Expression analyses of flower developmental genes in *Eschscholzia californica*

Expressionsanalyse von Entwicklungsgenen in *Eschscholzia californica*

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

Dem Fachbereich Biologie/Chemie

der Universität Bremen

vorgelegt von

Svetlana Orashakova

Bremen 2011

Reviewer: **Prof. Dr. Annette Becker**
Evolutionary Developmental Genetics
University of Bremen

Prof. Dr. Uwe Nehls
Section of Botany, Department of Ecology
University of Bremen

Examiner: **Prof. Ulrich Fischer**
Department of Marine Microbiology
University of Bremen

Dr. Andrea Krause
Department of Micobe-Plant Interactions
University of Bremen

Staff: **Sabrina Lange**
University of Bremen

Student: **Nils Engelke**
University of Bremen

Danksagungen

Ich möchte mich bei Prof. Dr. Annette Becker bedanken, dass sie mir die Möglichkeit gegeben hat meine Dissertation in ihrer Arbeitsgruppe zu machen.

Ich möchte Prof. Dr. Uwe Nehls danken, dass er das Zweigutachten meiner Dissertation übernommen hat. Ebenfalls bedanke ich mich bei Prof. Dr. Ulrich Fischer and bei Dr. Andrea Krause, dass sie eingewilligt haben, als Mitglieder der Prüfungskommission an meinem Dissertationskolloquium teilzunehmen.

Bedanken möchte ich mich insbesondere bei den Mitgliedern der AG Becker für die gute Zusammenarbeit, die Unterstützung und die hilfreichen Diskussionen im Laufe meiner Doktorarbeit. Ich danke Matthias, Sabrina, Aravinda and Robert für die angenehme Arbeitsatmosphäre, Hilfsbereitschaft und Freundschaft während unserer gemeinsamen Zeit. Ich bedanke mich auch bei Anna, Dawit, Amey, Kai und Tina.

Insbesondere möchte ich mich bei Matthias bedanken für seine volle Unterstützung und Aufmunterung. Ich danke ihm außerdem für die langen und interessanten Diskussionen zur denen er immer bereit ist.

Ich möchte mich auch bei meiner Familie und insbesondere bei meiner Mutter bedanken, die mich immer unterstützt haben.

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Abbreviations

A. asparagoides	*Asparagus asparagoides*
A. longifolia	*Asimina longifolia*
A. majus	*Antirrhinum majus*
A. officinalis	*Asparagus officinalis*
A. thaliana	*Arabidopsis thaliana*
A. trichopoda	*Amborella trichopoda*
A. vulgaris	*Aquilegia vulgaris*
AG	*AGAMOUS*
AGL11	*AGAMOUS LIKE11*
ALC	*ALCATRAZ*
AP	*APETALA*
AP2/EREBP	APETALA2/ethylene-responsive element binding protein
AuxREs	Auxin Response Elements
ARF	Auxin Response Factor
B. oleraceae	*Brassica oleraceae*
B. rapa	*Brassica rapa*
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BiFC	Bimolecular fluorescence complementation
BLR	*BELLRINGER*
bHLH domain	basic helix-loop-helix domain
BSA	bovine serum albumin
bp	base pairs
ca	carpels
C. poppy	California poppy
CRC	*CRABS CLAW*
DEF	*DEFICIENS*
DEPC	Diethylpyrocarbonate
DL	*DROOPING LEAF*
DZ	dehiscence zone
E. californica	*Eschscholzia californica*
E. elephas	*Elegia elephas*
ETT	*ETTIN*

euAP3 motif	eudicot *AP3* motif
FAR	*FARINELLI*
FBP	*FLORAL BINDING PROTEIN*
G. gnemon	*Gnetum gnemon*
GLO	*GLOBOSA*
g	gynoecium
I. floridanum	*Illicium floridanum*
IM	inflorescence meristem
IND	*INDEHISCENT*
J. ascendens	*Joinvillea ascendens*
kb	kilobases
L. longiflorum	*Lilium longiflorum*
LAS	*LATERAL SUPPRESSOR*
LFY	*LEAFY*
LEU	*LEUNIG*
M. grandiflora	*Magnolia grandiflora*
Mbp	mega base pairs
min	minutes
MYA	million years ago
N. benthamiana	*N. benthamiana*
NBT	Nitro blue tetrazolium chloride
NLS	nuclear localization sequence
N. advena	*Nuphar advena*
OCT	*OCTANDRA*
O. sativa	*Oryza sativa*
P. abies	*Picea abies*
P. hybrida	*Petunia hybrida*
P. persica	*Prunus persica*
P. radiata	*Pinus radiata*
P. somniferum	*Papaver somniferum*
P. trichocarpa	*Populus trichocarpa*
paleoAP3 motif	*paleoAPETALA3* motif
PAN	*PERIANTHIA*
PAT	polar auxin transport

pMADS2	*PETUNIA MADS BOX GENE2*
PI	*PISTILLATA*
PLE	*PLENA*
RBL	*REBELOTE*
RNase	Ribonuclease
RT	room temperature
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
S. angustifolia	*Streptochaeta angustifolia*
S. bicolor	*Sorghum bicolor*
S. lycopersicum	*Solanum lycopersicum*
SAM	shoot apical meristem
SEP 1/2/3/4	*SEPALLATA 1/2/3/4*
se	sepals
sec	seconds
SEU	*SEUSS*
SHP1/2	*SHATTERPROOF1/2*
SIL1	*SILKY1*
sir	*sirene*
STK	*SEEDSTICK*
STY	*STYLISH*
SQN	*SQUINT*
SPW1	*SUPERWOMAN1*
SPT	*SPATULA*
SUP	*SUPERMAN*
T. aestivum	*Triticum aestivum*
T. dioicum	*Thalictrum dioicum*
T. gesneriana	*Tulipa gesneriana*
TM6	*TOMATO MADSBOX GENE6*
tRNA	transfer RNA
UFO	*UNUSUAL FLORAL ORGANS*
ULT1	*ULTRAPETALA1*
VIGS	Virus-induced gene silencing
WUS	*WUSCHEL*
Z. mays	*Zea mays*

Summary

The combination and precise control of different organ identity programs underlies the flower development in angiosperms. Despite the enormous diversity in colour, shape and morphology, angiosperms share common flower architecture, suggesting an astonishing conservation of organ identity programs in angiosperm evolution since the flowering plants separated from the gymnosperms about 300 MYA. Even though the key genes in flower development share high conservation in expression and function, most of them have gained or lost expression/function due to multiple duplication events during angiosperm evolution with subsequent sub- or neofunctionalization in gene function. Generally, any change in the gene expression is a first hint for a gain or a loss of function. Thus, the examination of gene expression and the comparison of expression patterns between lineages is a starting point to get insight into the evolution of gene function. Studying the gene expression and function in phylogenetically important species such as *Eschscholzia californica (E. californica),* a representative of the earliest diverging basal eudicot lineage Ranunculales and an emerging model species for investigating flower development, contributes to our understanding about the genetics of floral organ development.

The orthologous gene expression patterns of key regulators in flower development of *A. thaliana* were examined in *E. californica*. The ortholog of the *A. thaliana* carpel developmental gene *CRABS CLAW (CRC)* displays conserved expression in the abaxial gynoecium wall and controls abaxial tissue differentiation of the carpel walls. The function of *EcCRC* in meristem termination is also conserved across *CRC*-like genes and is in concordance with the *EcCRC* expression at the base of the gynoecium. In addition, *EcCRC* has acquired novel functions in differentiation of the adaxial margin tissues placenta and ovules. In contrast to its function in meristem termination and abaxial tissue differentiation, *EcCRC* probably functions non-cell autonomously in placenta development/ovule initiation, probably from the carpel margins, where it is expressed.

It was revealed that *EScaAG1* and *EScaAG2*, the orthologous genes of the C-class organ identity gene *AGAMOUS (AG)* from *A. thaliana*, share the conserved expression of *AG* orthologous genes in floral meristem, carpels and stamens. The expression patterns of *EScaAG1/2* correlate with their conserved function in floral meristem termination, carpel and stamen identity. Additionally, the *AG* orthologs might have acquired a novel function in the control of stamen number in wild-type *E. californica* flowers.

SIR, the ortholog of the B-class organ identity gene *GLO* in *E. californica,* displays the conserved expression of B class genes in petals and stamens and also confers petal and stamen identity. Furthermore, *SIR* controls the expression of *EScaAG2*, but the *EScaAG1*expression is not dependent on *SIR*, suggesting the existence of B-dependent and B-independent expression C-class gene expression. Furthermore, a declining gradient of *EScaAG1* expression was observed in *E. californica* flowers, which has not been reported before. Also C-dependent B gene expression occurs in stamens, but not in carpels of *E. californica* flowers.

Finally, *EcSPT,* the ortholog of the carpel developmental gene *SPATULA (SPT)* from *A. thaliana,* displays continuous expression in the floral meristem and in the boundary region between carpels and stamens. The transient silencing of *SPT* via Virus-induced gene silencing (VIGS) caused the development of fruits in the *EcSPT*-VIGS plants, generally being shorter and developing fewer seeds than the untreated plants.

This work demonstrates that orthologous gene expression of developmental control genes is often highly conserved across angiosperm lineages, however also shifts in expression between orthologs arise by alteration in *cis*-regulatory elements that allow the gene function to evolve.

Zusammenfassung

Die Verknüpfung und die präzise Kontrolle von verschiedenen Organidentitätsprogrammen liegen der Blütenentwicklung der Blütenpflanzen zugrunde. Trotz der enormen Vielfalt in Farbe, Form und Morphologie, teilen die Blüten aller Blütenpflanzen eine gemeinsame Struktur. Das deutet darauf hin, dass sich eine erstaunliche Konservierung der Organidentitätsprogramme während der Blütenpflanzenevolution etabliert hat. Die Blütenpflanzen haben sich von den Nicht-Blütenpflanzen, auch Gymnospermen genannt, wahrscheinlich vor ungefähr 300 Millionen Jahren getrennt. Trotz des hohen Konservierungsgrades von Expression und Funktion der wichtigen Gene der Blütenentwicklung, haben die meisten von ihnen zusätzliche Expressionen/Funktionen bekommen oder auch vorhandene Expressionen/Funktionen verloren im Laufe der Evolution. Das geschieht als Konsequenz der mehrfachen Genvervielfältigung mit darauf folgende Sub- oder Neufunktionalisierung der Gene. Generell kann man postulieren, dass jede Änderung in der Expression von Entwicklungsgenen einen Hinweis auf zusätzlich evolvierte oder auch verlorene Genfunktionen darstellt. Deswegen sind die Untersuchungen der Genexpression sowie der anschließende Vergleich der Expressionsmuster von Ortholog-Genen aus verschiedenen Abstammungslinien ein Startpunkt in der Erforschung der Evolution der Genfunktion. Die Erforschung der Genfunktion und der Genexpression in repräsentativen Pflanzenarten wie z.b. *Eschscholzia californica* (*E. californica*, Kalifornischer Mohn), einem Mitglied einer der frühsten Linien der eudicotylen Pflanzen und eine neuartige Modellpflanze, trägt zu unserem Verständnis über die Genetik der Entwicklung von Blütenorganen bei.

