incubated for about 48 hours at 37°C with agitation. Subsequently, the stain solution was removed and replaced with 70% ethanol for twice in the subsequent 24 hours. Afterwards, the samples were observed for blue coloration.

4.6.4 *In situ* hybridization

The experiments were carried out by Svetlana Orashakova, Evolutionary Developmental Genetics Group, University of Bremen. The experimental procedure and the probe used for detection of *EScaAG1/2* expression were described previously (Orashakova et al., 2009; Yellina et al., 2010).

5 Results

The study has been carried out on two main objectives in order to understand the molecular genetics of carpel development in California poppy. In the first project, the functional characterization of *EScaAG* paralogues was carried out by deploying VIGS methodology. In the second project, a preliminary attempt was made for the establishment of *Agrobacterium*-mediated stable genetic transformation, where in high frequency somatic embryogenesis and plant regeneration system was achieved by using the unripe seeds as the new explant source in *E. californica.*

5.1 Functional analysis of *EScaAG* paralogues through VIGS

5.1.1 Sequence analysis of *AG* paralogues in *E.californica*

E. californica consists of two *AG* orthologues namely *EScaAG1* and *EScaAG2,* which show high sequence similarity at both untranslated regions (UTR), and open reading frames (ORF). They share about 75% similarity at the nucleotide level including the 5'UTR and ORF. Additionally, the translated amino acid sequences share about 81.7% similarity. When the two *EScaAG* sequences of *E.californica* were aligned against *AG* of *A. thaliana*, they exhibited an amino acid sequence identity of 66.6% and 61.1% respectively (Yellina et al., 2010). However, there are few differences between two sequences, the *EScaAG*2 nucleotide sequence showed a 40 bp insertion and 14 bp deletions in the 5' UTR, and a 15 bp deletion in the 3' coding region of *EScaAG1*. Based on these sequence dissimilarities paralogue specific primers were designed. The forward primer was designed by extending through an intron and the reverse primer was made spanning through a 15 bp nucleotide sequence deletion of *EScaAG1* at C-terminal end.

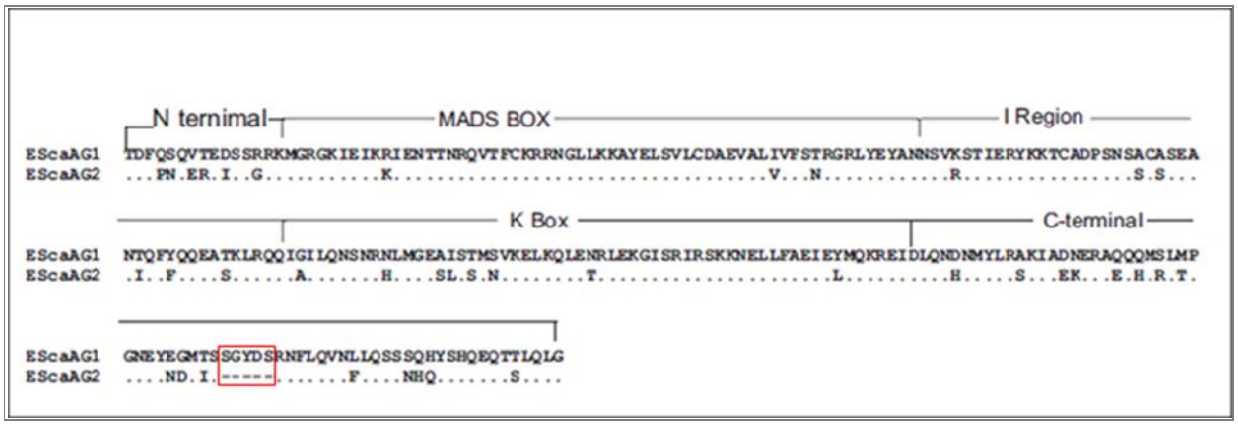

Fig. 6: Protein sequence alignment of *EScaAG1* and *EScaAG2*.

Identical amino acids of two paralogues are indicated by dots and dissimilar residues are indicated by the respective amino acids. The five amino acid deletion is marked by dashes with a red box at the C-terminal region of *EScaAG1*.

5.1.2 Expression analysis of *EScaAG* paralogues in *E. californica*

The two *EScaAG* paralogues showing high sequence similarity were examined further for their expression pattern. The expression pattern of two *EScaAG* paralogues in *E. californica* was analysed as a preliminary attempt through RT-PCR on cDNA pools isolated from different stages of the flower development, different parts of the flower at anthesis, and at various seed development stages. The paralogue specific primers were used to differentiate the expression pattern of two paralogues and the expression of *EcACTIN2 (EcACT2)* served as a control. The expression could be detected for both the paralogues; however, the *EScaAG*1 transcripts were present more abundantly than that of *EScaAG*2 (Suppl.Fig 1). The experiments have been carried out in triplicates. RT-PCR on floral organs at anthesis detects that *EScaAG1* and *EScaAG2* showed strong expression in stamens and carpels and an attenuated expression in sepals and petals (Suppl.Fig.1). Furthermore, both paralogous genes are expressed throughout the flower development, assayed in buds from 0-3 mm size, in young fruits and green seeds. However, the expression is decreased later on in the mature seeds (Suppl.Fig.1).

On the other hand, due to an overall low *EScaAG2* expression, RT-PCR expression profiles were inconsistent among the technical replicates for *EScaAG2* gene. To overcome this problem, a more sensitive technique, quantitative real time PCR (qRT-PCR) was deployed. It was carried out in four floral organs namely sepals, petals, stamens, and carpels of the flower at anthesis, young fruits, leaves, and buds of different developmental stages (Fig.7A). The differential expression pattern of *EScaAG1* and *EScaAG2* was established using paralogue specific primers. As evident from RT-PCR results**,** the overall expression level of *EScaAG2* was lower than that of *EScaAG1*. Both paralogues are highly expressed in the stamens and carpels of the mature flower and they show extremely low expression in sepals and petals. Moreover, *EScaAG2* showed stronger expression in the stamens compared to *EScaAG1* and *EScaAG1* showed its strongest expression in the carpels.

Additionally, *EScaAG1* and *EScaAG2* genes are expressed throughout the flower development extending from organ initiation to differentiation (bud stages 1 to 9 followed based on (Becker et al., 2005)). Furthermore, both genes are being strongly expressed in young fruits; however, show negligible expression in the leaves (Fig. 7A). In conclusion, the overall expression of *EScaAG1* was more distinct than that of *EScaAG2* in all the tested organs such as sepals, petals, carpels, all stages of buds, leaves and, young fruits, in contrast to the stamens, in which the expression of *EScaAG2* was 1.5 times higher than that of *EScaAG1* (Fig. 7A).

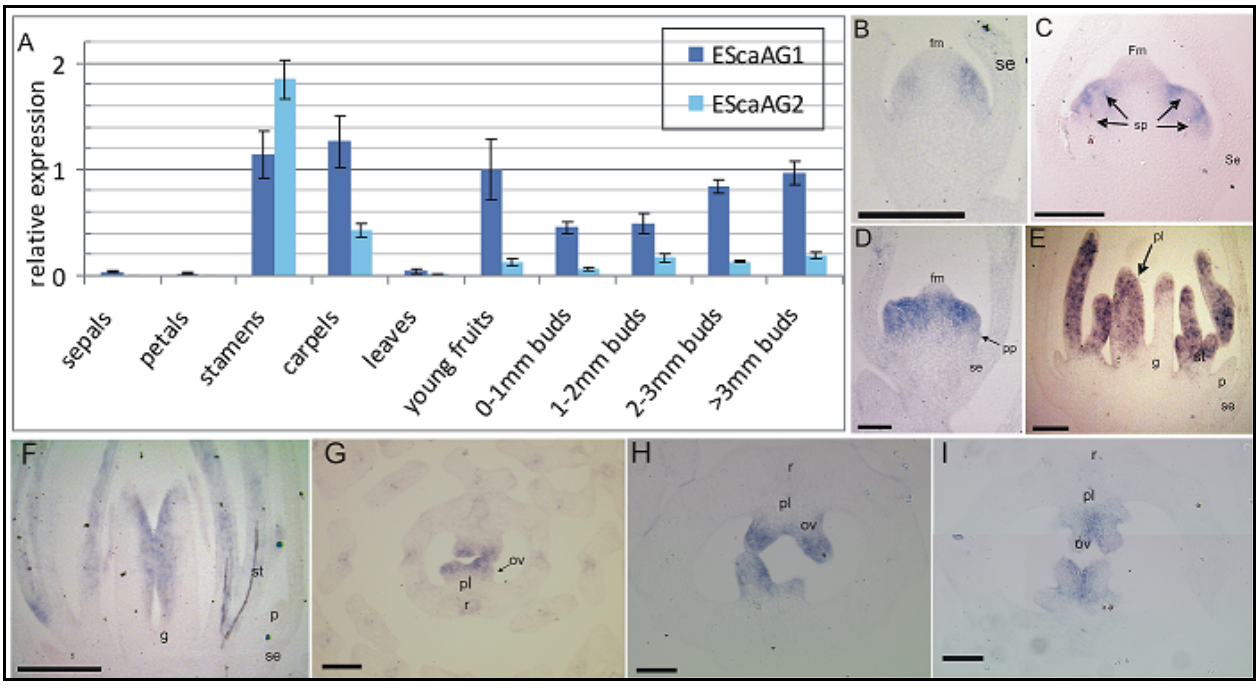

Fig. 7: Expression analysis of *EScaAG1* and *2* genes in wild type plants analyzed through qRT-PCR and *in situ* hybridization

A) qRT-PCR based relative expression analysis of *EScaAG1* and *EScaAG2* in various tissues of wild type *E. californica*. *Actin* and *GAPDH* were the reference genes. B-I) Expression domains of *EScaAG1/2* genes in bud stages from 2 to 9. B) longitudinal section of bud at stage 2; C) longitudinal section of bud at stage 3; D) longitudinal section of bud at stage 4) E: longitudinal section of bud at stage 6; F) longitudinal section of bud at stage 7; G) transverse section of bud at stage 7; H) transverse section of bud at stage 8; I) transverse section of bud at stage 9. All scale bars = 100 μm.

Abbreviations: fm, floral meristem; g, gynoecium; gw, gynoecium wall; ov, ovule; p, petal; pl,placenta; pp, petal primordium; se, sepal; sp, stamen primordium; st, stamen.

Additionally, *in situ hybridization* was carried out by Svetlana Orashakova to obtain more detailed spatio-temporal expression domains of *EScaAG1* and *2*. However, as the open reading frames and UTR's of *EScaAG1* and *EScaAG2* are highly similar, it was difficult to generate probes that could discriminate between both paralogous genes. Hence, the expression patterns observed were considered common to both paralogues and it is described later on as *EScaAG* gene expression.

The *EScaAG* gene expression was first observed in stage 2 buds before the initiation of the gynoecium and was visible as lateral domains in a few cells in the floral meristem where in the stamen primordia were found to be initiated later (Fig. 7B). In stage 4 buds, the expression was uniformly expanded in the floral meristem but was excluded from the central primordium where in the gynoecium developed later (Fig. 7C). By late stage 4, *EScaAG* expression was stronger in the boundaries between the stamen anlagen and weak expression at the tip in the floral meristem just before gynoecium initiated (Fig. 7D). In stage 6, strong expression was found in the region adjacent to the placenta, the apical part of the medial carpel wall and in the stamens (Fig. 7E). Later on in late stage 6, *EScaAG1/2* expression was restricted to the adaxial side of the gynoecium and in the stamens (Fig. 7F). In transverse sections of the developing flower bud, *EScaAG* expression was confined to the apical part of the ovules and it was absent in the placenta. In later stages of ovule development, the *EScaAG* expression was stronger on the adaxial side compared to the abaxial side (Fig. 7G, H, I). In summary, *EScaAG* genes were

expressed during initiation of floral meristem at stage 2, during early development of stamen and carpel primordia and later in the developing stamens and ovules.

5.1.3 Functional analysis of *EScaAG* genes in conferring stamen identity

Functional characterization of *EScaAG1* and *EScaAG2* genes was accomplished through Virus-Induced Gene Silencing (VIGS). *AGAMOUS (AG)* is involved in specifying stamen and carpel identity and floral meristem determinacy in *A. thaliana* (Yanofsky et al., 1990)**.** The down-regulation of *EScaAG1* and *EScaAG2* expression was carried out by infecting 1) a set of 120 plants with pTRV1 + pTRV2- *EScaAG*1, 2) 120 plants with pTRV1 + pTRV2-*EScaAG*2, 3) 120 plants with pTRV1 + pTRV2-*EScaAG1/AG2* and 4) the control treatments consisted of a set of 12 plants infected with pTRV1 + pTRV2-E, an empty vector as a negative control and 5) another set of 12 untreated wild type plants as a positive control. The first three flowers of each plant were scored and based on the strength of the silenced phenotype the flowers were categorized into four types: a) wild-type resembling flowers that did not show any obvious silencing effect. b) weak phenotypic flowers without any altered floral morphology except an increase in the stamen number. c) medium phenotypic flowers showing partial homeotic conversion of stamens into petals and flattened or normal gynoecium with or without enclosed ovules. d) strongly silenced flowers with no obvious stamens in the 3rd whorl along with an orange colored gynoecium with enclosed additional gynoecium or additional flower inside the 4th whorl.