Die Expressionsmuster von Orthologen der Schlüsselregulatoren der Blütenentwicklung aus *A. thaliana* wurden in *E. californica* untersucht. Das Ortholog des Fruchtblattentwicklungsgens *CRABS CLAW (CRC)* von *A. thaliana* zeigt konservierte Expression in der abaxialen Fruchtblattwand und kontrolliert dementsprechend die Differenzierung der abaxialen Gewebe des Fruchtblattes. Die Funktion von *EcCRC* in der Terminierung des Blütenmeristems ist ebenfalls hoch konserviert zwischen den *CRC*-ähnlichen Genen und entspricht der *EcCRC* Expression an der Fruchtblattbasis. Darüber hinaus hat *EcCRC* zusätzliche Funktionen, sowohl in der Spezifikation der adaxialen Gewebe des Fruchtblattes, als auch in der Placenta-Entwicklung und Ovuleninitation, herausgebildet. *EcCRC* funktioniert Zell-autonom in der Meristemterminierung und in der abaxialen Differenzierung der Fruchtblattwand. Im Unterschied dazu funktioniert *EcCRC* höchstwahrscheinlich nicht Zell-autonom in der Differenzierung der adaxialen Gewebe der

Fruchtblattwand und der Placenta-Entwicklung/Ovuleninitierung sonder reguliert möglichweise von den Fruchtblatträndern aus die adaxiale Gewebedifferenzierung, wo es auch exprimiert ist.

Es wurde gezeigt, dass die beiden Orthologe des *A. thaliana* C-Organidentitätsgens *AGAMOUS (AG)* in *E. californica, EScaAG1* und *EScaAG2*, die hoch konservierte Expression der *AG*-ähnlichen Genen im Blütenmeristem, dem Fruchtblatt (Karpell) und den Staubblättern (Stamina) teilen. Die Expression der beiden *AG* Orthologen entspricht ihren auch hoch konservierten Funktionen in der Blütenmeristemtermination, sowie in der Karpell und Stamina-Identität. Zusätzlich könnten *EScaAG1* und *EScaAG2* eine neue Funktion in der Kontrolle der Stamina-Zahl in der Mohn-Blüte erworben haben.

SIR, das Ortholog des B-Organidentitätsgens *GLO* ist exprimiert in Kronblättern (Petalen) und Staubblättern von *E. californica. SIR* hat konservierte Funktionen in der Kontrolle der Petalen und der Stamina-Identität. Außerdem hält *SIR* die Expression des C-Organidentitätsgen *EScaAG2* in den äußeren Staminawirteln aufrecht, während die Expression von *EScaAG1* unabhängig von *SIR* zu sein scheint. Das deutet darauf hin, dass B-abhängige und B-unabhängige C-Genexpression in *E. californica* existiert. Zusätzlich konnte gezeigt werden, dass ein abnehmender Gradient der *EScaAG1* Expression in der *E. californica* Blüte existiert, der vorher nie gezeigt werden konnte. Außerdem befindet sich die Expression von B-Organidentitätsgenen unter der Kontrolle der beiden C-Organidentitätsgene in den Karpellen, aber nicht in den Stamina.

Schließlich, *EcSPT,* das orthologe Gen des Karpellgens *SPATULA (SPT)* aus *A. thaliana* zeigt andauernde Expression im Blütenmeristem und an der Grenze zwischen Karpell und den Stamina. Die Reduktion der *EcSPT* Expression führt dazu, dass generell kürzere Früchte entwickelt werden, die zudem weniger Samen enthielten verglichen mit den unbehandelten Pflanzen.

Diese Arbeit zeigt, dass die Expression von orthologen Entwicklungsgenen oft hoch konserviert zwischen verschiedenen Blütenpflanzenlinien ist. Zusätzlich aber konnten Verschiebungen in der Expression zwischen Orthologen entstehen als Folge von Veränderungen in *cis*-regulatorischen Elementen, welche die Evolution von Genfunktion ermöglicht haben könnten.

1. Introduction

1.1 Floral organs and organ identity genes

Despite the enormous diversity in flower shape, colour and size, all angiosperm flowers share a common architecture and usually consist of four floral organ types. The development of the floral organs is a complex process involving floral meristem formation, establishment of organ identities and subsequent floral organ differentiation, and occurs by an accurately regulated genetic interplay of floral homeotic genes (ZIK and IRISH 2003). Flower organs originate from a floral meristem cell population and are arranged in concentric whorls (BOWMAN 1997; LENHARD *et al.* 2001). From outside to inside, whorl 1 consists of sepals, whorl 2 of petals, whorl 3 of stamens and whorl 4 of carpels. The developmental genes responsible for determination of the floral organ identities are transcription factors and belong to the MADS-box gene family. Detailed genetic studies, carried out extensively in *Arabidopsis thaliana (A. thaliana)* and *Antirrhinum majus (A. majus),* have led to the development of the almost universally applicable ABC model that explains the genetic control of floral organ determination by the combinatorial action of four classes organ identity genes (COEN and MEYEROWITZ 1991). According to the ABC model, class A genes specify sepal identity in the first whorl, A and B together specify petal identity in the second one, B and C are required for stamen identity in the third, and C alone establishes carpel identity in the central fourth whorl. Mainly, the floral homeotic genes belong to the biggest family of transcription factors in plants, the MADS-box gene family. The only exception is the A-class gene *APETALA2 (AP2),* which is a member of the AP2/EREBP (APETALA2/ethylene-responsive element binding protein) transcription factors' family (OKAMURO *et al.* 1997). Simultaneous loss-of-function of A, B and C floral homeotic genes lead to transformation of all floral organs into leaves (HONMA and GOTO 2001). On the other hand, co-expression of A, B and C class genes fails to convert leaves into floral organs indicating that the three classes of floral homeotic genes alone are not sufficient to determine the flower and an additional factor is required. The classical ABC model has been extended to ABCDE by including D and E class organ identity genes. Four E class genes have been identified in *A. thaliana* flower development, *SEPALLATA 1/2/3/4 (SEP 1/2/3/4).* They function redundantly in determining all floral organ identities. *sep1/sep2/sep3/sep4* quadruple mutants display a conversion of all floral organs into leaf-like structures demonstrated that E class genes are the missing factor required for successful floral organ formation (DITTA *et al.* 2004; PELAZ *et al.* 2000). An ectopic expression of a *SEP* gene with A, B and C class genes is sufficient to convert leaves

into floral organs (HONMA and GOTO 2001). Studies on *Petunia hybrida* (*P. hybrida*) have led to the discovery of a novel functional class of MADS-box genes, the D class genes (ANGENENT *et al.* 1995; COLOMBO *et al.* 1995). D-class genes are highly homologous to the C-class genes and control ovule development. In *P. hybrida,* D-class genes are represented by the paralogs *FLORAL BINDING PROTEIN7 (FBP7)* and *FLORAL BINDING PROTEIN11 (FBP11).* The orthologous gene to *FBP7* and *FBP11* in *A. thaliana* is *SEEDSTICK (STK)*, formerly known as *AGAMOUS LIKE11 (AGL11)* (PINYOPICH *et al.* 2003).

1.2. The carpel, a major innovation of angiosperms

Angiosperms and gymnosperms represent the extant seed plants. The female reproductive organ of angiosperms or flowering plants, the carpel, represents not only the most distinguishable characteristic between these sister groups, but also the most complex and innovative feature of angiosperms (ENDRESS 2001). The most ancient living seed plants, the gymnosperms, develop male (male cone) and female (female cone) reproductive organs on separated plants, whereas the evolutionary younger angiosperms have carpels and stamens (male reproductive organs) usually united in a bisexual flower. The carpels of most angiosperm species are fused into a gynoecium. When the carpels are fused from their inception, the fusion is termed 'congenital', whereas a carpel fusion, which occurs during development, is called 'post-genital'. An advantage of the carpel is that it encloses and protects the ovules, whereas in gymnosperms the ovules develop as naked structures. Furthermore, the carpel provides a sheltered environment for fertilization and its specialized tissues ensure successful pollination. At the time of pollen germination and growth, the selective mechanisms of self-incompatibility, operating on pollen, facilitate out-breading. This contributes to the enormous diversity of already exciting plant species and the creation of new ones, and determines the agronomical success of the angiosperms (SCUTT *et al.* 2006). After fertilization, the carpel tissues undergo structural changes and develop into a fruit, which protects the seeds, and facilitates their dehiscence and dispersal by using a variety of mechanisms in different species (SCUTT *et al.* 2006). All these advantages of the carpel are assumed to underlie the enormous evolutionary success of angiosperms.

But the evolutionary origin of the carpel still remains unclear. Goethe had hypothesised over 200 years ago that the carpels are actually modified leaves and that the vegetative leaf is the real ancestor of the floral organs (GOETHE 1790). A supporting evidence for this was the

complete transformation of the floral organs into leaf-like organs in the *sep1/ sep2/sep3/sep4* (DITTA *et al.* 2004).

1.3 Model plants for studying carpel development in angiosperms

In order to elucidate the molecular control of carpel formation in angiosperms, it is critical to compare the genetic mechanisms underlying carpel development in different angiosperm lineages. Angiosperms are divided into four major lineages, basal angiosperms, magnoliids, eudicots and monocots (Figure 1). The model plant *Amborella,* considered to be the earliest diverged angiosperm species, belongs to the basal angiosperms (KUZOFF and GASSER 2000; ZANIS *et al.* 2002). *Amborella* develops spirally arranged male and female flowers on separated plants. Generally, basal angiosperms have undifferentiated perianth consisting of identical floral organs with petal characteristics referred to as tepals. Also in magnoliids, most species exhibit an undifferentiated perianth, composed of identical organ types as only few species like *Asimina* and *Saruma* have a well-differentiated perianth, constituted of distinct sepals and petals (KIM *et al.* 2005). Monocots and eudicots represent sister lineages, which are thought to be arisen from a common ancestor (IRISH and LITT 2005). The monocot lineage includes the grasses and the non-grasses, while the eudicot lineage comprises two sister clades, the basal eudicots and the core eudicots, all considered to be arisen from a common precursor (ZAHN *et al.* 2006). According to Irish and Litt, the core eudicot lineage is subdivided into three groups, the rosids, the asterids and the Caryophyllids (IRISH and LITT 2005) (Figure 1). Within eudicots, most information about the molecular genetics governing carpel development comes predominantly from the rosid *A. thaliana,* whereas *A. majus* and *P. hybrida* are suitable model plants for studying carpel development in asterids. In monocots, most of the accumulated functional data are derived from the grass species *Oryza sativa (O. sativa)* and *Zea mays (Z. mays).*

The basal eudicot *Eschscholzia californica (E. californica)* is a representative of the Ranunculales order, similarly to the already established genetic model plant *Aquilegia vulgaris (A. vulgaris)*. Ranunculales are located at the base of the basal eudicot lineage and represents the earliest diverging eudicot order.

In this chapter, the morphogenesis and morphology of the carpel in *A. thaliana*, *E. californica* and *O. sativa* as representatives of core eudicots, basal eudicots and monocots, respectively, are described in details.

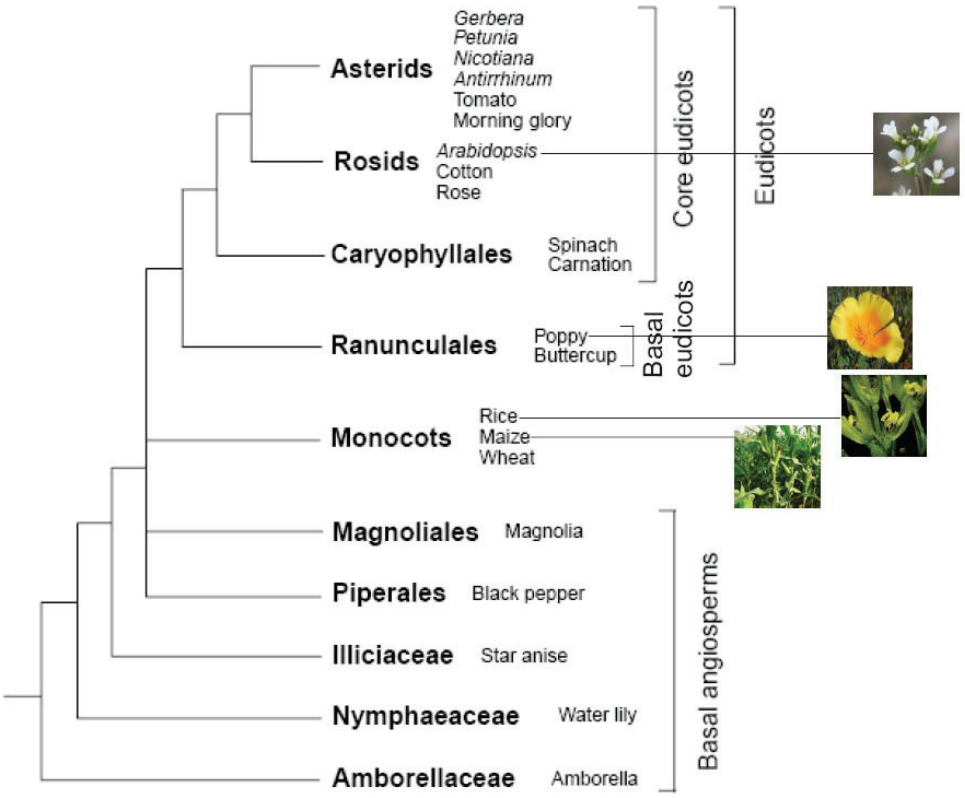

Figure 1: A simplified phylogeny of angiosperm plants.
In bold, order and family names are indicated, examples of well-known representatives of these clades are listed on the right side of each lineage and pictures of some model plants for molecular genetic analyses are included (modified from (IRISH and LITT 2005).

1.3.1 Carpel development in eudicot model systems

1.3.1.1 Morphology and morphogenesis of the carpel in *A. thaliana* and *E. californica*

In the last two decades, the core eudicot *A. thaliana,* a member of *Brassicaceae,* has been established as a model system for studying the molecular genetics of flower development. Almost all of the known genes participating in carpel development have been initially identified and characterized in *A. thaliana*.

The mature flower of *A. thaliana* has a simple structure, characteristic for *Brassicaceae*. It consists of four distinct floral organ types arranged in four concentric whorls. From outside to inside, the first whorl is composed of four sepals, the second of four petals, the third of six stamens, and the fourth of two lateral carpels congenitally fused into a central gynoecium (DINNENY and YANOFSKY 2005; FERRANDIZ *et al.* 1999). The non-reproductive organs sepals and petals are organized in a well-differentiated perianth.

The mature gynoecium of *A. thaliana* consists of two congenitally fused at the base carpels and shows three different axes of tissue organization, an apical-basal, a medial-lateral and an abaxial-adaxial axis. In longitudinal view and from top to base, the apical-basal axis is established (Figure 2) (BALANZA *et al.* 2006; FERRANDIZ *et al.* 1999). Along this, the following structures can be distinguished: an apical stigma, a short style, connecting the stigma to the ovary, an ovary, protectively bearing the ovules inside, and a short gymnophore at the base, which attaches the ovary to the flower (Figure 2A, B). The style and stigma made up the apical part of the gynoecium, while the ovary and gymnophore constitute the basal part. The stigmatic tissue consists of elongated cells, called stigmatic papillae, specialized in catching the pollen. The ovary is externally divided by the replum into two valves, corresponding to the two carpel walls. Internally, the ovary is divided by a septum, which is fused post-genitally (FERRANDIZ *et al.* 1999). A polysaccharide-rich tissue, termed transmitting tract, develops from the septum. The transmitting tract runs along the entire ovary as it starts from the style, goes throughout the centre of the stigma, and further through the septum. After pollination, the growing pollen tubes are guided by the transmitting tract to the unfertilized egg cells inside of the ovary, where the fertilization takes place.

In cross section, the tissues of the gynoecium wall show an abaxial-adaxial and a medial-lateral axis of tissue organization (Figure 2A and B). The two valves of the ovary, which are located laterally in the gynoecium, are joined to the presumptive replum region by tiny stripes of cells called valve (carpel) margins. The presumptive replum region differentiates abaxially into replum and adaxially into placenta. From the placenta, placental outgrowths develop, which bear the ovules on the tip. All these tissues, together with style, stigma, septum and transmitting tract arise from the carpel margins and therefore are collectively termed carpel marginal tissues. They occupy the medial plane of the gynoecium wall (Figure 2A and B).

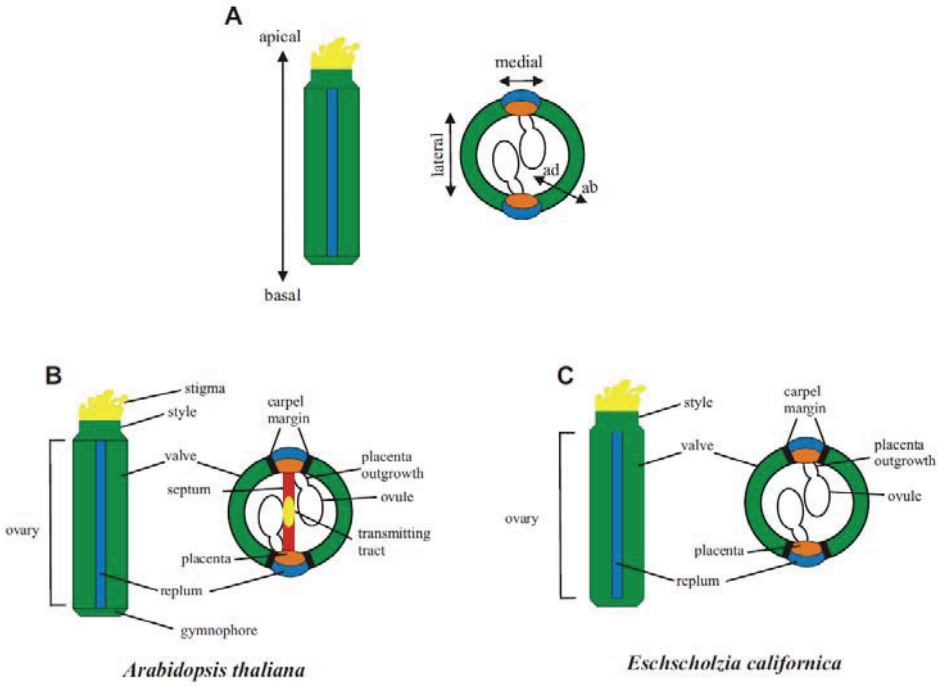

Figure 2: Schematic view representing the gynoecium axes **(A)**, and the tissue organization of mature gynoecia in *A. thaliana* **(B)** and *E. californica* **(C).**

(A) On the left side, a longitudinal view of the apical-basal axis is shown. On the right side, a cross section views the abaxial-adaxial and medial-lateral axes of the gynoecium (DINNENY and YANOFSKY 2005). (**B)** Longitudinal section on the left shows the tissue organisation along the apical-basal axis in *A. thaliana* gynoecium. On the right side, a transverse section illustrates the abaxial-adaxial and medial-lateral tissue organization. **(C)** On the right side, a longitudinal view of an *E. californica* gynoecium is drawn, indicating similar tissues organization along the apical-basal axis as in *A. thaliana*. On left, a transverse section through the ovary of *E. californica* shows the arrangement of the gynoecium tissues in the medial-lateral and abaxial-adaxial axes.

Abbreviations: ab, abaxial; ad, adaxial.

In *A. thaliana*, the gynoecium initiates as a single primordium at around stage 5-6 (stages according to (ALVAREZ and SMYTH 2002; SMYTH *et al.* 1990) (Table 1). It is the last floral organ produced from the floral meristem, and after its initiation the floral meristem is

terminated. In the following stages, the gynoecium elongates and the carpel tissues differentiate. During stages 8 and 9, valves, placenta, septum and ovules initiate (Table 1). Around stage 10-11, the gynoecium starts to close in the apical region, and style and stigma develop at the top. During stage 11-12, replum and transmitting tract differentiate (Table 1). In stage 13, known as anthesis the gynoecium reaches maturity (Table 1). After fertilization, the ovules develop into seeds and the ovary into a fruit. The dehiscent fruit of *A. thaliana,* termed silique or pod, is characteristic for many members of *Brassicaceae* (ROBLES and PELAZ 2005). It not only provides a save environment for seed maturation, but is also responsible for dispersal of the mature seeds. All tissue types of the mature fruit are initiated already in the gynoecium. After fertilization, the ovary cells start dividing and the fruit grows until reaching its final length. In the mature fruit, the region at the valve margins located between the valves and the replum undergoes changes and develops into a dehiscence zone (DZ) (BALANZA *et al.* 2006; ROBLES and PELAZ 2005). This starts before dehiscence with the lignification of the cells next to the valves and a lignified margin layer is formed. The DZ comprises not only the lignified margin cell layers, but also tiny separating strips of small cells marking the longitudinal plane of shatter at both valve margins and a patch of adjacent lignified cells. The internal most adaxial sub-epidermal cell layer of the valves adjacent to the valve margins also undergoes lignification. When the mature fruit dries, this lignified sub-epidermal layer and the patch of lignified cells provide a tension zone that serves as a spring-like mechanism to cause braking of the silique and releasing of the seeds (FERRANDIZ 2002).