In the first set of 120 plants inoculated with pTRV2-*EScaAG*1, a total of 239 flowers were analyzed, out of which 122 flowers (51%) showed homeotic conversion of stamens and carpels (Tab.1). Similarly, in the second set of the 120 plants infected with pTRV2-*EScaAG*2, 209 flowers were analyzed, out of which 118 flowers (56.4%) expressed homeotic transformation of reproductive structures. In the third set of 120 plants infected with the dual gene knock-down cassette pTRV2-*EScaAG*1/*AG2*, out of 261 flowers analyzed, 174 flowers (66.6%) showed homeotic transformations in the 3rd and 4th whorls of the flower. On the other hand, in all the 36 flowers observed from 12 plants of pTRV2-E had neither homeotic conversions nor signs of loss of floral meristem termination (Tab.1).

Table.1: **Summary of total phenotypes observed during the VIGS-*EScaAG* down-regulation**

	Phenotypes observed	pTRV1/ pTRV2-E	pTRV1/ pTRV2-EScaAG1	pTRV1/ pTRV2-EScaAG2	pTRV1/ pTRV2-EScaAG1+2
1	No. of inoculated plants	12	120	120	120
2	No. of analyzed flowers	36	239	209	261
3	No.of flowers showing phenotype in the 3rd and 4th whorls	0	122 (51.0%)	118 (56.4%)	174(66.6%)
3.1	No.of flowers with homeotic conversions in the stamens	0	67 (54.9%)	53 (44.9%)	113 (64.9%)
3.1.1	No.of flowers with transformation of all the stamens into petals	0	3 (4.4%)	8 (15%)	17 (15%)
3.1.2	No.of flowers showing only outer stamen whorls converted into petaloid organs	0	64 (95.5%)	0	0
3.1.3	No.of flowers showing only inner stamen whorls converted into petaloid organs	0	0	45 (84.9%)	0
3.1.4	No.of flowers showing only outer and inner stamen whorls converted into petaloid organs	0	0	0	96 (84.9%)
3.2	No.of flowers with alterations in the carpels	0	27 (22.1%)	31 (26.2%)	40 (22.9%)
3.2.1	No.of flowers with flattened green gynoecium	0	23 (85.1%)	26 (83.8%)	32 (80%)
3.2.2	No.of flowers with an orange pigmented gynoecium	0	4 (17.3%)	5 (16.1%)	8 (20%)
3.3	No.of flowers showing defects in the floral meristem termination	0	62 (50.8%)	80 (67.7%)	110 (63.2%)

E. californica wild-type flower consists of two fused sepals occupying the floral whorl one, four petals arranged alternatively in two consecutive whorls, varying number of 18 to 34 stamens

arranged in four to five whorls and central whorl containing a bicarpellate gynoecium (Becker et al., 2005). Downregulation of *EScaAG1/2* genes in *E. californica* resulted in homeotic conversion of stamens into petals in the 3rd whorl, loss of carpel identity into petal-like structures in the 4th whorl. Furthermore, loss of floral meristem determinacy was observed in two types of meristems, one in the ring meristem in third whorl and another one in the central floral meristem in the fourth whorl. Additionally, the ovule development was severely hampered in *EScaAG* down-regulated plants.

The down-regulation of *EScaAG* genes in VIGS treated plants was analysed by the quantitative RT-PCR (qRT-PCR) and phenotyping analysis. Both the analyses were confined to the first three flowers of each plant and avoided the later formed flowers as the frequency of putative knock-down phenotype is known to decrease in the successive flowers (Wege et al., 2007). The downregulation of *EScaAG* genes expression was examined by qRT-PCR on first buds of 1 to 3 mm diameter (corresponding to bud stages 2 to 9), in *EScaAG1, EScaAG2,* and *EScaAG1/2* VIGS silenced plants. The knock-down expression analysis of the first buds was correlated with the phenotype of the later formed 2nd and 3rd flowers of that plant. The scenario was found to be inconsistent with previous experiments wherein VIGS downregulated flowers of *E.californica* in 99% of the cases (n = 414), when the second flower of the plant showed a stronger phenotype, the first flower of that plant exhibited strongest phenotype (Orashakova et al., 2009; Wege et al., 2007). This persistent nature of phenotypes allowed to analyse the knock-down expression of a gene in the first buds through quantitative RT-PCR based on the phenotype of the second flower.

The differential expression pattern of *EScaAG* genes through qRT-PCR in VIGS-*EScaAG* treated plants revealed that both genes were down-regulated irrespective of the gene targeted for down regulation. This suggests that the observed phenotypes were resulted from overlapping effect of both *EScaAG* paralogues (Fig.8). Based on the qRT-PCR analysis, the expression of *EScaAG1* was reduced from 70% to 10% of its wild-type expression and *EScaAG2* expression was reduced from 25% to less than 5%. In case of the pTRV2-*EScaAG1/2* vector, both genes were reduced similarly to around 20%. Six out of seven plants buds selected for qRT-PCR analysis showed silencing of both *EScaAG1* and *EScaAG2* genes and one plant (pTRV2:AG2-1) treated with pTRV2- *EScaAG2* exhibited reduction of *EScaAG2* expression but increased *EScaAG1* expression relative to untreated plants. This might be due to the variability of VIGS experiments.

However, the rest of the six buds showed a significant reduction of expression of *EScaAG* genes as expected.

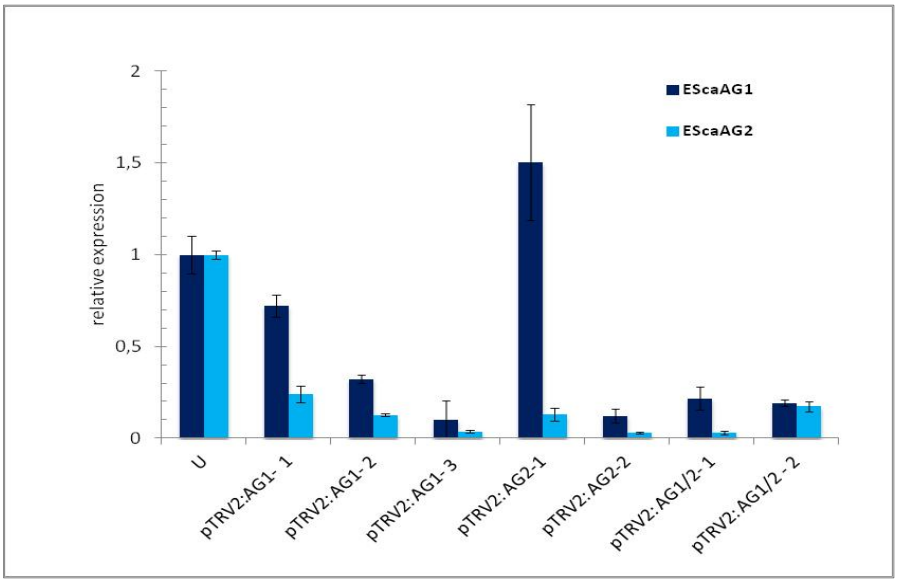

Fig.8: qRT-PCR analysis of first bud of *E. californica* untreated (U) and VIGS treated plants.

Individual plants treated with pTRV2-*EScaAG1,* pTRV2-*EScaAG*2 and pTRV2-*EScaAG1/AG2* respectively. The numbers at the end of each treatment on X-axis indicate the plant number of corresponding treatments. The relative expression of *EScaAG1* and *EScaAG2* are set at one in untreated plants.

In VIGS-*EScaAG* treated plants, the homeotic transformation of the 3rd whorl of stamens into petals has occurred in three ways. In the first case, i) complete conversion of all the stamens (4 to 5 whorls) into petals; ii) the second scenario comprised of partial conversion of only a set of stamen whorls into petaloid-like organs and the rest of the stamen whorls remained normal without any homeotic transformation; iii) the third case consists of an increase in the number of stamens without any homeotic conversions. Out of these three scenarios, first and third scenarios were occurred in both *EScaAG* paralogues irrespective of the gene down-regulated. On the other hand, the second scenario of partial homeotic conversion of only few whorls of stamens into petals occurred in a paralogue specific manner. In pTRV2-*EScaAG*1 infected plants, 64 flowers

(26.7%) expressed partial homeotic transformation of only the outer stamen whorls into petaloid organs (Fig.9 G, I), and three flowers (1.25%) showed a complete homeotic conversion of all stamens into petals (Fig.9 B).

In the down regulation of the pTRV2-*EScaAG*2 gene, 45 flowers (21.5%) exhibited homeotic conversion of only the inner whorl of stamens into petaloid organs (Fig.9 H, J) and 8 flowers (3.8%) exhibited complete homeotic transformation of all stamens into petals (Fig.9 C). Furthermore, in plants infected with pTRV2-*EScaAG1/AG2,* 96 flowers (36.7%) showed partial homeotic conversion of outermost and innermost whorls of stamens into petals with normal stamen morphology in the middle whorl(s) (Fig.9 K) and 17 flowers (6.5%) displayed complete homeotic transformation of all stamens into petals (Fig.9 C,D,F).

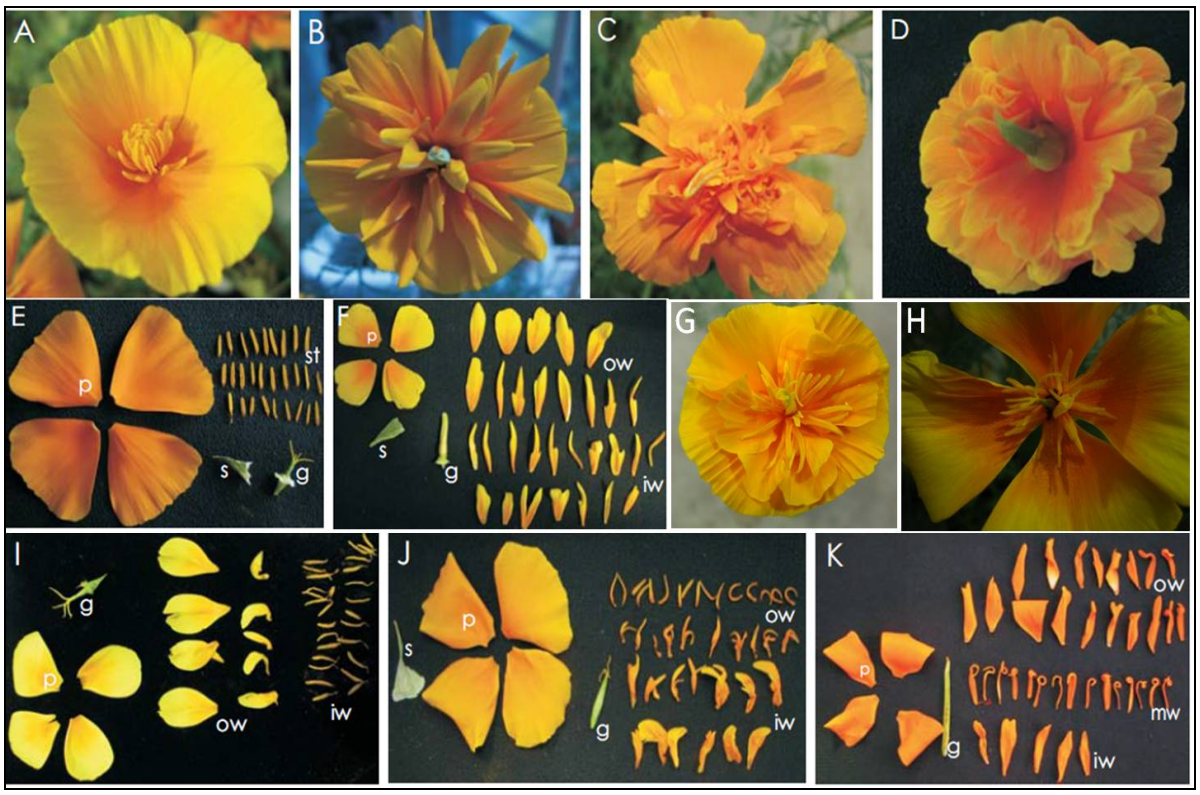

Fig.9: Phenotypes of VIGS-*EScaAG* treated plants with homeotic transformation of stamens into petals.

A, E: wild type flower; B, C, D: completely transformed stamens into petals in VIGS-E*ScaAG1/2* phenotypic flowers; G, I: partial transformation of outer whorl of stamens into petals in VIGS-*EScaAG1* phenotypic flowers; H, J: partial transformation of inner whorl of stamens into petals in VIGS-*EScaAG2* down regulated flowers; K:

transformation of outermost and innermost whorl of stamens into petals in *EScaAG1/2* down regulated flowers.

Moreover, the homeotic transformation of stamens occurred in a gradual transition ranging from mosaic staminoid-petaloid structures to complete petal like organs (Suppl.Fig.2 M). On the other hand, 36 flowers from the plants infected with pTRV1 and pTRV2-E were analysed and all of them had shown normal stamens and carpels as of wild type (Suppl.Fig.2 L). Homeotic transformation of neither stamens nor carpels was observed.

5.1.4 VIGS-*EScaAG* genes display a loss of carpel identity

The wild type gynoecium in *E. californica* is green coloured cylindrical structure. Down regulation of *EScaAG* function in California poppy has resulted in the transformation of carpels into petal-like structures. The homeotic transformation of the gynoecia has occurred in two forms, either into a (i) flat green gynoecium or (ii) flat orange gynoecium (Fig.10 B, C, D) in the 4th whorl. The former appears as flat and slender compared to wild type gynoecia, the latter type was flat, more petal like with orange color. In addition to that, both flat green and flat orange gynoecia were either empty or enclosed very few ovules. In some other instances, the flat green gynoecium had multiple gynoecia enclosed inside.

Fig.10: Homeotic transformations of the gynoecium in VIGS-*EScaAG* treated plants.

A: Green and solid gynoecium of wild type plant on the left side and flat green gynoecium without ovules on the right side; B: flat green gynoecium; C: flat orange gynoecium without ovules; D: petal like gynoecia of VIGS treated plants

In case of pTRV2-*EScaAG*1 inoculated plants, 24% of 239 flowers showed flat green gynoecia and 12.7% displayed orange petal like gynoecia. In case of pTRV2-*EScaAG*2 down regulated plants, 31.4% of 209 flowers exhibited flat green gynoecium and 21% flowers showed orange gynoecia. Whereas in pTRV2-*EScaAG1/2* double knockdown plants, 36% of 261 flowers showed flat green and 20.5% flowers displayed orange gynoecia (Suppl.Fig.3).

The flat green gynoecium was similar to wild type gynoecium in its external morphology; however, orange petal-like gynoecium consisted of striated regions of orange- green colors. The mosaic pattern of gynoecium was further analysed through Scanning Electron Microscopy (SEM) to deduce the cellular structure. In general, the petal surface of wildtype flower in *E.californica* consists of parallelly spaced long narrow tubular cells (Fig.11 A) and the carpel surface consists of small compact cells with scattered stomata (Fig.11 B) (Becker et al., 2005). SEM micrographs of flat orange gynoecia in VIGS-*EScaAG* flowers demonstrated the mosaic pattern of long, narrow, and tubular petal cells running beside the small, compact cells of carpel surface with scattered stomata (Fig.11 C, D). These observations indicated that the orange-pigmented gynoecia of *EScaAG1/2* downregulated flowers had not only a partial petal-like pigmentation but also acquired the characteristics of petal-like cell surface and thereby confirming the partial transformation of gynoecia into petal-like organs.

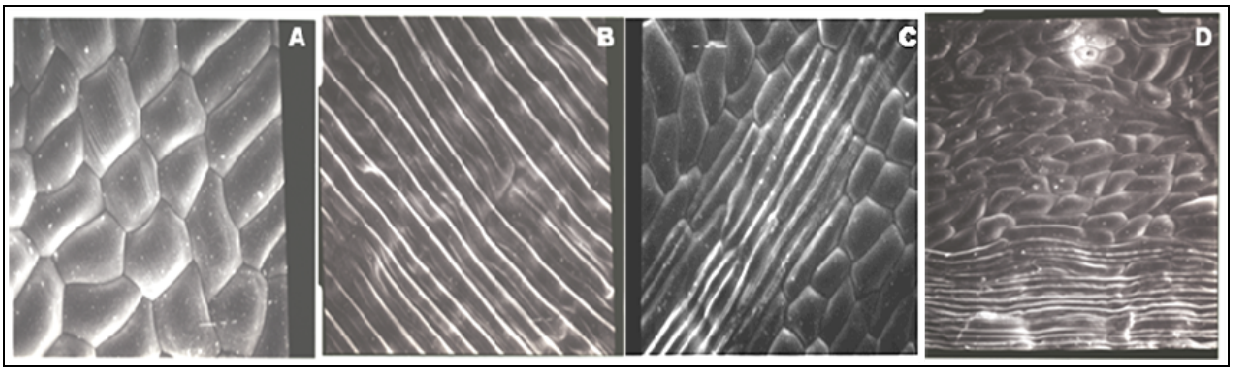

Fig 11: Scanning Electron Micrograph (SEM) analysis of surface structure of petaloid gynoecium

A: the wild type carpel tissue; B: wild type petal surface; C& D: petaloid gynoecium in the central floral whorl of VIGS-*EScaAG* plants showing long, narrow tubular petal cells running parallel to small, compact carpel cells containing stomata.

The orange petal-like gynoecia of *EScaAG* down-regulated plants with inferred petal cellular characteristics were further tested for the expression domains of B- class genes. qRT-PCR was conducted to further analyse the hypothesis that the expression domains of floral homeotic B-genes were expanded to the central gynoecium whorl of *EScaAG1* and *2* VIGS treated plants. Three B-class genes namely *EScaDEF1, EScaDEF2*, and *EScaGLO* and two C- class genes: *EScaAG1* and *EScaAG2* of *E. californica* were examined. Two types of tissues were selected for analysis; i) carpels at anthesis stage of the flower (mature carpels) and ii) carpels at pre-anthesis stages (young carpels). The samples were collected from untreated, pTRV2-E mock plants, pTRV2-*EScaAG1, EScaAG2* and *EScaAG1/2* treated plants. qRT-PCR expression studies have shown that the C-class genes were expressed upto the expected level in the carpels at young and mature stages in the wild-type flowers. However, the B-class gene ortholog, *EScaDEF1* was expressed comparatively at a higher level than expected in the carpels of untreated plants, while the expression of other two B-class genes *EScaDEF2* and *EScaGLO* were hardly detectable in the gynoecium.

Subsequently, the expression of B and C- class genes were recorded in the gynoecia of VIGS treated plants. The relative expression of all analyzed genes in the gynoecia of untreated plants at pre-anthesis was normalized to one (Fig.12 A). In the gynoecia of VIGS treated plants (Fig.12 B), the expression of *EScaAG1* was reduced to about 20-50% and *EScaAG2* expression was highly reduced in most of the gynoecia. On the contrary, VIGS-*EScaAG* treatments had not influenced *EScaDEF1* expression in the gynoecia and it showed same level of expression in the gynoecium as in the wild type gynoecium. However, there was 5.8 to 17.7 fold increase in the expression of *EScaDEF2* as compared to the expression in untreated gynoecia. Additionally, *EScaGLO* transcripts were also increased significantly upon silencing the C- function genes by 2.2 to 5.7 times in the *EScaAG1* and *EScaAG2* VIGS treated plants. These expression analyses indicated that in the central whorl, with reduction of expression of *EScaAG* genes, there was a significant increment in the expression of two B-function genes *EScaDEF2* and *EScaGLO* in

EScaAG1 and *EScaAG2* down-regulated plants compared to the untreated or mock-treated plants.

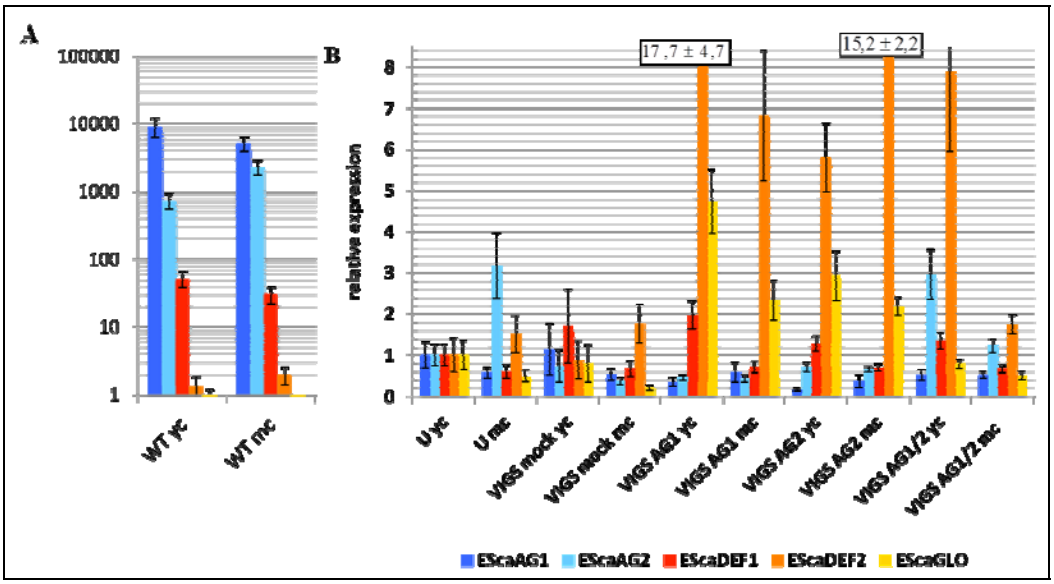

Fig.12: qRT-PCR expression analysis in young and mature carpels.

A: qRT-PCR of *EScaAG1, EScaAG2, EScaDEF1, EScaDEF2,* and *EScaGLO* in the gynoecia of wild-type plants; B: qRT-PCR of *EScaAG1, EScaAG2, EScaDEF1, EScaDEF2,* and *EScaGLO* in the gynoecia of VIGS treated plants.

Abbreviations used here are yc- young carpel, mc- mature carpel, U- untreated plants.

5.1.5 *EScaAG1* and *EScaAG2* both regulate floral meristem determinacy

Downregulation of *EScaAG* genes affects the floral meristem determinacy in various degrees based on the strength of the gene silencing. A strong reduction of *EScaAG* gene expression has resulted in the severe phenotypic changes and a slight reduction of *EScaAG* transcripts produced weak phenotypes. The strong phenotypic flowers exhibited prolonged floral meristem activity in the form of either additional floral organs or carpel-like structures, or additional gynoecium enclosed inside the normal gynoecium in the 4th whorl (Fig.13 B- J).

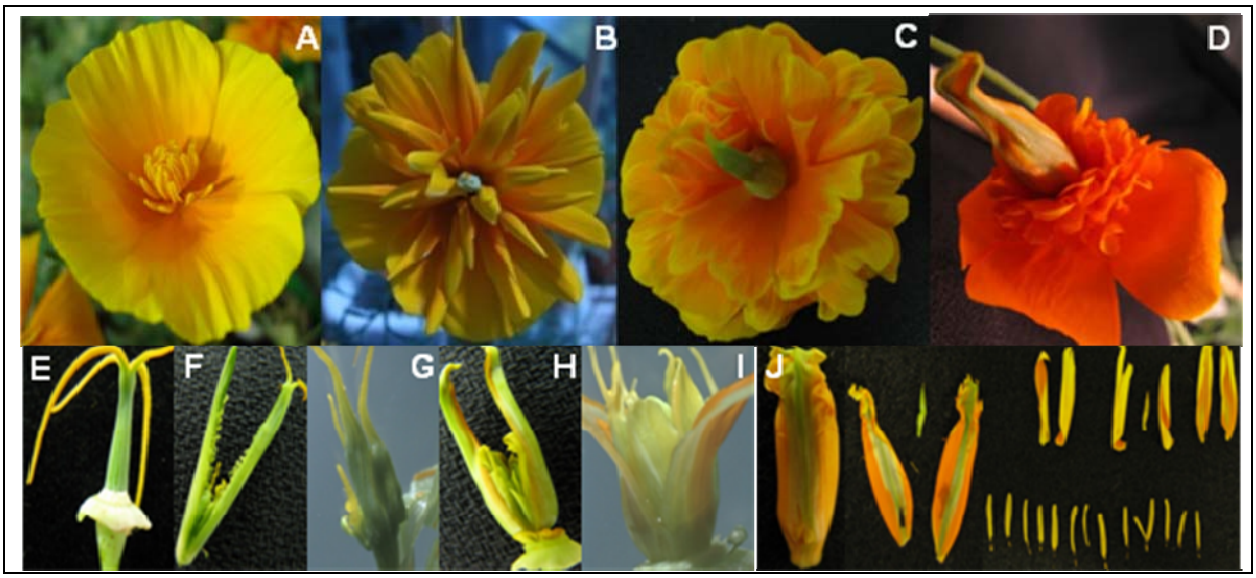

Fig. 13: Loss of floral meristem determinacy in VIGS-*EScaAG* flowers.

A,E: wild type flower and gynoecium; B,C,D:VIGS-*EScaAG* flowers showing enclosed multiple gynoecia inside the petaloid gynoecium; F,G,H,I: gynoecium enclosing additional carpel-like structures; J:enclosed additional flower inside the gynoecium in the 4th whorl.

In very weak phenotypic flowers, there were no obvious homeotic organ conversions. However, a significant increase in stamen number in the VIGS-*EScaAG* treated plants was observed (Tab 2). Under normal conditions, *E. californica* flowers produce 18 to 26 stamens that are arranged in 4-5 consecutive whorls. Here, the untreated or wild type plants that were grown under the same conditions as the VIGS treated plants on an average 26.3 stamens were produced per flower. Whereas in VIGS-*EScaAG1* treated plants without any homeotic conversions 31.9 stamens were produced, while in VIGS-*EScaAG2* treated plants 33.4 stamens were produced in each flower and in plants treated with *EScaAG1*/*AG2,* around 35.5 stamens per flower produced.