E. californica Cham. or California poppy (*Papaveraceae*) is a basal eudicot species in the Ranunculales order. It is an emerging model plant for detailed investigations of evolutionary developmental genetics. This is due to its key phylogenetic position as a representative of the earliest diverging eudicot lineage and the accumulation of functional data in the recent years (BECKER *et al.* 2005; CARLSON *et al.* 2006b; ORASHAKOVA *et al.* 2009; WEGE *et al.* 2007; YELLINA *et al.* 2010; ZAHN *et al.* 2006; ZAHN *et al.* 2010). *E. californica* has a diploid genome with 1078 Mbp per haploid chromosome set (BENNETT *et al.* 2000). It is also easily cultivated and can be transgenically manipulated. Furthermore, owing to the highly efficient employment of Virus-induced gene silencing method (VIGS) in *E. californica,* it represents an excellent object for studying gene functions and gene interactions (ORASHAKOVA *et al.* 2009; WEGE *et al.* 2007; YELLINA *et al.* 2010). Additionally, the Floral Genome Project (FGP) has provided a large number of expressed sequence tags (EST) of flower developmental genes (CARLSON *et al.* 2006a; ZAHN *et al.* 2010).

Similar to *A. thaliana*, the *E. californica* constitutes of four distinct floral organ types organized into four concentric whorls. The first whorl consists of two sepals, the second of four petals, the third includes variable number of stamens and the central whorl is composed of two carpels congenitally fused into a gynoecium (BECKER *et al.* 2005). Longitudinal view of the *E. californica* gynoecium shows the same tissue organization along the apical-basal axes as in that of *A. thaliana* (Figure 2C). In *E. californica*, the transition between stigma, style and ovary is rather continuous. In transverse view of a mature gynoecium, the two valves (carpels) are joined to the presumptive replum region in the (carpel) margins. The presumptive replum region enclosed between both valves differentiates into a replum an abaxial replum and an adaxial placenta. Two placental outgrowths arise from the placenta bear the ovules on the tip and grow inwards the gynoecium cavity. According to the medial-lateral axis, both carpel walls have lateral position, whereas the presumptive replum region, placenta outgrowths and ovules are located medially in the gynoecium wall. In contrast to *A. thaliana*, transmitting tract and septum do not develop in *E. californica*. Instead, pollen tubes grow throughout the placental (BECKER *et al.* 2005).

In *E. californica*, the gynoecium initiates as a single primordium in the centre of the flower in stage 5 (stages according to (BECKER *et al.* 2005) (Table 1). During stage 6, the gynoecium elongates intensively. The two placental regions develop inward of the gynoecium and this results into a central hollow with narrow centre, separating the gynoecium into two carpel cylinders with completely free tips. Stage 7 is marked by ovule primordia initiation (Table 1). The gynoecium grows laterally. In a cross section of gynoecium in stage 8 is visible that each carpel develops five longitudinal ridges on its abaxial site (BECKER *et al.* 2005). Inside the ovary, the ovule primordia elongate. In the ovary wall, tiny strips of lignified cells marking the position of dehiscence are formed along the valve/replum border. Stage 11 is marked by anthesis (Table 1). After fertilization, the gynoecium develops into a fruit, which encloses and protects the seeds (BECKER *et al.* 2005). During stage 12, the capsules elongate and in stage 13 they reach maturity and dry out (Table 1). The dry capsules dehisce explosively from the bottom to the top at stage 14 as both valves remain attached to the style (COOK 1962).

Table 1 Floral developmental stages in *A. thaliana* (according to (ALVAREZ and SMYTH 2002; SMYTH *et al.* 1990) and *E. californica* (BECKER *et al.* 2005). The strike (-) marks no data available or absence of such event in the development.

Key events in flower development	Stages in *A. thaliana*	Stages in *E. californica*
Meristem formation	Stage 1	Stage 1
Sepal primordia appears	Stage 3	Stage 2
Petal primordia appears	Stage 5	Stage 3
Stamens initiate	Stage 5	Stage 4
Gynoecium initiation	Around stage 5-6	Stage 5
Placenta inception	Stage 8	Stage 6
Septum inception	Stage 8	-
Ovule primordia initiation	Stage 9	Stage 7
Male meiosis	-	Stage 8
Female meiosis	-	Stage 9
Style and stigma appear	Stage 11	Stage 11
Replum differentiation, transmitting tract develops	Stage 11, 12	-
Anthesis	Stage 13	Stage 11
Fruit (capsule) formation and elongation	Stage 17	Stage 12
Fully elongated capsule dries out	Stage 18	Stage 13
Capsule opens and seeds disperse	Stage 19, 20	Stage 14

1.3.2 Carpel development in monocot model systems

1.3.2.1 Morphology and morphogenesis of the carpel in *O. sativa*

The grasses represent a large family including app. 10 000 different plant species in the monocotyledonous plants (monocots), characterized by an enormous morphological, genetic and ecological diversity. *O. sativa* and *Z. mays* belong to the most important crop plants in the world and are highly appropriate model systems for investigation the genetic control of

diverse developmental aspects due to fully sequenced genomes, availability of mutants and molecular tools. Additionally, *O. sativa* can be transformed relatively easy, whereas in *Z. mays* many essential genes have been isolated in the past several decades via employment of transposable elements (BOMMERT *et al.* 2005; ITOH *et al.* 2005). All these advantages enable the identification and characterization of orthologous genes associated with development and morphology. Moreover, although most grasses develop a unique flower structure distinct from that in eudicots, the reproductive organs are similar (GARRIS *et al.* 2005; YAMAGUCHI *et al.* 2004). The grass inflorescence consists of structural units called spikelet, which comprise variable number of flowers (florets). The maize spikelet comprises two florets, whereas the rice spikelet bears just a single one. Generally, the grass floret consists of a lemma, a palea, two lodicules, tree to six stamens and a pistil (gynoecium). The palea/lemma and the lodicules are specific to grasses flower structures and occupy the first and second whorl of the flower, respectively (Figure 3) (ZHANG *et al.* 2007).

Maize and rice differ in the types of inflorescence meristem (IM) they develop. Maize forms two distinct types of IM, the terminal tassel (male inflorescence) and the ear (female inflorescence). The terminal tassel produces male flowers and the ear gives rise to female flowers as both types of flowers develop on the same plant (BOMMERT *et al.* 2005; MCSTEEN *et al.* 2000). Initially, tassel and ear develop similar bisexual flowers on both inflorescences, which later undergo sex determination. This results in arrested development of the pistil in the tassel florets and of the stamens in the ear florets, and in their subsequent degeneration (IRISH and NELSON 1989). In the ear, only one of the two florets in the spikelet is fertile. The maize pistil consists of tree fused carpels, which differ from each other. The two abaxial carpels are sterile and fused into a silk, which elongates. The third carpel, which is the fertile one, elongates just enough to cover the developing ovule. On the contrary, rice elaborates just one type of IM, which produces a bisexual floret with equally developed stamens and a pistil in the spikelet (ITOH *et al.* 2005).

In *O. sativa*, the carpel differentiates into a stigma, style and ovary, similar to the eudicot flowers described above, but within the ovary, just a single ovule develops. Furthermore, the carpel does not differentiate into transmitting tract and septum (YAMAGUCHI *et al.* 2004). After the carpel primordium initiates on the lemma side of the floral meristem, it elongates and encloses the floral meristem, which remains undifferentiated. In contrast to *A. thaliana*, the floral meristem is not consumed by the carpel primordia, but gives rise to the placenta and ovule (COLOMBO *et al.* 2008). Pollination and fertilization take place immediately after flower opening (ITOH *et al.* 2005).

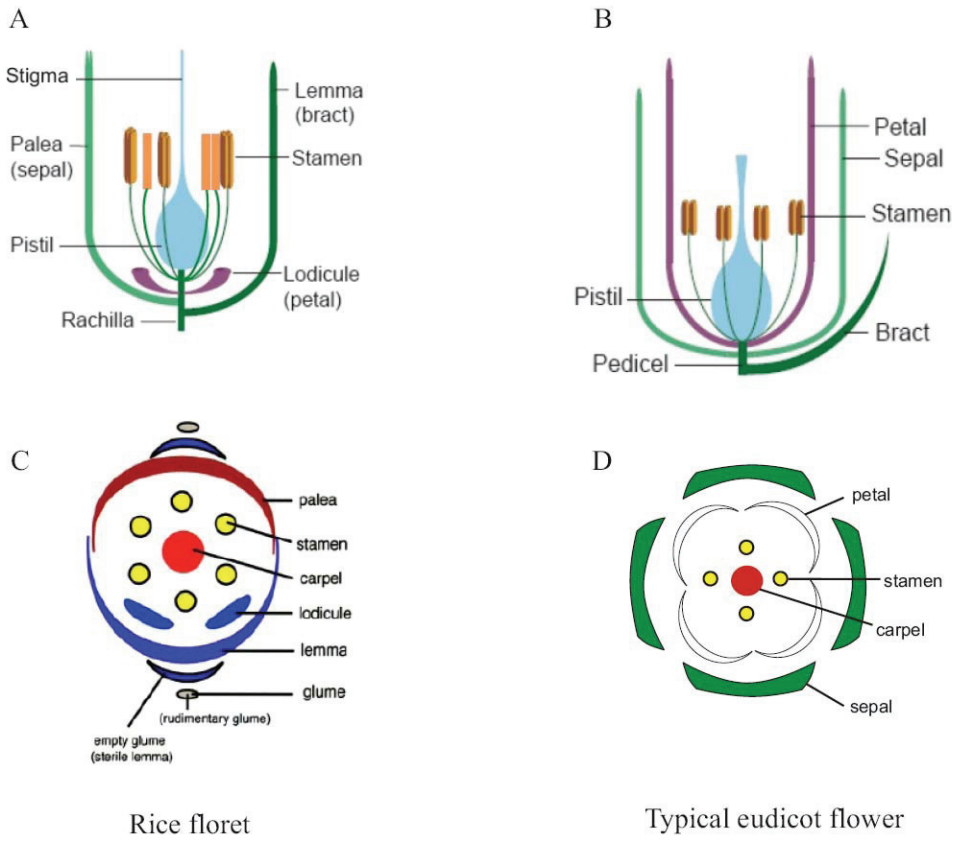

Figure 3: Depiction of the rice flower and a typical eudicot flower**.**
Schematic longitudinal views of rice floret **(A)** and eudicot flower **(B)**. Schematic transversal views of rice floret **(C)** and eudicot flower **(D)** (ITOH *et al.* 2005; MCSTEEN *et al.* 2000).

1.4 Genes in carpel development

In this chapter, the expression, function and interactions of key genes in carpel development of representative angiosperm species will be described. Detailed information on carpel developmental genes comes exclusively from the core eudicots *A. thaliana, A. majus* and *P. hybrida*, whereas most information within monocots is derived from genetic studies in *O. sativa* and *Z. mays*.

1.4.1 *CRABS CLAW (CRC)*-like genes

The *CRABS CLAW (CRC)* gene belongs to the YABBY gene family, which is a small plant-specific family of transcription factors. In the core eudicot *A. thaliana*, the YABBY gene family includes six members, which promote abaxial cell fate in lateral organs, i.e. cotyledons, leaves, sepals, petals, stamens and carpels (BOWMAN and SMYTH 1999b; ESHED *et al.* 1999; SAWA *et al.* 1999; SIEGFRIED *et al.* 1999; VILLANUEVA *et al.* 1999). All family members share the same protein structure and contain two conserved domains, a zinc finger C_2C_2 and a YABBY domain (BOWMAN and SMYTH 1999b). The zinc-finger domain is a serine/proline rich domain located at the N-terminus (MACKAY and CROSSLEY 1998). Many zinc fingers are involved in DNA-binding, whereas others are associated with protein-protein interactions (BOWMAN and SMYTH 1999a; MITCHELL and TJIAN 1989). The YABBY domain is a helix-loop-helix domain, positioned at the C-terminus. Its two helices show similarity to the HMG box, which is a conserved DNA-binding domain of about 80 amino acids, found in a large family of eukaryotic proteins (BAXEVANIS and LANDSMAN 1995).

The *CRC* gene controls different aspects of the carpel development in *A. thaliana* as establishment of the abaxial polarity of the carpel walls, carpel growth and carpel fusion. Additionally, it is required for nectary formation and plays a role in meristem termination (ALVAREZ and SMYTH 2002; BOWMAN and SMYTH 1999b).

The *CRC* expression is confined to carpels and nectaries. It commences at their initiation and is maintained throughout the entire development. However, the transcripts' accumulation of *CRC* in the gynoecium changes dynamically throughout developmental stages (BOWMAN *et al.* 1999). Initially, *CRC* is expressed along the entire carpel walls at stage 6, but is excluded from the medial regions of the gynoecium. In a longitudinal section of a gynoecium at stage 7-8, the hybridization signal is restricted to the abaxial (outer) site of the carpel walls embracing also the carpel tips. In a cross section through the gynoecium, *CRC* is further abaxially expressed in the carpel walls, but the *CRC* expression persists also in the abaxial side of the presumptive replum region, remaining excluded from its adaxial side (BOWMAN and SMYTH 1999a). The hybridization signal resembles a regular circle occupying the abaxial side of the gynoecium. Shortly after, *EcCRC* display additional domains of expression in the adaxial regions of the carpel walls and in four internal patches adjacent to the regions, where the placenta develops. During stage 9, the *CRC* expression is maintained only in the abaxial carpel walls. The *CRC* expression is excluded from placenta and ovules throughout all developmental stages.

The strong *crc-1* mutants of *A. thaliana* exhibit defects in carpel development and nectary formation (ALVAREZ and SMYTH 1999; BOWMAN and SMYTH 1999b). The mature mutant gynoecium in *crc-1* is wider and shorter than that in wild-type, and the carpels are unfused in the apical region. The amount of the apical tissues style and stigma is reduced. Occasionally, an additional carpel arises medially between both lateral carpels in the fourth whorl, and an ectopic ovule arises outside of the ovary. The *crc-1* gynoecium consists of fewer, but larger cells than the wild-type gynoecium. It seems that vascular differentiation occurs earlier in the *crc-1* gynoecium (ALVAREZ and SMYTH 2002). The septum is not fused in the apical part of the gynoecium, although the transmitting tract cells develop normally. Furthermore, nectary development is completely abolished in the *crc-1* mutants. Replum, placenta, septum, transmitting tract and ovules develop normally, but the ovule number per gynoecium is reduced. The *crc-1* mutants develop shorter siliques than wild-type plants as these are unfused at the apex and form less seeds (BOWMAN and SMYTH 1999a).

Lee and colleges identified five conserved regulatory regions (modules) in the 5' upstream regions of *CRC*-like genes from three *Brassicaceae* species, including *A. thaliana,* suggesting that the regulation of *CRC*-like gene expression is conserved across *Brassicaceae* (LEE et al. 2005a). These are probably associated with the control of *CRC*-like gene expression in carpels and nectaries. Furthermore, the authors identified several CArG boxes, which are binding sites for MADS box proteins and putative LEAFY (LFY) binding sites. *LFY* is a transcription factor required to specify the lateral meristem as floral and it appears to induce nectary development inside of the flower (BAUM *et al.* 2001).

The *CRC* expression in *A. thaliana* is controlled by organ identity genes. One of these is the C-organ identity gene *AGAMOUS (AG). AG* is a main determiner of floral meristem determinacy, and carpel and stamen identities in *A. thaliana* (see next chapter). In *crc-1 ag* +/- mutants, ectopic stamens and carpels arise in the fourth whorl (ALVAREZ and SMYTH 1999). *AG* is obviously not required for initial activation of the *CRC* expression because of persisting *CRC* expression, when *AG* is mutated. Probably, the later *CRC* expression is dependent to some extend on *AG*, due to the spatially modified *CRC* expression and the down regulation of its expression in absence *AG* (BOWMAN and SMYTH 1999b; GOMEZ-MENA et al. 2005).

Bowman and Smyth deduced a possible negative regulation of the *CRC* expression in the outer floral whorl by the A-class gene *AP2*, due to elevated *CRC* expression in the *ap2* mutant (BOWMAN and SMYTH 1999a).

The B-class floral homeotic genes in *A. thaliana*, *PISTILLATA (PI)* and *APETALA3 (AP3),* normally negatively regulate *CRC* expression the third whorl. *CRC* is expressed in the ectopic carpels, which develop in the third whorl of *pi-1*and *ap3-3* mutant flowers (BOWMAN and SMYTH 1999a).

Additionally, *LEUNIG (LEU)* a putative transcriptional co-repressor in *A. thaliana,* which encodes a glutamine-rich protein*,* was shown to suppress the *CRC* expression in the outer whorl of wild-type flowers (BOWMAN and SMYTH 1999a).

In monocots, the information on *CRC*-like genes is based extensively on detailed studies on its single ortholog in *O. sativa, DROOPING LEAF (DL)* (YAMAGUCHI *et al.* 2004). *DL* is initially expressed in the regions of floral meristem, where carpel primordia will develop. After carpel primordia inception, *DL* is uniformly expressed there, but without being expressed in the enclosed by the carpel floral meristem, from which the ovule arise. *DL* is not expressed also in the developing ovule. Furthermore, in contrast to *A. thaliana*, *DL* expression is present also in leaves. Mutation in *DL* causes a complete homeotic conversion of carpels into stamens in the severe *dl* mutants. Over-expression of *DL* affects the midrib formation and results in leaf blades curled toward (YAMAGUCHI *et al.* 2004).

1.4.2 *AG* orthologous genes

The ABC model determines the specification of carpel identity as a result of C class organ identity gene expression (COEN and MEYEROWITZ 1991). The C class organ identity gene in *A. thaliana AG* belongs to one of the biggest families of transcription factors, the MADS-box family. The MADS-box genes encode DNA-binding proteins conserved in plants, fungi and animals, which control diverse developmental processes (SCHWARZ-SOMMER *et al.* 1990). The term MADS comes from the first identified members of the family: the yeast gene *MCM1*, the plant genes *AGAMOUS* and *DEFICIENS*, and the mammalian gene, *SERUM RESPONSE FACTOR*. All MADS-box proteins share a highly conserved MADS domain of approx. 60 amino acids at the N-terminus, which is required for DNA-binding. All MADS-box proteins in the ABC model belong to the $MIKC^C$ type (YANG and JACK 2004). *In vitro,* the MADS-box proteins recognize and bind via the MADS domain to a nucleotide consensus sequence CC-$(A/T)_6$-GG termed CArG box, as homo- or heterodimers (RIECHMANN *et al.* 1996). The CArG box is located in the promoter region of numerous genes, which expression is regulated by MADS-box genes (THEISSEN *et al.* 2000; TILLY *et al.* 1998). Additional to the MADS domain, the majority of the plant MADS box proteins also share a less conserved I

(intervening) and a more conserved K (keratin-like) domains. The K-domain is not present in MADS proteins of animals and fungi, and in plants the K-domain is reported to be involved in protein-protein dimerization (JACK 2001; RIECHMANN *et al.* 1996; SCHWARZ-SOMMER *et al.* 1992; TRÖBNER *et al.* 1992). The C-terminal domain of MADS-box genes is highly variable in sequence and structure between family members and is probably associated with higher-order complex formation among different MADS-box proteins (EGEA-CORTINES *et al.* 1999; HONMA and GOTO 2001). Within the C-terminus, different conserved motifs, characteristic for members of different MADS-box subfamilies of transcription factors have been identified (KRAMER *et al.* 2003; KRAMER *et al.* 1998; ZAHN *et al.* 2006).

In *A. thaliana,* the *AG*-like genes *AG, SHATTERPROOF1/2 (SHP1/2)* and *SEEDSTICK (STK,* formerly *AGL11)* are members of the *euAG*, *PLE* and *AGL11* lineages, respectively (MA *et al.* 1991). *SHP1* and *SHP2* are paralogs and resent duplicates*,* which control the development of dehiscence zone and the pod shattering in *A. thaliana* fruits (LILJEGREN *et al.* 2000). *STK* is a D-class gene in *A. thaliana* and is considered to be an ortholog of *FBP7 (FLORAL BINDING PROTEIN7)* and *FBP11 (FLORAL BINDING PROTEIN11)* in *P. hybrida* (ANGENENT *et al.* 1995; COLOMBO *et al.* 1995; ROUNSLEY *et al.* 1995). Several duplication events are evident in the *AG* subfamily (BECKER and THEIßEN 2003; KRAMER *et al.* 2004; ZAHN *et al.* 2006). The first one occurred early in angiosperms after they diverged from gymnosperms and led to the *AG* and *STK* lineages, which include genes controlling stamen/carpel identity (C lineage) and ovule identity (D lineage), respectively (KRAMER *et al.* 2004; ZAHN *et al.* 2006). Within the C-lineage, another major, but more recent duplication event, took place early in core eudicot evolution before their divergence into rosids and asterids. This gave rise to the *euAG* and *PLENA (PLE)* clades, which contain *AG* and *SHP1/2*, respectively. Although *PLE* is the orthologous gene to *SHP1/2* in *A. majus,* it functionally resembles rather *AG* than the *SHP* genes (BRADLEY *et al.* 1993; DAVIES *et al.* 1999). Furthermore, *PLE* and *AG* represent relatively ancient paralogous lineages within core eudicots, with *AG* being the ortholog of the *A. majus FARINELLI (FAR)* gene, which is also a member of the *euAG* lineage (KRAMER *et al.* 2004; ZAHN *et al.* 2006). C-like genes have been found in species from all angiosperm lineages and in gymnosperms, but not in non-seed plants which suggests that they arose 300 MYA in the common ancestor of gymno- and angiosperms (BECKER and THEIßEN 2003).