Table.2: Number of stamens in wild-type and *EScaAG* treated plants

	Untreated/pTRV1 and pTRV2-E treated	EScaAG1 VIGS treated	EScaAG2 VIGS treated	EScaAG1/2 VIGS treated
No. of flowers analyzed	28	244	242	333
No. of flowers without homeotic conversions	28	93	123	92
Average no. of stamens in flowers without homeotic conversions	26.2 ± 1.9	29* ± 4.1	28.2* ± 2.9	28.6* ± 4.5

* Significant change to untreated control plants (ANOVA test)

5.1.6 *EScaAG* paralogues regulate ovule identity

The down-regulation of *EScaAG1* and *EScaAG2* paralogous genes has further shown that they regulate the ovule identity. The VIGS-*EScaAG* downregulated phenotypic plants enclosed only sparse number of ovules inside the gynoecium. In weak phenotypic flowers, where no morphological defects were observed have shown normal ovule development. However, in medium phenotypic flowers, sparse number of ovules were produced compared to the wild type situation. Whereas in the severe phenotypic flowers with gynoecium as flat green structure or flat orange or petal-like structures, severe defects in the ovule development was observed. Neither the flat gynoecium nor the petal-like gynoecium enclosed the ovules, both types of gynoecia were completely empty without any ovules.

The D- class gene SEEDSTICK (*STK)* and *SHATTERPROOF* (*SHP1/2*) are responsible for specifying ovule identity in *A. thaliana*. In *E.californica,* the *STK* orthologue is *EScaAGL11*. Impaired ovule development in the *EScaAG1/2* down-regulation background hint that *EScaAG* genes might be involved in specifying the ovule identity along with *EScaAGL11* while *SHP* genes are absent in *E. californica* (Zahn et al., 2006).

5.2 Establishment of somatic embryogenesis and regeneration of unripe seeds in *E. californica*

For any plant species, some explant tissues are more amenable to transform than other tissues and at the same time, highly transformable tissues may not be amenable to regeneration. Development of protocols to transform a particular tissue is always associated with establishment of plant regeneration protocols for that same tissue. In the current project, unripe seeds were selected as a new explant source because of its ease of isolation and high amenability to regeneration. Thus as a first step, plant regeneration protocol was established using unripe seeds.

5.2.1 Seeds of a defined stage serve as explants for *E. californica*

The development of an efficient transformation system is a prerequisite for functional genomics studies of any crop species. To optimize the conditions for *Agrobacterium*-mediated DNA transfer of *E. californica*, establishment of callus induction and plant regeneration through somatic embryogenesis is mandatory. The explants that are amenable to regeneration through somatic embryogenesis were subsequently exposed to *Agrobacterium tumefaciens* for genetic manipulation of the same.

Furthermore, the optimal developmental stage is an important aspect for the selection of tissue to be used as an explant and therefore, different explant sources such as cotyledons and hypocotyls were selected and followed the published protocol in order to produce the embryogenic calli and regenerated plants through somatic embryogenesis (Park and Facchini, 2000). Cotyledons were excised from germinated seedlings after five days and incubated on primary callus induction medium (CIM) to induce the callus production. Hypocotyls were isolated by cutting below the cotyledons and incubated on CIM. In about 4-6 weeks, both explants were started producing the calli. The calli were transferred to somatic embryo induction medium (B5 medium containing1 mg L^{-1} NAA, 0.5 mg L^{-1} BAP and 8 g L^{-1} Phytoagar) as stated in the methodology (Park and Facchini, 2000). However, only few numbers of somatic embryos were regenerated from each callus during six weeks of incubation time (Table 3). Afterwards, the somatic embryos were transferred to plant regeneration medium (hormone free B5 medium and 8 g L^{-1} Phytoagar) and incubated for 4-8 weeks in order produce individual plantlets. Though the somatic embryos

developed healthy shoot system, root formation was completely absent even after eight weeks of incubation on plant regeneration medium. Therefore, individual plantlets could not be recovered even after several months of incubation due to lack of root system. In addition to that, aseptic isolation of cotyledons and hypocotyledons was highly laborious and tedious.

Table 3: **Regeneration of *E. californica* through somatic embryogenesis by using cotyledons and hypocotyledons as explants**

Experiment number	Explant used	No. of explants used	No. of calli produced	No. of somatic embryos produced	No. of plants regenerated
1	Cotyledons	200	69	4	0
2	Cotyledons	440	263	4	0
3	Cotyledons	760	167	0	0
4	Cotyledons	60	20	1	0
5	Hypocotyledons	340	191	0	0
6	Hypocotyledons	562	280	0	0
7	Hypocotyledons	52	28	2	0

All these reasons have led to the establishment of stable transformation of *E. californica* in our laboratory using a new explant source. Unripe seeds or immature seeds were selected as new explant source of *E. californica* and tested their callus induction potential. The immature seeds gave rise to embryogenic calli on CIM in 2-4 weeks of incubation time. However, consistent behaviour of callus induction was absent from the unripe seeds. Therefore, optimization of immature seed stage was found to be necessary in order to achieve stable transformation at a less laborious way. The ideal stage of the seed was determined based on different selection criteria. At first, the fruits were selected based on different sizes such as 3 to 5 cms, 5 to 9 cms and 9 to 12 cms. However, this parameter did not assist much to select an optimum stage of the seed. Secondly, maturity index of fruits was taken into consideration and based on that the fruits were categorized into green seeds and black seeds. Black seeds were always germinated into normal

seedlings, whereas green seeds produced calli sporadically. Thereafter, the green seeds were analysed further at different stages of their development.

Afterwards, days after anthesis (DAA) was selected as a third criteria and the fruits containing green seeds were collected at different days after anthesis (DAA) spanning from 10 to 27 DAA. These seeds were observed under light microscope and were categorized based on the significant changes in the seed structure (Table 4). At 10-14 DAA, the seeds contained light green, shiny seed coat and were filled with a translucent liquid endosperm (Fig. 14 A, B, C). There was no embryo-like structures visible under the light microscope possibly because of its small size. At 15-18 DAA, the seeds were characterized by a green and shiny seed coat with enclosed milky white liquid endosperm (Fig.14 D, E, and F). Consequently, the seeds collected during 20-24 DAA were green in colour with irregularly textured seed coat and without any shining. The seeds at this stage consist of milky but solid endosperm. However, the embryonic structures were still invisible under microscope (Fig.14 G, H, I). From 25 DAA onwards, the seeds were having a greenish-brown seed coat with irregular texture and without shining (Fig.14 J, K, and L). The endosperm was solid and milky. At this stage, the embryo has grown large enough and visible under light microscope as a heart-shaped differentiated structure (Fig.14 J).

Table 4: Characterization of seeds through light microscopic observation based on DAA

	DAA	**Thickness of fruit coat (mm)**	**Nature of endosperm**	**Description of seed coat**
1	10-14	0.2 to 0.5	Colour less & liquid consistency	Light green, shiny and smooth textured
2	15-19	0.6 to 0.9	Milky white & liquid consistency	Greenish, shiny, irregular textured
3	20-24	0.9 to 1.0	Milky white & solid consistency	Dark green and irregular textured
4	25-27	0.95 to 0.5	Milky white & solid consistency	Brownish green and irregular texture

Fig.14: **Microscopic examination of unripe seeds at different DAA**.

A, B, C: seeds of stage 1 having a liquid endosperm; D, E, F: seeds of stage 2 showing a milky, liquid endosperm; G, H, I: seeds of stage 3 having a solid and milky endosperm; J, K, L: seeds at stage 4 having a solid and milky endosperm with a differentiated heart-shaped embryo indicated by an arrow.

Afterwards, the morphological differences of seeds observed under a light microscope at different DAA were correlated with the callus induction efficiency.

5.2.2 Callus induction

About 20 seeds were collected from four representative developmental stages, spanning from 10 to 27 DAA and incubated on CIM for four weeks in order to define the optimal stage of the seed. Out of 40 seeds incubated at two developmental stages spanning from 10-19 DAA, only 3 % seeds produced calli and the remaining seeds were un-reactive without any response of either germination or callus production. On the other hand, at 20-24 DAA, on an average out of three technical replicates about 53% of the seeds produced white, embryogenic calli after two weeks of incubation on CIM medium (Table 5). Whereas the seeds from 25-27 DAA, on an average 11.5% calli produced and rest of the seeds were either germinated into normal plantlets on CIM medium or remained unresponsive during the four weeks of incubation.

The morphological examination of seeds at different DAA through light microscopy combined with the callus induction efficiency revealed that the seeds at 20-24 DAA produced high frequency of embryogenic calli. Furthermore, when the seeds from 20-24 DAA were analysed in detail, the seeds at 22 DAA were produced about 83 % of calli. This hints that 22 DAA is the optimum time frame in order to produce high-frequency of callus in *E. californica.*

Table 5A: Callus induction efficiency of unripe seeds at different DAA

	DAA	Number of seeds incubated	Average number of calli produced	Percentage of callus induction (%)
1	10-14	20	0	0
2	15-19	20	0.6	3
3	**20-24**	**20**	**10.6**	**53**
4	25-27	20	2.3	11.5

Table 5B: Callus induction efficiency of unripe seeds during 20- 24 DAA

	DAA	Number of seeds incubated	Average number of calli produced	Percentage of callus induction (%)
1	20	20	8.3	41.5
2	21	20	11.3	56.5
3	**22**	**20**	**16.6**	**83**
4	23	20	13.6	68
5	24	20	8.6	43

Nevertheless, due to practical difficulties in collection and subsequent processing of seeds at 22 DAA, the unripe seeds were collected in a time window of 20-24 DAA for subsequent experiments

5.2.3 Somatic embryogenesis and root induction of regenerated shoots/ plantlets

The root formation was completely absent in the regenerated plantlets after following the published protocol. Therefore, root induction medium was optimized by supplementing the plant regeneration medium (B5 medium, 8 g L^{-1} Phytoagar) with five different types of rooting hormones. The treatments as described in **Table 6** consisting of three hormone supplements namely NAA (**1-Naphthaleneacetic acid**), BAP (**6-Benzylaminopurine),** and GA3 (**Gibberellic acid)** enriched in the basic B5 medium at different concentrations. About five somatic embryos were taken for each treatment and incubated for one month. However, no roots were produced in any of the treatments.

Thereafter, the vitality of sucrose in the root induction was realized (through personal communication by Prof.Dr.Wolfgang Heyser, Plant physiology, University of Bremen) and eventually sucrose was added to the basic B5 medium at a concentration 30 g L^{-1} (Fig. 15). The

five somatic embryos incubated on plant regeneration medium supplemented with sucrose produced roots in two weeks of incubation time.

Subsequently, the importance of sucrose was realized in tissue culture media for somatic embryogenesis and root formation (Iraqi and Tremblay, 2001; Kamenicka, 1998) Therefore, sucrose was added at 30 g L^{-1} to SEIM also. The addition of sucrose has shown enormous effect on somatic embryogenesis by producing 70 somatic embryos per calli.

Table 6: **Optimization of rooting through the addition of supplements in the PRM**

B5 medium + concentration of hormone	**No.of shoots incubated/plate**	**No.of roots produced/shoot**
Basic B5 medium		
No hormones	5	No response
NAA (mg/L)		
0.5	5	No response
1.0	5	No response
1.5	5	No response
2.0	5	No response
BAP (mg/L)		
0.1	5	No response
0.2	5	No response
0.3	5	No response
0.4	5	No response
NAA+BAP (mg/L)		
0.1+0.3	5	No response
0.2+0.5	5	No response
0.3+0.8	5	No response
0.4+1.0	5	No response
½ MS+NAA (mg/L)		
0.5	5	No response
1.0	5	No response
1.5	5	No response
2.0	5	No response
IAA (mg/L)		
0.1	5	No response
0.3	5	No response
0.5	5	No response
0.8	5	No response
IAA+GA3 (mg/L)		
0.1+0.3	5	No response
0.2+0.5	5	No response
0.3+0.8	5	No response
0.4+1.0	5	No response
Sucrose (g/L)		
30	5	7-10

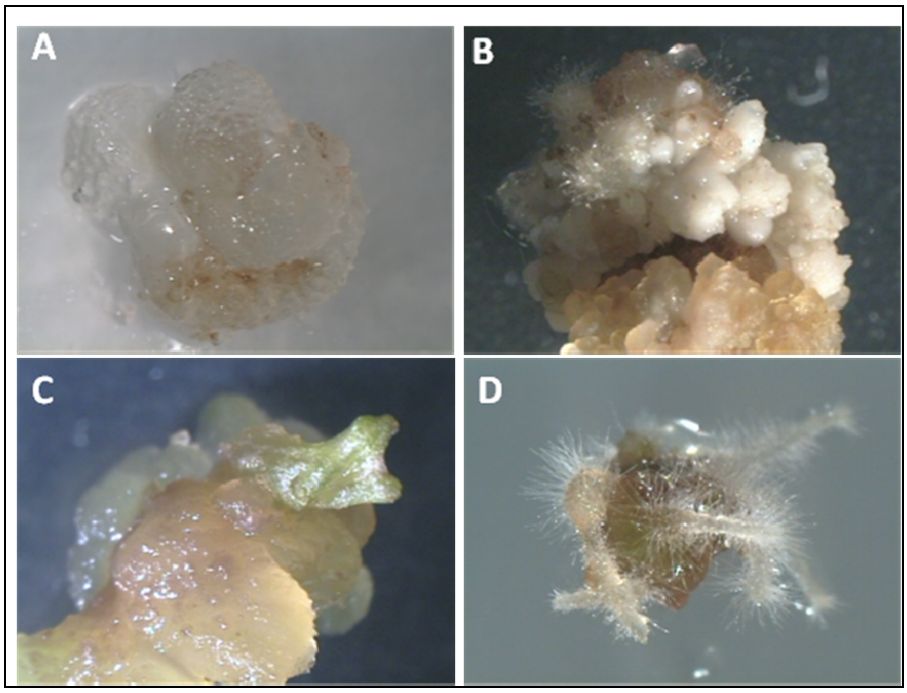

Fig 15: High-efficiency somatic embryogenesis and root induction through addition of sucrose to the growing media