The *AG* gene of *A. thaliana* is the first identified and fully characterized C-class gene. Its expression is initially uniformly distributed in the entire floral meristem of flowers at stage 3 (DREWS *et al.* 1991). During stages 5-7, the *AG* gene is strongly expressed in the carpel and stamen primordia. At later stages (stage 9 and 12), the *AG* expression further persist in carpels

and stamens (DREWS *et al.* 1991; YANOFSKY *et al.* 1990a). A strong hybridization signal is also present in the ovules, since they initiate at stage 9, and it is maintained there until stage 14, when fertilization takes place (BOWMAN *et al.* 1991a). Expression data on *AG* in stages 8, 10 and 11 are not available.

In *A. majus,* both *PLE* and *FAR* similarly expressed in the floral meristem and subsequently in the developing stamen and carpel primordia (DAVIES *et al.* 1999). In later developmental stages, *PLE* is expressed strongly in ovules and weaker in carpel walls and placenta, while *FAR* expression is weak in ovules and strong in placenta and both genes are further expressed in stamens.

Also *AG* orthologs within core eudicots show expression in the floral meristem, stamen and carpel primordia and subsequently in the developing stamens and carpels. Similar expression patterns to those of *AG* are reported for the paralogs in *Populus trichocarpa (P. trichocarpa)*. *P. trichocarpa* is a rosid species outside of *Brassicaceae*, which has two C-class genes arisen by a duplication event within the *Populus* lineage (BRUNNER *et al.* 2000). Both are also expressed in the floral meristem and subsequently in the developing stamens and carpels. The expression patterns of representatives of rosids and asterids indicate that C-class gene expression is highly conserved across core eudicots.

In the monocot *AG* subclade, several duplication events have occurred independently of those in the core eudicots (ZAHN *et al.* 2006). In the grasses *O. sativa* and *Z. mays,* these resulted into the C-genes *OSMADS3* and *OSMADS58*, and *ZAG1* and *ZMM2*, respectively. *OSMADS3* and *OSMADS58* display expression exclusively in whorls three and four, but the temporal distribution of their transcripts differs between paralogs (YAMAGUCHI *et al.* 2006b). The expression of *OSMADS3* commences in the floral meristem of the third and fourth whorls and is highly evaluated shortly before stamen and carpel primordia arise. After their inception, the expression disappears, and *OSMADS3* is strongly expressed only in the region of the floral meristem, where the ovule subsequently arises. Once the ovule primordium develops, the *OSMADS3* expression disappears also from there. The initial expression of *OSMADS58* coincides temporally with the *OSMADS3* expression in the regions of the floral meristem, where stamen, carpel and ovule primordia originate (YAMAGUCHI *et al.* 2006b). But in contrast to *OSMADS3*, *OSMADS58* remains expressed in the developing stamens, carpels and ovules throughout their entire development. In difference, the C-class genes in *Z. mays, ZAG1* and *ZMM2* display spatially overlapping, but not identical expression (MENA *et al.* 1996). Transcripts of *ZAG1* and *ZMM2* are present in carpels and stamens, but with different abundances. *ZAG1* is stronger expressed in carpels, whereas *ZMM2* shows higher transcript

abundance in stamens. The overlapping expression of *ZAG1* and *ZMM2* show that they are might be partially redundant in function, whereas the different intensity of their expression hints to a different contribution of each of them to stamen and carpel development. It was suggested that the C-class genes in monocots have arisen by a gene duplication preceding the divergence of the grasses. The C-class genes are divided into two subclasses based on similarity in the protein sequences, subclass I and II (YAMAGUCHI *et al.* 2006b). The two *AG* ortholog genes in *Z. mays ZMM2* and *ZMM23,* which are closely related to each other, have been classified together with *OSMADS3* to subclass I (YAMAGUCHI *et al.* 2006b). *ZAG1* is most closely related to *OSMADS58* and both are members of subclass II.

Generally, the expression patterns of *AG* orthologs correlate very well with their function. The strong loss-of-function *agamous* mutant, *ag-1,* develop multiple sterile flowers, which display a full homeotic conversion of stamens into petals and carpels into sepals, appearing in a spiral pattern (BOWMAN *et al.* 1989; BOWMAN *et al.* 1991b). Additionally, an ectopic flower develops in the third whorl of *ag-1* mutants. Similarly, *ag-3* displays a homeotic conversion of stamens into petals and carpels into sepals, whereas the weaker *AG* mutant allele *ag-4* results only in the conversion of carpels into sepals (SIEBURTH *et al.* 1995b). The evaluated number of floral organs in the third and fourth whorls of *ag-1* mutants demonstrates the function of *AG* in the termination of the activity of the floral meristem. Within the *A. thaliana* flower, the carpel is the last organ, which initiates and after its inception, the floral meristem is terminated. In difference, in *ag-1* mutants, the floral meristem does not terminate after establishment of the fourth whorl, but continues producing organs. In contrast, *ple-1* mutants display a third whorl composed of petaloid/staminoid organs and fourth whorl made up of sepaloid/petaloid/carpeloid organs, whereas in *far* mutants, only pollen development is aborted (DAVIES *et al.* 1999). The *ple-1/far* double mutants exhibit a petaloid third whorl organs, a homeotic transformation of carpels into petals and an additional flower in the fourth whorl. *PLE* confers carpel identity, whereas both *PLE* and *FAR* redundantly control stamen identity and floral meristem determinacy (BRADLEY *et al.* 1993; DAVIES *et al.* 1999). The single and double mutant phenotypes of *PLE* demonstrate that it is functionally more similar to *AG* than to *SHP1/2*. The *SHP1/*2 genes*,* members of the *PLE* lineage, are expressed in ovules and function redundantly with *AG* in the ovule development of *A. thaliana* (LILJEGREN *et al.* 2000). Also *STK,* which belongs to the *AGL11*-gene lineage, functions redundantly whit *AG* and *SHP1/*2 in ovule development (ROUNSLEY *et al.* 1995). Hence, ovule identity is controlled by the combinatorial action of C and D organ identity genes, which indicates that

absolute separation of D and C lineage function is not universally applicable (KRAMER *et al.* 2004).

Subfunctionalization has occurred independently also in the monocot C-gene lineage. *OSMADS3* and *OSMADS58* display a partial functional redundancy in controlling floral meristem determinacy and carpel and stamen development, but both contribute differently to these aspects. *OSMADS58* is stronger involved in the regulation of meristem determinacy and carpel morphogenesis than *OSMADS3*, whereas both are required for specification of stamen identity with a stronger contribution of *OSMADS3* (YAMAGUCHI *et al.* 2006b). Loss-of-function *osmads3-3* mutants exhibit an increased carpel number in the floral centre and a partial homeotic transformation of stamens into lodicules, whereas *osmads58-s1* silenced plants develop multiple carpels with severely affected morphology in the centre and a partial transformation of stamens into lodicules. *ZAG1,* similarly to *OSMADS58*, regulates floral meristem determinacy in *Z. mays*, while *ZMM2* might be required to promote stamen development (MENA *et al.* 1996). The function of *ZMM23* still needs to be investigated.

The intron/exon structure of C-class genes is highly similar (ZHANG *et al.* 2004). Within the large second intron of *AG*-like genes, functionally important *cis*-elements are located (SIEBURTH and MEYEROWITZ 1997). One of these is a conserved 70-bp element found in *AG*-like genes of eudicots and monocots, required for the late-stage expression of *AG*. Another conserved element in the second intron of all dicot C-genes, with the exception of *PLE,* is the aAGAAT box, which function still remains to be investigated (HONG *et al.* 2003). Furthermore, the second intron of *AG* contains binding sites for numerous transcription activators and repressors of its expression, such as *LFY, WUSCHEL* (*WUS*), *AP2, AP1, PERIANTHIA (PAN), UNUSUAL FLORAL ORGANS (UFO), LEU, SEUSS* (*SEU)* and *BELLRINGER (BLR)* (BAO *et al.* 2004; BUSCH *et al.* 1999; DEYHOLOS and SIEBURTH 2000; GREGIS *et al.* 2006; LIU and MEYEROWITZ 1995; LOHMANN *et al.* 2001; SIEBURTH and MEYEROWITZ 1997; SRIDHAR *et al.* 2004). Information about the control of C-gene expression comes exclusively from *A. thaliana*. In *A. thaliana*, *LFY* can bind directly to the second intron of *AG*, whereas a deletion of the *LFY* binding site in the second intron of *PLE* affects stamen development in *A. majus* (BUSCH *et al.* 1999; CAUSIER *et al.* 2009; LOHMANN *et al.* 2001).

The *AG* expression seems to be activated by different genetic pathways, in which *LFY* and *AP1* play important and partially overlapping roles (LIU and MARA 2010). Recently, it was hypothesized that LFY activates *AP1* in the early floral meristem (LIU and MARA 2010). Once activated, AP1 activates the *LFY* cofactor *SEP3,* probably indirectly through direct suppression of expression of genes, required for the transition of shoot meristem into floral

meristem (GREGIS *et al.* 2008; LIU *et al.* 2009; LIU *et al.* 2007; SRIDHAR *et al.* 2006). The LFY/SEP3 than induces the *AG* expression (CASTILLEJO *et al.* 2005; LIU *et al.* 2009). Once activated, *AG* auto regulates its own expression, probably via an AG/SEP3 complex, and suppresses the *AP1* expression (GOMEZ-MENA *et al.* 2005; LIU and MARA 2010). *LFY* binding site is found also in *OSMADS3* and *OSMADS58*, suggesting a function of the *LFY* ortholog in *O. sativa* in the control of C-class gene expression (CAUSIER *et al.* 2009).

Another direct activator of the *AG* expression is the WOX-domain transcription factor *WUS* (LAUX *et al.* 1996b; MAYER *et al.* 1998). *WUS* maintains a central stem cell population in the shoot and floral meristem. It binds to the second intron of *AG* and induces its expression in the centre of the floral meristem (LENHARD *et al.* 2001; LOHMANN *et al.* 2001). After stage 6, *AG* possibly in concert with other factor(s), represses the *WUS* expression and terminates the floral meristem. *PAN* and *UFO* are also *LFY* cofactors and direct activators of the *AG* expression (CHUANG *et al.* 1999; LOHMANN *et al.* 2009).

In contrast, *LEU, SEU, AP2* and *BLR* are transcriptional repressors of *AG* in the first and second floral whorls (BAO *et al.* 2004; BOWMAN *et al.* 1991b; LIU and MEYEROWITZ 1995). *LEU* and *SEU* function in combination to suppress *AG* expression (FRANKS *et al.* 2002). The LEU and SEU proteins interact physically with each other in yeasts and *A. thaliana* protoplasts and are able to repress transcription there through a chimeric DNA-binding domain (SRIDHAR *et al.* 2004). It was hypothesized that a putative complex, including SEU and LEU proteins, is associated with the direct or indirect transcriptional repression of *AG* (FRANKS *et al.* 2006; FRANKS *et al.* 2002).

It has been hypothesized that the floral organ identities are determined by the combinatorial action of the MADS-box proteins and that the different combinations of MADS-box proteins activate different groups of target genes in each floral whorl (HONMA and GOTO 2001). In *A. thaliana*, multimeric complexes including the B-class proteins APETALA3 (AP3) and PISTILATA (PI), the SEP3 protein and the AG protein are able to bind DNA, and this led to the postulation of the 'floral quartet' model (THEISSEN and SAEDLER 2001). The protein quartets consist of two dimers, which recognize and bind to two different CArG-boxes within the promoter region of the target gene. According to this model, carpel identity is defined by a 'quartet' including AG and SEP proteins, whereas the protein complexes, determining stamen identity, contains PI, AP3, AG and SEP proteins.

1.4.3 *SPATULA (SPT)*-like genes

The basic-helix-loop-helix (bHLH) genes are members of a large family of transcription factors found in plants and animals, where they control diverse developmental processes (BUCK and ATCHLEY 2003). In *A. thaliana*, bHLH transcription factors are associated with various processes like anthocyane synthesis, trichome formation, and light signalling (BAILEY *et al.* 2003; HEIM *et al.* 2003). All bHLH genes share a highly conserved bHLH domain, composed of a DNA-binding basic domain at the N-terminus and two α-helices separated by a variable loop region (helix-loop-helix, HLH). The basic domain confer specify in DNA target recognition, whereas the α-helices are associated with homo- and heterodimerization. bHLH proteins bind DNA as dimers and most of them recognize the symmetric E-box (CANNTG) or one of its variants, the G-box (CACGTG) located within the DNA upstream promoter region of target genes (HEIM *et al.* 2003; LI *et al.* 2006; PATTANAIK *et al.* 2008).

SPT in *A. thaliana* is the founder and so far the only *SPT* gene, for which both detailed expression and functional data are available (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; HEISLER *et al.* 2001). In *A. thaliana,* the closest relative of *SPT* bHLH gene is *ALCATRAZ (ALC). SPT* and *ALC* share 51 identical residues out of 62, including the bHLH domain and its surrounding regions (HEISLER *et al.* 2001; RAJANI and SUNDARESAN 2001). *ALC* defines the separation layer in the dehiscence zone in *A. thaliana* fruit (RAJANI and SUNDARESAN 2001). *SPT* and *ALC* probably had arisen by a recent duplication event in the *Brassicaceae* ancestor (GROSZMANN *et al.* 2008).

In *SPT*-like genes, a conserved bipartite nuclear localization sequence (NLS) was identified (GROSZMANN *et al.* 2008). Two further highly conserved domains with predicted secondary structure have been found in the eudicot *SPT* proteins, an amphipathic helix located closely to the N-terminus of the protein, and an acidic domain placed upstream close to the bHLH domain (GROSZMANN *et al.* 2008). These two domains are not found outside of eudicot *SPT*-like genes. It was suggested that the acidic domain mediates the function of SPT in activating downstream target gene expression. The role of the amphipathic helix still needs to be investigated, but it has been shown that such structures are often associated with protein-protein interactions, possibly due to its proximity to the bHLH domain. Additionally, nine amino acids placed downstream of the bHLH domain were supposed to form a beta strand. The role of the beta strand also needs further elucidation, but it might support the two helix of HLH in the protein dimerization processes (GROSZMANN *et al.* 2008).

In *A. thaliana*, *SPT* is expressed in the centre of the floral meristem (HEISLER *et al.* 2001). In stage 6 and after the gynoecium developed, *SPT* is expressed at the apex of the carpel

primordia and along the carpel margins. At stage 8, *SPT* transcripts are present in the adaxial side of the presumptive replum region. Between stages 9-11, *SPT* transcripts are detected within the developing septum, stigma and transmitting tract. *SPT* expression is found in the ovule primordia at stage 10, and is further maintained there. In a gynoecium at stage 13, *SPT* is present in the entire valves, but is excluded from the vascular bundles. In the silique, *SPT* is expressed in the valve margins and in the neighbour cells, where the DZ will be established. Subsequently, expression of *SPT* is present in the DZ. Outside of the gynoecium, *SPT* is widely expressed in different tissues throughout vegetative and reproductive development (HEISLER *et al.* 2001). *SPT* expression is detected in petals, stamens, seeds and young leaves but not in sepals.

In *A. thaliana, SPT* regulates the growth of carpel margins and the deriving from them style, stigma, septum and transmitting tract (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002). The loss-of-function *spt-2* mutants display abnormalities in the carpels and fruits. Until stage 6, the gynoecium of the strong *spt-2* mutants is indistinguishable from wild-type gynoecium. The first defects appear around stage 7. The gynoecia of the *spt-2* mutants are narrower, but longer than in wild-type, and its apical part is wider. Additionally, the carpels are not fused in the stylar region and the transmitting tract tissue is completely missing. The development of style, stigma and is impaired and the ovule number is reduced. Later in the fruits, which are shorter than wild-type siliques, a reduced seed set, restricted to the apical part of the siliques, is produced (ALVAREZ and SMYTH 1999; HEISLER *et al.* 2001).

A novel function of *SPT* in the cold germination of *A. thaliana* seeds has been reported a few years ago (PENFIELD *et al.* 2005). Penfield and colleagues demonstrated that *SPT* is a key regulator of seed germination as response to light and temperature by repressing the gibberellin biosynthesis.

Recently, Groszmann and colleagues have show that two main sub-regions located within the *SPT* upstream promoter sequence are required for the overall *SPT* expression (GROSZMANN *et al.* 2010). These contain binding sites for tissue-specific enhancers and silencer. Within the upstream promoter region of *SPT*, putative Auxin Response Elements (AuxREs) were identified (GROSZMANN *et al.* 2010). These are binding sites for Auxin Repose Factors (ARFs) and have the conserved sequence TGTCTC. Previously, indirect evidences suggested that *SPT* in *A. thaliana* is possibly connected with the auxin levels in the gynoecium and this probably occurs through binding of ARFs to AuxREs within the *SPT* promoter. Nemhauser and colleagues proposed that the establishment of the apical-basal patterning early in the development is dependent on an auxin gradient (NEMHAUSER *et al.* 2000). Based on this

hypothesis, auxin is synthesized at the apical part of the gynoecium and subsequently transported downstream, generating a declining gradient from the top to the base of the gynoecium. Furthermore, high levels of auxin in the apical part induce the development of the apical tissues style and stigma, intermediate levels determine the ovary, and low levels at the gynoecium base promote gymnophore formation (NEMHAUSER *et al.* 2000). An inhibition of the polar auxin transport (PAT) impairs the establishment of proper apical-basal patterning and results in elongated style and stigma, a reduced ovary and an extended gymnophore. When an inhibitor of PAT is applied to the apex of *spt* mutant gynoecia, the wild-type phenotype is almost restored, indicating that *SPT* very likely participates in the auxin transport from the apical to the basal gynoecium regions or may control negatively the PAT down from the apical regions (NEMHAUSER *et al.* 2000; STALDAL and SUNDBERG 2009). *ETTIN (ETT)*, a member of the ARF family, probably negatively regulates *SPT*. It was proposed that *ETT* controls the auxin levels in the gynoecium and elaborates the boundaries between style and ovary, and ovary and gymnophore (NEMHAUSER *et al.* 2000; SESSIONS *et al.* 1997). The gynoecia of *ett* mutants display defects in the development of the same apical tissues affected also in the *spt* mutants, style and stigma. Putative AuxREs have been found also in *BoSPT* and *BrSPTa/b,* the *SPT* homologs in *Brassica oleraceae (B. oleraceae)* and *B. rapa,* respectively (GROSZMANN *et al.* 2010).

It was also suggested that the *SPT* expression is positively regulated by *INDEHISCENT (IND)* (GROSZMANN *et al.* 2008). *IND* is a bHLH transcription factor and controls the development of the dehiscence zone in *A. thaliana* siliques (LILJEGREN *et al.* 2004). An atypical E-box representing a potential binding site for *IND* located closely to one of the mutated AuxREs was identified within the *SPT* promoter sequence. Specifically, the *SPT* expression in the indehiscence zone is abolished in *ind* mutant siliques (GROSZMANN *et al.* 2010). *IND* might mediate the *SPT* interaction with auxin since it was demonstrated that *IND* promotes the auxin efflux from the precursor cells (SOREFAN and OSTERGAARD 2007).

In respect to the polar auxin transport, an interaction between *SPT* and *STYLISH1 (STY1)* was supposed. *STY1* controls the establishment of style and stigma in *A. thaliana* and *SPT* and *STY1* expression overlaps in the apical regions of the gynoecium (HEISLER *et al.* 2001; KUUSK *et al.* 2006). Furthermore, the *sty1-1spt-2* double mutants develop gynoecia without any stigmatic tissues and a strong reduction in the style (KUUSK *et al.* 2006). The expression of *STY2,* the paralog of *STY1,* is increased by an ectopic expression of *SPT* in *A. thaliana,* but *STY2* is expressed normally in *spt-2,* demonstrating that, if *SPT* directly activates *STY2* expression, that occurs in concert with other transcription factors (GROSZMANN *et al.* 2008).

In *A. thaliana*, *SPT* expression in the sepals is negatively regulated by the A-class gene *APETALA2 (AP2)*. In *ap2-2* mutants, ectopic *SPT* expression in the sepals causes the appearance of cell types characteristic for septum, transmitting tract and stigma, indicating that *AP2* prevents *SPT* expression in wild-type sepals (HEISLER *et al.* 2001).
SlSPT, the *SPT* homolog in *Solanum lycopersicum (S. lycopersicum),* is able to complement the defects in the fruits of *spt-2* mutants in *A. thaliana* (GROSZMANN *et al.* 2008). The complemented *spt-2* mutants develop siliques with a wild-type appearance and these form an increased seed set equally distributed along the fruit, similarly to wild-type. Based on this, it was concluded that *SlSPT* is able to provide completely the *SPT* function in the gynoecium development of *A. thaliana.*

1.5 *GLOBOSA (GLO)*-like genes in petal and stamen development

B-class floral homeotic genes are key regulators of the identity and development of the second and third floral whorls across angiosperms. They are members of the MADS-box gene family. The first B-class homeotic genes were almost simultaneously identified and functionally characterized in the model core eudicots *A. thaliana* and *A. majus. A. thaliana* has two B-genes, *PISTILATA (PI)* and *APETALA3 (AP3),* while in *A. majus* the B-genes are *GLOBOSA (GLO)* and *DEFICIENS (DEF)*. *PI* is the paralog of *AP3* in *A. thaliana* and *GLO* is the paralog of *DEF* in *A. majus. GLO* and *DEF* are the orthologs of the *A. thaliana PI* and *AP3*, respectively. The B-proteins share the characteristic structure of $MIKC^C$ type MADS-box proteins, but have a variable C-terminus. Numerous gene duplications have occurred within the *DEF/GLO* subfamily across angiosperm clades. It was hypothesized that a key duplication event has occurred in angiosperms after their split from the gymnosperms, but before their diversification into the extant angiosperm lineages and led to the *PI* and *paleoAP3* gene clades (HERNANDEZ-HERNANDEZ *et al.* 2007; KIM *et al.* 2005; KRAMER *et al.* 1998; KRAMER and IRISH 2000; THEISSEN *et al.* 2000; ZAHN *et al.* 2005). Another major duplication has occurred within the *paleoAP3* lineage close to the base of core eudicots and led to two paralogous *AP3* sublineages, *euAP3* and *TM6* present in the extant core eudicots (KRAMER *et al.* 1998; KRAMER and HALL 2005; ZAHN *et al.* 2005). The *TM6* sublineage is named after the *TOMATO MADSBOX GENE6* (PNUELI *et al.* 1991). *TM6* genes have been found in some *Solanaceae* spices, but not in *A. thaliana* and *A. majus*. The *euAP3* and the *TM6* genes differ in their C-terminus as the *euAP3*-genes contain in the C-terminus a motif

called *euAP3* domain, while the *TM6* genes have a *paleoAP3* motif instead (KRAMER *et al.* 2006). The *paleoAP3* domain of the *TM6* genes shares some sequence similarity with the ancestral *paleoAP3* motif detected in the *paleoAP3* type genes characteristic for basal eudicots, monocots and basal angiosperms. It was shown that the *euAP3* motif has evolved from the ancestral *paleoAP3* domain via a frameshift mutation (KRAMER *et al.* 2006; VANDENBUSSCHE *et al.* 2003).