5.3 *Agrobacterium*-mediated genetic transformation of *E. californica*

About 1000 unripe seeds at stage 3 (20-24 DAA) were collected and inoculated with four types of gene constructs as given below and 171 unripe seeds were untreated without any inoculation to serve as a positive control.

i) *Agrobacterium* GV3101 strain containing pMLBART_35S:EcCRC overexpression construct of *EcCRC*

ii) *Agrobacterium* GV3101 strain containing pHELLSGATE12_35S:hpRNAi:EcCRC knock-down expression construct of *EcCRC*

iii) *Agrobacterium* GV3101 strain containing pMLBART:GFP reporter gene for inducing constitutive expression of *GFP*.

iv) *Agrobacterium* GV 3101 strain containing pCX35S:GUS reporter gene for constitutive expression of *GUS*

The seeds after infection and co-cultivation have been grown on the CIM+ selection medium. The selection agent was added based on the vector construct used for the infection, either 10 mg L^{-1} Basta for pMLBART, 50 mg L^{-1} Paromomycin for pHELLSGATE12, or 10 mg L^{-1} Hygromycin against pCX35S and were incubated at 25 °C in the dark. The embryogenic calli were produced in about two weeks (Fig16 B). Subsequently, the resistant calli were transferred to SEIM and incubated for about four weeks. Afterwards, the SE's were transferred to PRM and incubated for one more month in the light conditions. The SE's were developed shoots and root system along with secondary somatic embryogenesis (Fig.16 D, E).

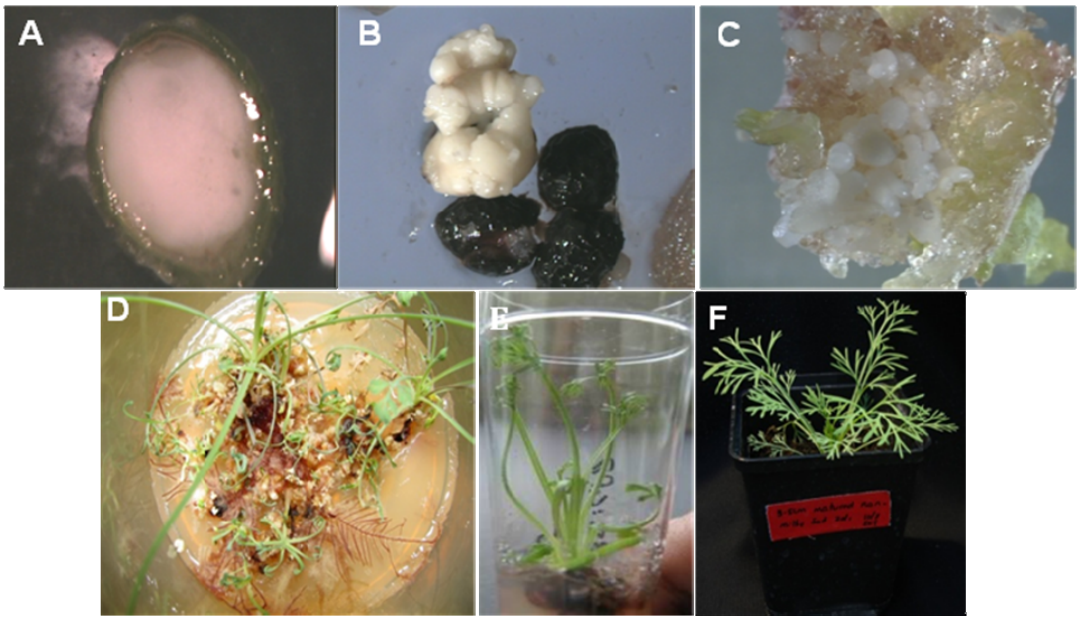

Fig.16: *Agrobacterium*-mediated stable transformation of *E. californica* through somatic embryogenesis and plant regeneration by using unripe seeds as a new explant source.

A: unripe seeds collected from stage 3; B: embryogenic calli produced in 2 weeks; C: somatic embryogenesis; D: plant regeneration; E: on regeneration medium with Gelrite; F: individual transgenic plant after hardening.

When the seeds were collected in a time frame of 20- 24 DAA, the percentage of callus production was about 52%, however, when the seeds were collected at a timeframe of 22 DAA, the callus induction efficiency was increased to 85- 90% in the untreated conditions. However,

due to *Agrobacterium* treatment, only 22% of the resistant calli were produced from pMLBART_35S: EcCRC overexpression construct and about 56% of resistant calli were recovered from pMLBART_35S: hpRNAi-EcCRC knock-down expression construct that were able to produce somatic embryos (Tab 7). Whereas about 59.2% of resistant calli were produced from the pCX35S:GUS construct, and about 41% calli produced from 35S::GFP construct and all the resistant calli were moved to the SEIM+ Sel medium.

Table 7: Transformation and regeneration of efficacy of unripe seeds in *E. californica*

Construct used	**pMLBART-35S:EcCRC**	**pHELLSGATE12-35S:EcCRC**	**pMLBART-35S:GFP**	**pCX35S:GUS**
No of seeds used	217	148	334	129
No.of seeds produced calli	45	32	65	27
No of calli produced SEs	10	18	27	16
No of plants regenerated	6	15	20	15
No of positive transformants	1	0	0	3
Percentage of Regeneration (%)	0.4	0	0	2.3

Genotyping of putative transformants was carried out by isolating the cDNA from leaf tissues of the wild-type plants and six putative transgenic plants transformed with *EcCRC* overexpression construct pMLBART_35S: EcCRC. It has been shown that *EcCRC* is not expressed in the leaves of wild-type *E. californica* plants (Fig.17 a) (Orashakova et al., 2009).

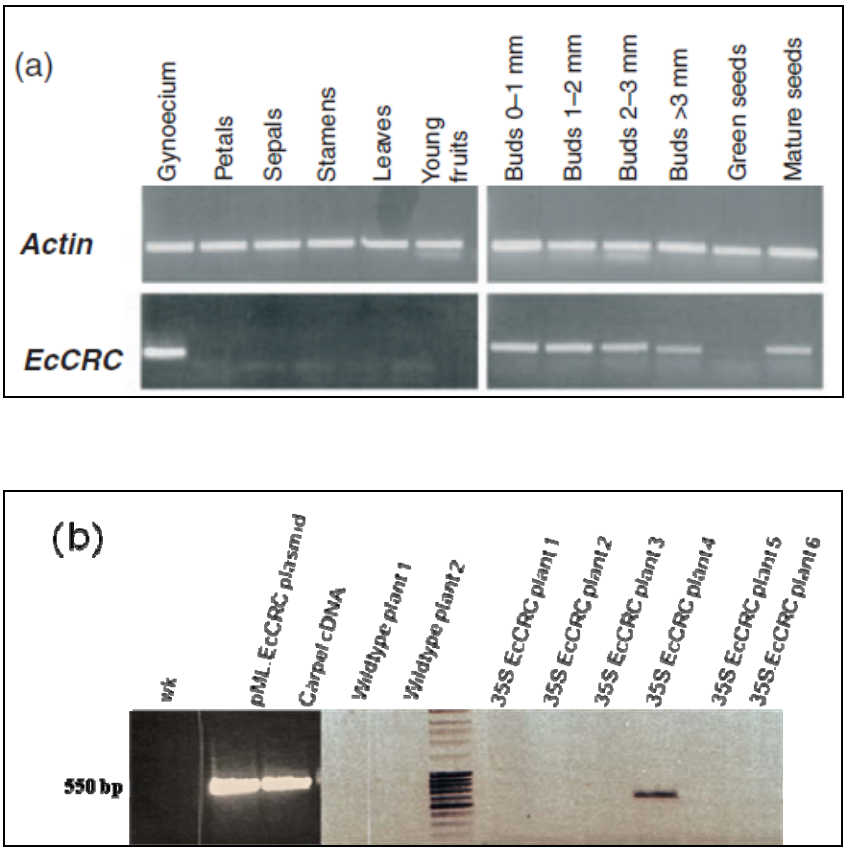

Fig.17: **Genotyping of putative transformants using RT-PCR on leaf cDNA**

(a) *EcCRC* expression profile in different tissues of wild type plant (b) RT-PCR on leaf cDNA of putative pMLBART_35S:*EcCRC* over expression lines.

Therefore, *EcCRC* gene specific primers were used to amplify a partial sequence of the 550 bp fragment from the cDNA isolated from leaf tissues of wild type and putative transformants of pMLBART_35S:EcCRC over expression lines. The result showed that out of the six independent lines of pMLBART_35S:EcCRC, only 1 (line 4) plant has shown to be positive (Fig.17 b). However, no altered phenotype was observed in the fruit or leaf morphology.

Further, the fruits were embedded in the FAE fixation solution and histology sections were observed under microscope for changes in the tissue organization of the fruits. However, there were no alterations in the tissue sections made from the fruits of pMLBART-35S:*EcCRC* overexpression lines (data not shown). This might be due to low copy number insertion. Usually low copy number insertion results in a low expression of the gene and it is obvious that if the

expression of a targeted gene does not reduce below a certain critical threshold level, that could not result in a mutant phenotype (Prelich, 2012). Also, there were no positive putative transformants observed from knock-down *EcCRC* construct and from the constitutive expression of pMLBART_35S:GFP construct. In contrast, in the constitutive expression lines of *GUS* construct, a few calli have shown positive signal after Gus staining. However, the signal was very week (Fig.18).

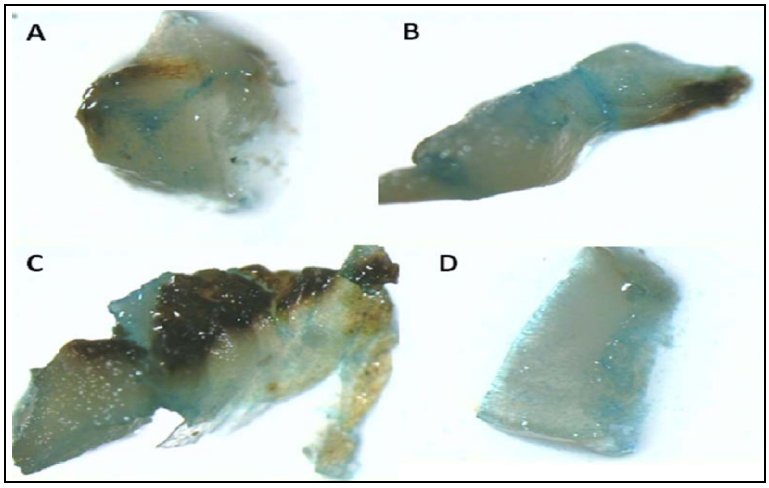

Fig.18: Histochemical GUS expression analysis of *E.californica* calli transformed with pCX35S:*GUS* construct through *Agrobacterium* using unripe seeds as explants.

6 Discussion

Homeotic mutants provide valuable source of information on molecular networks regulating plant development during evolutionary process. The present study focussed on the comparison of degree of conservation of C class genes in distantly related major plant groups such as *A. thaliana, O. sativa* and *E. californica*. The functional characterization of carpel developmental genes in California poppy was undertaken by deploying two genetic tools: *Agrobacterium*-mediated stable genetic transformation and virus-induced gene silencing (VIGS). Rapid and highly efficient VIGS methodology has been the main tool for detailed analysis of *EScaAG1* and *EScaAG2* paralogues in *E.californica*. The two *EScaAG* paralogues of *E.californica* were found to be recent duplicates of the *AG* clade with an estimated age of 51 million years as calculated by a penalized likelihood approach (Sanderson, 2003). This divergence time was obtained by using a maximum likelihood tree for the *AG* subfamily (Shan et al., 2009), calibrated with taxon ages as reported (Moore *et al*., 2007). It has long been suggested that several duplication events occurred in the *AG* clade (Becker and Theißen, 2003; Jager et al., 2003; Theissen et al., 1996). The duplication event in *E.californica* is found to be within the basal eudicots and is independent of a duplication event of eu*AG* genes in core eudicots (Zahn et al., 2006).

6.1 Functional analysis of *EScaAG1* and *EScaAG2* gene paralogues using VIGS

6.1.1 High sequence similarity at nucleotide and protein levels

The *EScaAG1* and *EScaAG2* paralogues are highly similar throughout the ORF and UTR regions. They shared 81.7% sequence similarity in the open reading frame and were 75.5% identical when the 5'UTR was included. Furthermore, both paralogues share 69% similarity when the two amino acid sequences were compared. It is generally believed that the duplicated genes will not survive for a long time in the genome unless they diversify either in expression and/or function (Liu et al., 2010). *In situ* hybridization have shown that *EScaAG1* and *EScaAG2* have similar expression patterns in stamens and carpels and *EScaAG1* is being expressed at much higher levels compared to *EScaAG2* (Zahn et al., 2006). However, no reduced constraint on *EScaAG2* was deduced from the analysis of the ratio of synonymous to non-synonymous nucleotide substitutions on the branch leading to *EScaAG2* (Shan et al., 2009). A recent shift in

constraint on *EScaAG2* may also not be detectable (Leebens-Mack and dePamphilis, 2002). Moreover, the molecular evolutionary analyses indicate that both *EScaAG1* and *EScaAG2* have been evolved under selective constraint for much of the approximately 50 million years since their duplication. Based on the above considerations, it has been inferred that both *EScaAG1* and *EScaAG2* genes are selectively maintained in the lineage leading to *E. californica*.

6.1.2 Differential expression pattern of *EScaAG* paralogues

From the RT-PCR and qRT-PCR expression studies, the general inference drawn is that the expression level of *EScaAG2* is lower than that of *EScaAG1* in all the tissues where it is being expressed. However, in stamens, *EScaAG2* is being expressed stronger than *EScaAG1*. Additionally, further qRT-PCR expression analysis in different stamen whorls has revealed more surprising results that there is a gradual increment of *EScaAG1* expression from the outer whorl of stamens to inner whorl of stamens and in the carpels. On the other hand, *EScaAG2* expression is stronger in the inner whorl of stamens compared to outer whorl of stamens and carpels (Lange et al., 2013). In conclusion, *EScaAG2* show its peak expression in the inner whorl of stamens and *EScaAG1* show its maximum expression in the carpels.

6.1.3 Homeotic conversions of VIGS-*EScaAG* paralogues genes

In *E. californica*, the two *AG* orthologous *EScaAG1* and *EScaAG2* act as C-function genes. In pTRV2-*EScaAG1* severe phenotypic flowers, the reproductive organs were homeotically converted into petaloid organs, and further perianth organs developed inside the fourth whorl. Hence, it can be suggested that *EScaAG1* is required to specify the identity of the stamens and carpels and to confer floral meristem determinacy. On the other hand, when the second paralogue *EScaAG2* was downregulated, the severe phenotypic flowers have shown homeotic conversion of stamens into petals and carpels into petaloid organs, and loss of floral meristem determinacy. In case of *EScaAG1/AG2* double gene knockdown, the phenotypic flowers revealed a complementary interaction of two genes and the whole scenario demonstrates that they are partially redundant. However, due to high sequence similarity between the two paralogues, the VIGS method could not be able to silence the paralogues individually and it was demonstrated

by qRT-PCR that in each single gene knock-down, there was a slight downregulation of the second gene too (Fig 8).

Nevertheless, in VIGS-*EScaAG1* medium phenotypic flowers, only the outer whorls of stamens were converted into petals and the inner whorls of stamens remained as stamens (Fig.9 G, I). In contrast, the inner whorls of stamens were converted into petals and outer whorl of stamens remained unchanged in VIGS-*EScaAG2* downregulated flowers (Fig.9 H, J). Moreover, in the VIGS-*EScaAG1/AG2* double gene knock-down medium phenotypic flowers, both the outermost and innermost whorls of stamens were converted into petals or petaloid organs and middle whorls remained as stamens (Fig.9 K). This is in correlation to their expression patterns, as *EScaAG2* show strongest expression in the inner whorl stamens and *EScaAG1* is strongly expressed in the outer whorl of stamens.

Besides that, the stamen identity in general is specified by the combination of B and C- class genes in the wildtype flowers, whereas in *sei-1* mutant flowers, the stamen identity is completely lost (Lange *et al*., 2013). Expression analysis of *EScaAG* paralogous genes through qRT-PCR in the *sei-1* mutant has shown that the expression of *EScaAG2* is strongly reduced compared to *EScaAG1* (Lange *et al*., 2013). Based on the deviated spatial distribution of the homeotic conversions of stamens into petals in different whorls (Fig.9 G-I), and differential expression pattern of the two paralogues in the stamens and carpels (Lange *et al*., 2013), a new dimensional role of *EScaAG2* in stamen development, mainly in the inner whorl of stamens is suggested. This kind of expression difference between two *AG* paralogues along with sub-functionalization was also proposed in *Thalictrum thalictroides* with a new dimensional role of *ThtAG2* in ovule identity (Galimba and Di Stilio, 2015). The distinct floral morphology of *E. californica* with variable number of spirally arranged stamens and developmental mechanism of ring meristem in promoting additional stamen whorls is proposed in this study.

Downregulation of *EScaAG* genes lead to homeotic conversion of carpels into flattened orange petaloid gynoecium with cell surface structure typical for petals (Fig.14 C, D). Whereas in *A. thaliana*, the *ag* mutant exhibit homeotic conversion of stamens into petals, carpels into sepals and this is due to antagonistic nature between A and C class genes; when C class genes are absent, the position is occupied by A class genes and results in the sepal identity in the 4th whorl (Coen and Meyerowitz, 1991). In *A. thaliana*, *SUPERMAN* (*SUP*) is a cadastral gene that prevents the expansion of expression domains of the B- function genes *AP3* and *PI* into the

fourth whorl and therefore carpels are converted to sepal-like organs (Sakai et al., 1995b). In *A. majus,* the *ple-1/far* double mutant exhibits homeotic conversions of stamens into petals, and carpels into petals (Schwarz-Sommer et al., 1990). The homeotic transformation of carpels into petaloid structures instead into sepals is due to an expansion of the B- function genes into the fourth whorl as a result of a C- function reduction. In case of *A. majus*, the putative *SUP* orthologue *OCTANDRA (OCT)* requires *PLE* or *FAR* to exclude B- function gene expression from the fourth whorl. Hence *OCT* function depends on the *PLE/FAR*, while *SUP* in *A. thaliana* acts independent of *AG* (Davies et al., 1999). The scenario of homeotic conversion of carpels into petal-like structures due to down-regulation of *EScaAG 1/2* paralogues is more similar to *A. majus* and is in contrast to *A. thaliana*. Moreover, the homeotic conversion of carpels into a petaloid gynoecium coincides with the expansion of the expression domains of two B class genes *EScaDEF2* and *EScaGLO* into the central floral whorl of *EScaAG1* and *EScaAG2* down-regulated plants. On the other hand, the third B- class gene *EScaDEF1* is expressed in the gynoecia of untreated and wild-type plants and its expression levels were unaffected by the reduction of C- class gene expression in the VIGS treated plants (Fig.12). These findings suggest that though the *EScaDEF1* expression is independent of class C gene expression, *EScaDEF2* and *EScaGLO* are negatively regulated by *EScaAG* genes in the central floral whorl. Hence, it can be proposed that the negative regulation of B- function genes in the fourth whorl may involve the activation of an unknown cofactor that could positively regulated by *EScaAG1* and *2* genes to restrict B- function expression to the second and third whorls in the wild type plants. Thus, the regulation of California poppy B- function genes in the 4th whorl is more similar to *A. majus* compared to *A. thaliana*.

This type of C- class dependent regulation of B- class genes was not observed in monocots also. In rice, the downregulation of *AG* homologues, *OSMADS58* and *OSMADS3* result in neither the transformation of carpels into lodicules nor the expansion of the expression domains of the B-class genes into the 4th whorl (Yamaguchi et al., 2006). This suggests a scenario of C-dependent B-gene expressions in the central 4th whorl and B-dependent C-expression (at least *EScaAG2*) in the 3rd whorl. This whole scheme hint three possibilities for the evolution of class C-dependent regulation of class B gene expression (i) This type of regulation had evolved before the monocot and eudicot lineages diverged but was lost independently, in lineages leading to Arabidopsis and rice (ii) The C-dependent regulation of B- expression evolved once in the eudicots before the

divergence of Ranunculales and was lost in the lineage leading to Arabidopsis after their split from the asterids. (iii) Class C genes were recruited twice independently, once in the lineage that led to *E. californica* after it diverged from the rest of the dicots, and a second time in the lineage leading to Antirrhinum after its divergence from the lineage leading to Arabidopsis. Owing to lack of C-class homeotic mutants from basal angiosperms or non-grass monocots, all three possibilities are equally considerable. The C-class dependent B- gene expression in *E. californica* as a representative of a basal eudicot lineage and *A. majus*, a member of the asterids clade might be more ancestral scenario compared to the C- independent regulation of B-class genes in *A. thaliana.*

6.1.4 *EScaAG* genes regulate the termination of meristem activity in both stamen and carpel whorls

A reduction of *EScaAG1* and *EScaAG2* functions in *E. californica* contributes to defects in floral meristem termination in both the stamen and the carpel whorls, albeit in a more complex pattern. The loss of meristem determinacy in *E. californica* was demonstrated as enclosure of an additional flower inside the gynoecium in the fourth whorl (Fig. 19 A) and which is similar to the phenomenon observed in *A. majus*. However, such a phenomenon observed is in contrast to the scenario in *A. thaliana,* where the additional flower is enclosed inside the third whorl (Davies et al., 1999).

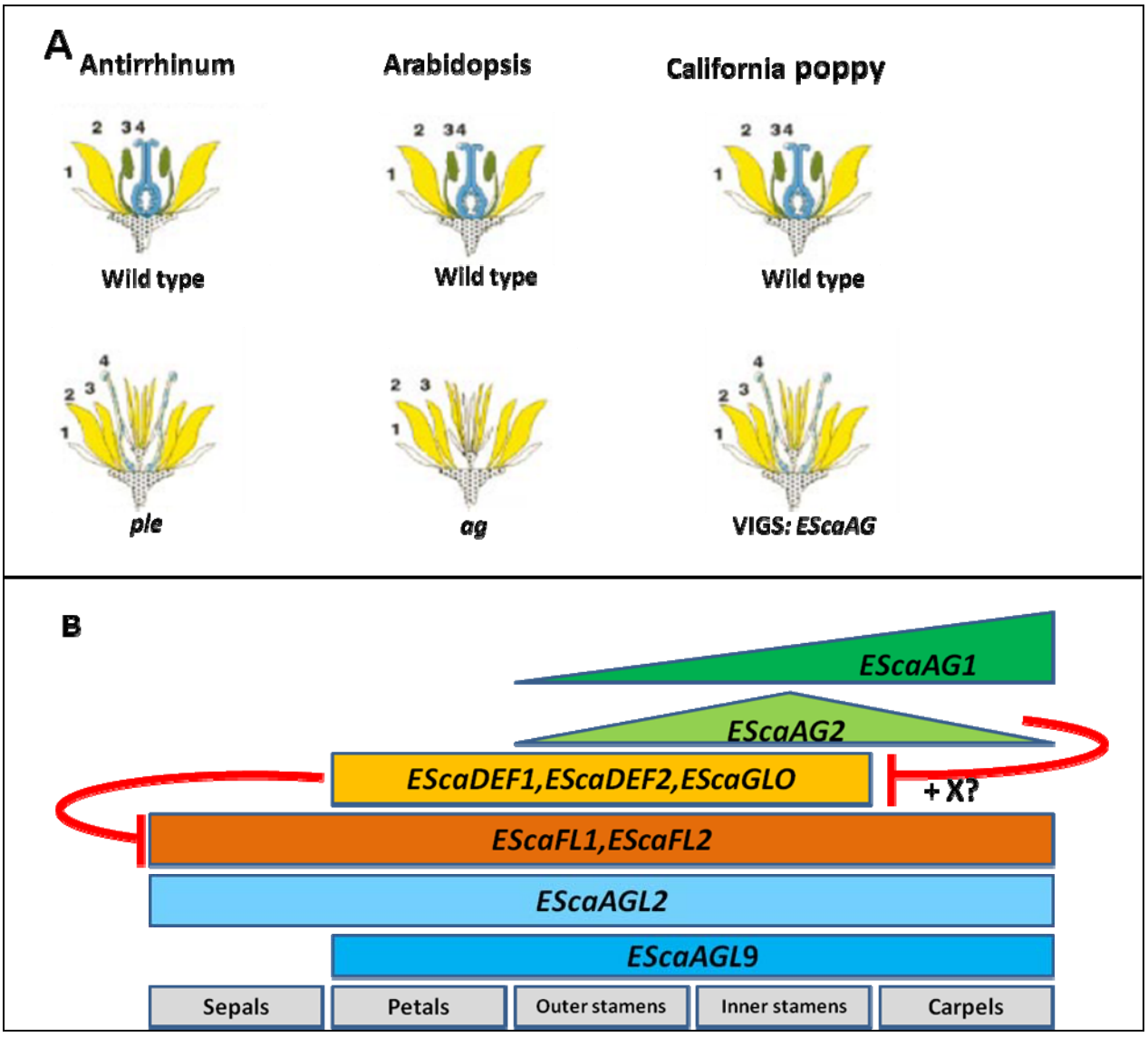

Fig.19 A: **Schematic representation of flower structure in Antirrhinum, Arabidopsis, and California poppy**.

The whorl numbers are highlighted to indicate the *AG* phenotypic differences in three species. The *ple* mutant in *A. majus* encloses additional flower inside the 4th whorl; in case of *A. thaliana, ag* mutant exhibits additional flower enclosed in the 3rd whorl, whereas in the case of *E.californica*, the scenario was more similar to *A. majus* with an additional flower encircled inside the 4th whorl.

B: Hypothetical model of C class dependent regulation of B class gene expression in *E. californica*.

The ABCE model of *E.californica* consisting of the B-class genes (yellow boxes) *EScaDEF1, EScaDEF2*, and *EScaGLO* that are supposed to be expressed in the second and third whorl. Out of two C class genes (green boxes), *EScaAG1* is expressed in a gradual increasing manner from outer whorl stamens to central whorl, whereas *EScaAG2* is expressed highest in the inner whorl of stamens. Two E class genes *EScaAGL2* (blue boxes) is expressed in all whorls and *EScaAGL9* is expressed in petals, stamen and carpel whorls (Viaene et al., 2010). Red bars indicate repression of gene expression and repression mechanism of *EScaAG1* and *EScaAG2* expression to the reproductive whorls of the flower is still enigmatic. Whereas the repression of *EScaDEF2* and *EScaGLO* by the C class gene *EScaAG2* is direct or mediated by a co-factor.

In addition to loss of floral meristem determinacy in the 4th whorl in *E. californica*, there was an indeterminacy in the 3rd whorl of VIGS-*EScaAG1/2* treated plants. About 38 to 40 floral organs were present in *EScaAG1/2* down-regulated plants in the 3rd whorl irrespective of the severity of the phenotype (Fig. 9 E,F,I,J,K). Moreover, the average number of stamens in the weak phenotypic flowers without any homeotic transformations was also found to be more than that of wild-type plants (Tab.2). These observations support the inferences drawn from *A. thaliana* and *A. majus* that even a mild reduction in C-class protein affects floral meristem determinacy (Causier et al., 2009; Mizukami and Ma, 1995). The morphogenesis of *E. californica* flowers differ from most core eudicots in a way that the innermost stamen whorls are still being formed when the central gynoecium is initiated. A ring of cells with meristematic activity is still maintained around the gynoecium while the central floral meristem is consumed in the process of gynoecium initiation. This study suggests that a mild reduction in *EScaAG1/2* expressions is sufficient for a prolonged meristem activity in this ring shaped meristem that produces additional stamen whorls in *EScaAG1* and *2* VIGS-treated flowers. The influence of pTRV2-*EScaAG1/2* on the stamen whorls is especially interesting as the number of stamens in the wild type *E. californica* are variable, ranging between 18-34 even under identical conditions and constant light (Becker et al., 2005). In *E. californica*, the number of stamens generally varies with the stature of the plant. Healthy plants produce more number of stamen whorls under well-grown

conditions, which has been also reported in *Stellaria media* (chickweed) (Haskell, 1949). The production of extra number of stamens from the ring meristem is hypothesized here to be determined by the quantity of *EScaAG2* expression in *E. californica* flowers. Even the slight differences in the timing and dose of *EScaAG2* transcript abundance between plants could result in variations in the number of stamens in the wild type plants. This might indicate a stature-dependent regulation of *EScaAG2* floral homeotic gene in the ring like meristem. Moreover, a direct link could exist between floral homeotic gene action and male fecundity in natural populations. This additional function of the *EScaAG2* in *E. californica* in the zone of meristematic activity around the gynoecium might represent a more general mode of function for class C genes in the large sub-group of angiosperms with several stamen whorls and often varying stamen numbers. The duration of class C gene activity in the meristems generating these stamen whorls might also determine the stamen number in these species. In general, zygomorphic flowers are more efficient in pollen transfer and needs less pollen. On the other hand, actinomorphic flowers with cross pollination mechanism needs more pollen grain in order to achieve more seed set (Walker-Larsen and Harder, 2000). However, the functional evolution of such morphological changes as increase in stamen number needs to be analyzed further.

In *A. thaliana, SUP* regulates the floral organ number and the *sup* mutation prompts the development of extra whorls of stamens inside the third whorl and which is at the expense of carpels (Hiratsu et al., 2002; Sakai et al., 1995a). In *A majus,* the *oct* mutation also exhibits a similar phenotype of extra whorl of stamens as *sup* in *A. thaliana*. Moreover, the *ple/far* double mutant of *A majus* and *ag/sup* double mutant of *A. thaliana* also causes the similar phenotype. This indicates that *PLE/FAR* share the functionality of *OCT* in this role (Davies et al., 1999; Schwarz-Sommer et al., 1990). In *E. californica,* the stamen number was enhanced in VIGS-*EScaAG1/2* phenotypic flowers but without the loss of carpel identity and is due to the presence of ring meristem. The extra numbers of stamens are produced in the inner whorls of stamens at the expense of ring meristem instead of consuming the central meristem. Owing to high expression pattern of *EScaAG2* in the inner whorl of stamens, it can be postulated that *EScaAG2* might be playing the main role in sharing the functions of *SUP* or *OCT* rather than *EScaAG1* in *E.californica*.

Furthermore, it can be speculated that the two types of floral meristems in *E.californica* flower, one is in the border of 3rd and 4th whorl as ring meristem and the other one in the central dome as

central floral meristem are regulated differentially by two *EScaAG* genes. Depending on their highest expression pattern, it can be postulated that *EScaAG1* is responsible for FMD in the central meristem and *EScaAG2* is responsible for the FMD in the ring meristem.

Additionally, the dose-dependent regulation of AG protein produces different range of phenotypes in *A. thaliana*, a mild loss of AG protein results in loss of reproductive organ identity and a severe loss of protein results in FM indeterminacy. Therefore, it has been suggested that high amount of AG protein is required to confer FMD and low amount of AG protein is sufficient to specify stamen and carpel identity (Sieburth et al., 1995). Hence, the small amount of protein loss leads to FMD in the central whorl without any homeotic transformations and indicates that FMD requires ample amount of protein compared to reproductive organ identity. Whereas in *E .californica*, a small amount of AG protein reduction resulted in the loss of ring meristem identity in the 3rd whorl without any homeotic conversions, medium amount of loss of AG protein resulted in sacrificing the reproductive organ identity and severe loss of AG protein resulted in the loss of FMD in the 4th whorl. This indicates that ring meristem requires high amount of protein compared to central FM and organ identity in *E. californica*.

Furthermore, the severe reduction of EScaAG protein in VIGS-*EScaAG1/2* flowers showed the loss of stamen identity and FMD, the carpelloid characteristics are still remained to be maintained. This might indicate that there are more carpel developmental genes acting in an *EScaAG* independent pathway. In *A. thaliana*, *agap2shp1shp2* quadruple mutant exhibited the complete absence of carpel features (Pinyopich et al., 2003). However, *SHATTERPROOF1/2* (*SHP1& SHP2*) genes are not present in *E. californica* (Zahn et al., 2006). On the other hand, the severe phenotypic VIGS-*EScaAG1/2* flowers with flat orange gynoecia were completely devoid of ovules consistently and the ovules were replaced with carpelloid structures enclosed inside the gynoecium. This is in correlation with the scenario in *A. thaliana* where *AG* play crucial role in ovule identity along with D- class gene *AGL11* (Pinyopich et al., 2003). This indicates that the ovule identity in *E. californica* is also determined by the interaction of *EScaAG1/2* and *EScaAGL11*. As the VIGS could not downregulate the complete expression of *EScaAG*, the residual amount of expression might be sufficient to specify the carpel identity or further cofactors involved in an *EScaAG* independent pathway needs to to be studied further through complete knockout mutants for *EScaAG* paralogues genes.

6.2 Somatic embryogenesis and plant regeneration using immature seeds of *E. californica*

A reliable and efficient regeneration system is a prerequisite for genetic manipulation of any plant species. Although there are successful transformation protocols available for *E. californica,* the direct application of published protocols could not be reproduced and further, the isolation of stated explants was highly time-consuming, laborious, and unsuccessful. Therefore, optimization steps have been developed for *E. californica* var *aurantiaca*. The plants were grown under controlled conditions in a greenhouse and immature seeds were collected to induce callus production. The stage of unripe seeds, the duration of the callus induction period, somatic embryogenesis, and root induction were established to optimize a regeneration protocol.

The developmental stage of the explant is a crucial factor for the *in vitro* culture and regeneration of plants. The quality of callus induction and regeneration capacity is strongly dependent on the developmental stage of the explant. A good observation parameter reflecting the physiological state of the explant is DAA. The seeds at 22 DAA had the highest regeneration potential and proved to be a suitable stage for *in vitro* culture of California poppy. However, based on seasonal variations, growing conditions, and pollination timings, a time window of around 22 to 24 DAA proves to be optimum for producing embryogenic calli. On the contrary, the seeds collected outside this time frame were found to be either unreactive or produce non-embryogenic calli or germinated into normal seedlings after four weeks of incubation.

Additionally, hormone free basic B5 medium could not induce roots and results in unsuccessful plant regeneration through somatic embryogenesis and therefore, culture medium was established for callus induction, somatic embryogenesis, and root formation by adding 30 g/L sucrose. Sucrose is one of the most important carbon sources in the micropropagation of plants mainly for somatic embryogenesis and root induction (Iraqi and Tremblay, 2001; Kamenicka, 1998). The addition of sucrose to the SEIM has been resulted in the production of 70 somatic embryos per calli. Moreover, there was a continuous production of secondary somatic embryos from the remaining calli. On the whole, a highly efficient regeneration system of California poppy was achieved by culturing unripe seeds at 22 DAA on B5 medium containing 30 g/L sucrose in all the growing media.

6.3 Establishment of *Agrobacterium tumefaciens* -mediated transformation

Although there are a number of publications available describing successful *Agrobacterium*-mediated transformation of California poppy by using various explants (Apuya et al., 2008; Park and Facchini, 2000), the ability to transform California poppy using *Agrobacterium tumefaciens* is currently restricted, and this might be partly due to the lack of clearly-written transformation protocols.

Agrobacterium-mediated transformation was carried out using unripe seeds collected at 22 to 24 DAA, inoculated for 20 min with *Agrobacterium* culture containing respective plasmid (OD=1.0) and co-cultivated for 2 days on CIM. Later on, the seeds were cultured on CIM + Timentin with potassium clavulanate (100 mg/l**)** + plant selection for 2-4 weeks, and sub-cultured for every 2 weeks interval on fresh medium. Then, the embryogenic callus was transferred to the regeneration medium. Although the transient expression of the *uidA* gene was observed, the *GUS* expression was low. Furthermore, in the *EcCRC* over expression putative transformants, though the transgene integration was confirmed through RT-PCR on leaf cDNA in one independent line, further detection by southern blotting was not successful. On the other hand, the putative transgenic line didn't show any altered phenotype. This might be due to low copy number insertion. These results suggest that though the unripe seeds were proven to be the good explant source for high throughput somatic embryogenesis and plant regeneration, *Agrobacterium* mediated genetic transformation still needs to be optimized further.

In order to increase the transformation efficiency, one of the possible routes is exposing the slightly wounded explants with *Agrobacterium* infection medium at different concentrations (OD_{600} 1.2-2.0). Additionally, infection should be carried out for different time periods in order to induce better inoculation and allowing the *Agrobacterium* to pass through different tissues of the seed to reach the embryo, as the embryo is present as a few cells deep inside the endosperm in California poppy. Another parameter that can further improve the transformation efficiency is changing the co-cultivation period. Longer co-cultivation periods can help the *A. tumefaciens* to infect the seeds in a more efficient way. However, all the mentioned parameters should be tried in a gradual manner with comparisons to the already established protocol, otherwise, too longer infection may cause the over growth of the *A. tumefaciens* and too shorter conditions could lead to poor transfer of *Agrobacterium* and there by poor transformation efficiency.

6.4 Advantages and disadvantages of VIGS and Stable transformation

Stable genetic transformation is one of the important molecular tools for the genetic engineering of plants. Stable transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium is the method of choice because of its exceptional ability to transfer the gene of our interest in the T-DNA region of the tumour-inducing (Ti) plasmid into the nucleus of target explant cells, where it is stably integrated into the host genome and transcribed (Hoekema et al., 1983; Petit and Tempé, 1978). Therefore, it is one of the attracted and most widely accepted techniques in the molecular breeding for improvement of agriculturally important and medicinal crops. Additionally, for functional genomics studies, as a reverse genetic tool, stable genetic transformation plays a pivotal role. Its role is more important in the functional characterization of perennial plants.

At the same time, stable transformation has its disadvantages. It is highly laborious and tedious because of tissue culture process. Furthermore, establishment of regeneration protocol for each genotype and individual explant source is tiresome. As an example, in case of barley, the whole process of regeneration from explant to generating plantlets takes about 10 months duration (Bartlett et al., 2008).

On the contrary, Virus-induced gene silencing (VIGS) has become a powerful technology in recent years for functional characterization of genes in a broad range of species. VIGS is an efficient system to analyze the gene functionality for both forward and reverse genetic studies. In the reverse genetic approach, it takes advantage of post transcriptional gene silencing (PTGS) phenomenon of the plant. PTGS is an RNA based silencing system of the plant which uses the plant innate defence system to down regulate the expression of gene of our interest (Watson et al., 2005).

Several dicots species were successfully infected by TRV based viral vectors. The main advantage of TRV based vectors is that they can infect meristamatic cells, hence can assist in functional genomics studies of flower and fruit development and aid in the evolutionary developmental genetic studies (Ratcliff et al., 2001; Wege et al., 2007). On the other hand, introduction of virus into plants is easy and thus established well in several model species. Furthermore, VIGS avoids the laborious tissue culture-based plant transformation procedures

and thus applicable to many recalcitrant species such as peanut (Tiwari et al., 2015), maize, barley, grapevine, pea, soybean, and so many other dicots (Becker and Lange, 2010). Wheat is the most recalcitrant cereal species to tissue culture *in vitro* (Shah et al., 2009). Stable transformation is still limited to a few responsive varieties with quite different transformation frequencies such as the model spring genotype 'Bobwhite' (Cheng et al., 1997; Hu et al., 2003). Usually, gene functional analysis is carried out through knockout mutants. Due to the polyploidy state of wheat genomes, functional redundancy of homologous genes hinder further analysis (Lawrence and Pikaard, 2003). In this case, VIGS has become the best alternative for simultaneous knockdown of expression of multiple related gene copies in polyploidy wheat (Manmathan et al., 2013).

Furthermore, partial sequence information is sufficient to silence a specific gene. VIGS is well suited to plants, where a complete knock-out or a mutation is lethal in embryo and seedling of sexually reproducing plant species (Burch-Smith et al., 2006). Additionally, it has been shown that VIGS can co-silence extremely redundant genes simultaneously by using a highly identical sequence.

Nevertheless, there are few limitations of VIGS methology, such as it cannot differentiate the functionality of highly redundant genes. At the same time, upon VIGS inoculation, depending on the penetrance of the virus, wide range of phenotypes were observed and hence large number of plants needs to be analysed (Becker and Lange, 2010). Hence VIGS can be used as a rapid and preliminary methodology to study the first hand functionality of a gene, which is followed by a stable genetic transformation results in confirmation of the gene function in detail. Stable transformants also provide further source material to study the interaction partners.

7 Conclusions and outlook

Gene function analysis of *EScaAG1* and *EScaAG2* in *E. californica* has brought out some interesting findings. Though *EScaAG2* showed similar expression pattern as *EScaAG1* through *in situ hybridization*, the present study through qRT-PCR expression analysis coupled with functional characterization has shown the importance of *EScaAG2* in stamen organ identity mainly in the inner stamen whorls and ring meristem activity.

Additionally, the second intron in *AG* acts as the promoter region and carries various *cis*-regulatory elements. The promoter region of *AG* in *A. thaliana* consists of several CArG box variants, multiple MYB binding sites and a single *LFY* binding site. CArG boxes are the binding sites for other MADS box proteins in order to form homo or hetero dimers. Hence *cis*-regulatory elements of *EScaAG1* and *2* needs to be analysed individually in order to understand the functional evolution of *EScaAG2* after gene duplication.

Besides that, the cadastral cofactors which are involved in the restriction of expansion of B class genes into 4th whorl along with *EScaAG1/2* is remaining as a blank still as there is no *SUP* orthologue identified in *E. californica.*

An another important gene involved in carpel development in *E. californica* is *EcCRC*, which regulates the central floral meristem determinacy in *E. californica*. Therefore the gene cascade and molecular mechanism involved in the two types of floral meristems in *E. californica* can explain the subfunctiuonalization of two *EScaAG* genes.

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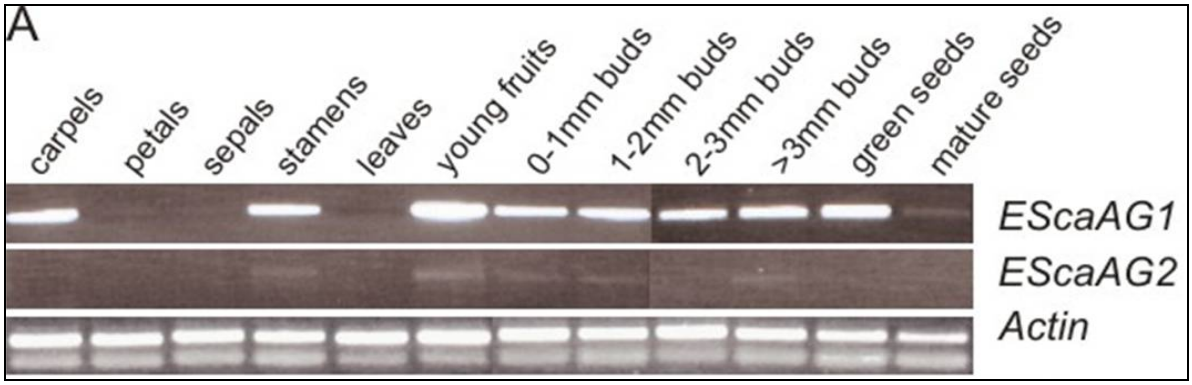
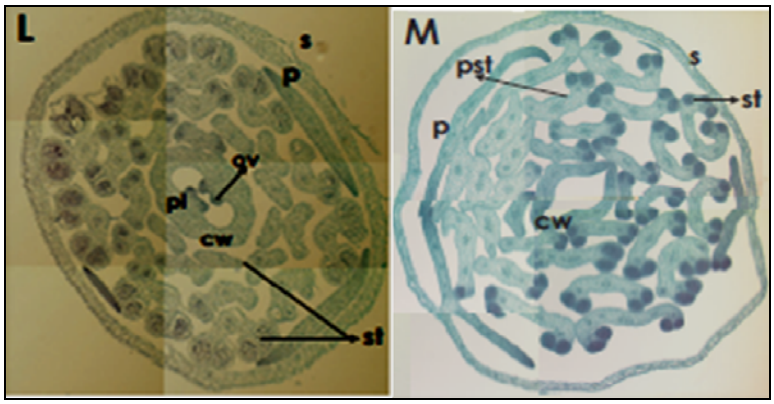
9 Appendix

9.1 Suppl.Fig.1: Relative expression analysis of *EScaAG* paralogues in *E. californica*



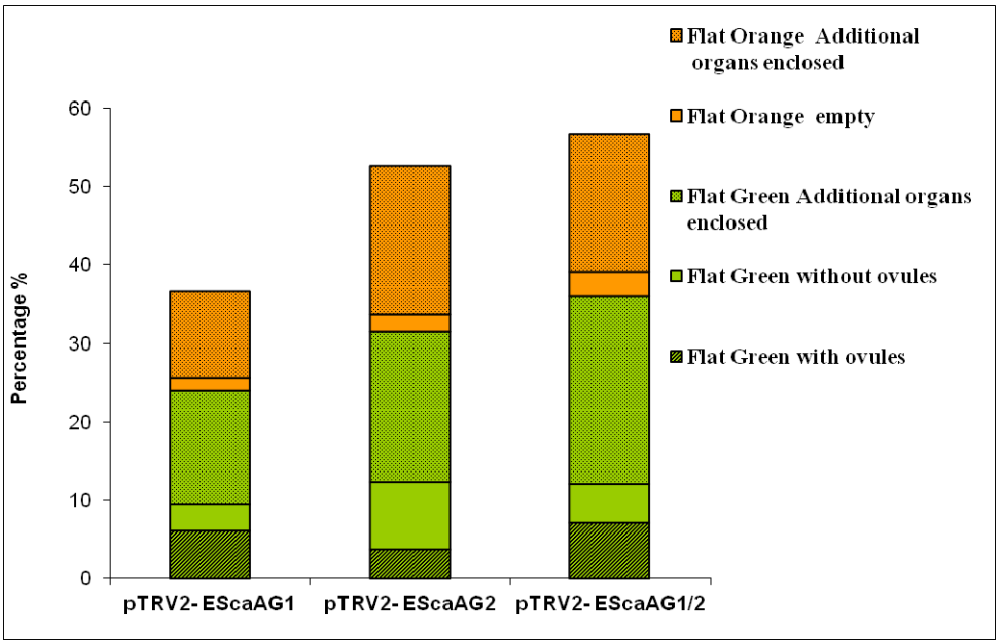
RT-PCR expression analysis of two *EScaAG* genes in floral organs at anthesis, various floral developmental stages, leaves, young fruits, and seeds is shown. The expression of *EcACTIN2 (EcACT2)* served as a control expression in all RT-PCR experiments.

9.2 Suppl.Fig.2: Histological section of VIGS-*EScaAG* phenotypic flower



(L) Transverse section of a flower of an untreated plant. (M) Transverse section of a flower from an *EScaAG1* VIGS treated plant showing homeotic conversion of stamens into petals, petaloid- stamen mosaic structures, malformed stamens, the central gynoecium showing lack of tissue differentiation, ovules, and placenta.
Abbreviations: p: petals; pst: petaloid stamens; s: sepals; st: stamens; cw: carpel walls.

9.3 Suppl.Fig 3: Percentage of homeotic transformation of gynoecia in VIGS: *EScaAG* treated plants



The X-axis denotes the various EScaAG down regulated plants and the Y-axis represents the percentages of different carpel identity phenotypes observed (pTRV2-*EScaAG1*, n=239; *EScaAG2,* n=209, *EScaAG1/2,* n=261 flowers). The green color symbolizes the occurrence of flat green gynoecia; the orange color symbolizes flat orange gynoecia. Stripes indicate gynoecia enclosing ovules, plane color indicates a gynoecium lacking ovules, and the dotted pattern indicates additional organs enclosed by the gynoecium.

9.4 Suppl Table: List of primers used

Primer	Sequence	Purpose
Actin2RTQfw	TTACAATGAGCTTCGTGTTGC	RT-PCR reference gene
Actin2RTQrev	CCCAGCACAATACCTGTAGTAC	RT-PCR reference gene
EcAG2RTFwprimer	GAGAGAGAGAGAGAGAGAGAA TGAGAA	RT-PCR paralogue specific primer for *EScaAG2*
EcAG1RTRevspan	CCTAGAGTCATAACCAGAAGAA GTC	RT-PCR paralogue specific primer for *EScaAG1*
EcAG2RTRevspan	CGCTAGAAATCATGTCGTTGTAT TCG	RT-PCR paralogue specific primer for *EScaAG2*
P27-5	GGGATGACGCACAATCC	sequencing primer for pHELLSGATE12
P27-3	GAGCTACACATGCTCAGG	sequencing primer for pHELLSGATE12
EcCRCRNAi forwardpri	CACCGGCTTTCATCAGGGTTTTT G	For making RNAi-knock down expression construct
EcCRCRNAi reversepri	CGATGCGGTATATCAGGATG	For making knock down expression construct
GFPforward	ATGCCACATACGGAAAGCTC	wild type GFP primer
GFPreverse	GGGTCTTGTAGTTCCCGTCA	wild type GFP primer
EcCRCihRNAifor	CACCGGACTACCTTTCTCACACT GAGC	For making RNAi-knock down expression construct
EcCRCihRNAiRev	CCCTGATGAAAGCCACTGAT	For making RNAi-knock down expression construct
EcAG1RTFW2	GCAGATCCCTCAAATTCTGC	RT-PCR paralogue specific primer to *EScaAG1*
GAPDH QRT Fw	GCTTCCTTCAACATCATTCC	Reference gene primer for qRT- PCR
GAPDH QRT Rev	AGTTGCCTTCTTCTCAAGTC	Reference gene primer for qRT- PCR
ACTIN-136-F	AAGAGCTCGAAACTGCCAAG	Reference gene primer with UPL probe from Roche for qRT-PCR
ACTIN-136-R	CATCGGGAAGCTCGTAATTT	Reference gene primer with UPL probe from Roche for qRT-PCR
EcAG1 QRTFw1	AGAAGAGGGAGATTGATTTGC	*EScaAG1* primer for qRT-PCR
EcAG1QRTRev1	AAGTTCCTAGAGTCATAACCAG	*EScaAG1* paralogue specific

		primer for qRT-PCR
EcAG2 QRT Fw	CGAAACTAGATTAGAGAAAGGC	*EScaAG2* primer for qRT-PCR
EcAG2 QRT Revspan	CGCTAGAAATCATGTCGTTGTATTCG	*EScaAG2* paralogue specific primer for qRT-PCR
EcDEF1-132-F	GGATGGGAGAGGATTTGGAT	*EScaDEF1* primer with UPL probe from Roche
EcDEF1-132-R	TTCCAGATTTTGCTCAAGACTTC	*EScaDEF1* primer with UPL probe from Roche
EcDEF2RTQfor2	ATTTGGTGGAGGAGATGATGAG	EScaDEF2 primer for qRT-PCR
EcDEF2RTQrev2	TTTTGAAGATTGGGATGGCTA	EScaDEF2 primer for qRT-PCR
EcGLORTQfor2	TCTAGCACTGGCAAGATGTC	EcGLO primer for qRT-PCR
EcGLORTQ rev2	TTGATTCTATCCACTTCAGCAC	EcGLO primer for qRT-PCR