GLO orthologous genes across angiosperms are expressed predominantly in the second and third floral whorls, regardless of the floral organs developing there. Their expression is detected since very early developmental stages in the floral meristem, in carpel and stamen primordia and is maintained in the developing petals and stamens during flower development. In *A. thaliana* and *A. majus*, *GLO*-like genes are constantly expressed in petals and stamens since their inception (GOTO and MEYEROWITZ 1994; TROBNER *et al.* 1992). But *GLO* and *PI* are differentially expressed in the floral meristem. In stage 3 flowers, *PI* is expressed in the cells of the floral meristem, which will give rise to petals, stamens and carpels, as the fourth whorl expression disappears before the carpel initiation at stage 5 (GOTO and MEYEROWITZ 1994). In *A. majus*, *GLO* is expressed only in the cells of the floral meristem that will give rise to the petals and stamens, but not in the centre of the meristem (TRÖBNER *et al.* 1992).

Among basal eudicots, multiple duplication events are evident in both *GLO* and *DEF* clades (KRAMER *et al.* 1998). *PI* orthologs within the most basal eudicot order Ranunculales seems to be products of numerous relatively recent duplications (KRAMER *et al.* 2003). In Ranunculales, besides the characteristic petal and stamen expression, *GLO* orthologous genes also show variable expression in first and/or fourth whorls throughout different developmental stages (DREA *et al.* 2007; KRAMER *et al.* 2003; KRAMER *et al.* 2007b; KRAMER and IRISH 2000). Among monocots, the expression of *GLO* orthologs is also present in second and third floral whorls, although second whorl organs are different in this lineage compared to eudicots. In the second whorl of grass monocots like *O. sativa* and *Z. mays*, lodicules develop, whereas in non-grass monocots, the two outer whorls are composed of tepals, which are organs with combined sepal and petal futures. The two grass genera *Streptochaeta* and *Anomochloa*, considered being the most basal grass monocots, do not have lodicules. Instead, both develop different and distinct from each other organs outside of the stamens, *Streptochaeta* develops bracts and *Anomochloa* has hairy structures (WHIPPLE *et al.* 2007). In both species, expression of *GLO* orthologs is present in the second and third whorls. Within the grass monocots *O. sativa* and *Z. mays,* expression of *GLO* homologs is found additionally to the second and third whorls, also in the fourth whorl, but is always excluded from the first one (KANG *et al.* 1998;

MUNSTER *et al.* 2001; WHIPPLE *et al.* 2007; YADAV *et al.* 2007). In the extant non-grass monocots, *GLO* orthologs show variable expression. For example, in *Asparagus officinalis (A. officinalis)* transcripts of *GLO*-like genes are detected only in second and third whorls, whereas transcripts of *GLO* orthologs in *Tulipa* are observed in all floral whorls (KANNO *et al.* 2003; PARK *et al.* 2003a; PARK *et al.* 2003b).

As the conserved expression patterns indicate, the *GLO*-like genes have conserved functions in specifying petal/lodicule and stamen identities and controlling their entire development. In the strong *pi-1* mutants of *A. thaliana,* the petal and stamen identities are lost. The *pi-1*flowers develop a second sepal whorl instead of petals and the stamens in the third whorl are completely absent (BOWMAN *et al.* 1989; BOWMAN *et al.* 1991b). Instead, the third whorl of *pi-1* mutants is occupied by ectopic carpeloid structures, fused to the central gynoecium. Similar mutant phenotype was observed in *A. majus,* when *GLO* was mutated. In the *glo-1* mutants, the sepals in the first whorl are not affected and the petals are transformed into sepaloid structures. In the stamen whorls, a variable number of ectopic gynoecia develop and these fuse to the central gynoecium (SOMMER *et al.* 1990; TROBNER *et al.* 1992).

Similarly, in monocots, *GLO*-like genes confer organ identity in the second and third whorls of the flower. When *GLO* orthologs in *O. sativa* are silenced, this affects the lodicule and stamen development in whorl two and three, respectively (CHUNG *et al.* 1995; KANG *et al.* 1998; YADAV *et al.* 2007). Similar to core and basal eudicots, in grass monocots no obvious phenotype in the fourth floral whorl was ever observed, although *GLO* orthologs are expressed there.

Generally, the expression patterns *AP3* and *DEF* and the phenotypes of their loss-of-function mutants resemble those characteristic for *PI* and *GLO* genes. *AP3* and *DEF* are expressed in petals and stamen whorls of *A. thaliana* and *A. majus* flowers, respectively (GOTO and MEYEROWITZ 1994; JACK *et al.* 1992). Mutation in *AP3* in *A. thaliana* and *DEF* in *A. majus* cause mutant phenotypes similar to those described for *pi* and *glo.* In both *ap3* and *def* single mutants*,* the petals are homeotically converted into sepals and the stamens into carpels (JACK *et al.* 1992; SOMMER *et al.* 1990).

The *AP3* orthologs within basal eudicots are more similar to the genes of the *TM6* lineage of core eudicots than to the *euAP3* lineage (KRAMER *et al.* 1998). Multiple independent gene duplications within the *AP3* clade have also occurred within the basal eudicots of the ranunculids. Based on the phylogenetic position, three distinct *AP3* lineages are present within the ranunculids, *AP3-I, AP3-II* and *AP3-III* which have arisen by major duplication events and were probably present in the last common ancestor of Ranunculales before they

split from their sister basal eudicot lineages (KRAMER *et al.* 2003). The basal eudicot orthologs within the *AP3* clade are expressed not only in petals and stamens but similar to their *PI*-like paralogs also occasionally in sepals and/or carpel (KRAMER *et al.* 2003; KRAMER and IRISH 2000). The expression in the sepals, often observed for *AP3* orthologs in basal eudicots can be attributed to the petaloid organs developing in their perianth.

Within monocots, gene duplication events also led to numerous *DEF*-like genes (KANNO *et al.* 2003; MONDRAGON-PALOMINO *et al.* 2009; TSAI *et al.* 2004). In *Orchidaceae* (orchids), even four distinct paralogous *DEF*-like gene clades are found, which presumably have arisen by at least three gene duplications at around 62 MYA (MONDRAGON-PALOMINO *et al.* 2009). In the grasses *O. sativa* and *Z. mays*, only single *DEF* orthologs are present. In *Z. mays,* the putative *DEF* ortholog *SILKY1 (SIL1)* is expressed early in the regions of the floral meristem, where lodicule and stamen primordia initiate (AMBROSE *et al.* 2000). Subsequently, it is continuously expressed in lodicules and stamens throughout during the entire development. In contrary to the *GLO* orthologs in *Z. mays*, *SIL1* is no expression in carpels at any developmental stage. Also the *AP3* ortholog in *O. sativa*, *SUPERWOMAN1 (SPW1),* is expressed in lodicule and stamen primordia, and subsequently in the developing lodicules and stamens, but not in the carpels (NAGASAWA *et al.* 2003). In the mutant *spw1-1* floret, the lodicules are homeotically transformed into palea-like structures, whereas the stamens are converted into carpels. A similar loss-of-function flower phenotype is observed in the *si1* mutants of *O. sativa*. The lodicules are transformed into palea/lemma-like organs and the stamens into carpels (AMBROSE *et al.* 2000).

The expression levels of *AP3 (DEF)* and *PI (GLO)* genes are dependent on each other although the initial expression of *PI* (*GLO)* is dispensable of that of *AP3* (*DEF*), and the other way around (GOTO and MEYEROWITZ 1994; HONMA and GOTO 2000; JACK *et al.* 1992; TROBNER *et al.* 1992). The activation of B gene expression in *A. thaliana* occurs in a similar manner as the activation of *AG* expression (LIU and MARA 2010). LFY activates the *AP1* expression. Subsequently, AP1 activates the LFY cofactor *SEP3,* and LFY/SEP3 than induces the *AP3* and *PI* expression (LIU and MARA 2010). Also UFO is a cofactor of LFY in the activation of B gene expression (LEVIN and MEYEROWITZ 1995; WILKINSON and HAUGHN 1995). The LFY protein can bind directly to the promoter region of *AP3,* whereas *UFO* does not have DNA-binding affinity to the *AP3* promoter. Furthermore, UFO and LFY can interact directly with each other (CHAE *et al.* 2008; LAMB *et al.* 2002). The activation of *AP3* expression occurs through binding of LFY/UFO to the *AP3* promoter. Subsequently, AP3 and PI negatively regulate the *AP1* expression (LAMB *et al.* 2002; NG and YANOFSKY 2001;

SUNDSTROM *et al.* 2006; WELLMER *et al.* 2004). This negative regulation is probably directed by binding of the PI protein to a CArG-box within the *AP1* promoter region (WELLMER *et al.* 2004). Also the other A-class gene in *A. thaliana, AP2,* is a possible regulator of the *PI* expression, although the nature of this regulation still needs to be elucidated (GOTO and MEYEROWITZ 1994).

As predicted by the ABC model, further modulation of *PI* and *AP3* expression is achieved by the C-class gene *AG*. It has been reported that the *AP3* expression is regulated by *AG* (GOMEZ-MENA *et al.* 2005). Also *in vitro*, AG and AP3 proteins interact with each other (HONMA and GOTO 2001). Furthermore, DEF- and GLO-like proteins function as obligate heterodimers (RIECHMANN *et al.* 1996). These bind to CArG boxes in the *AP3* promoter region and reinforce their own expression. In *A. thaliana*, the *AP3* autoregulation occurs directly, whereas the *PI* autoregulation is probably indirect (HONMA and GOTO 2001). The *AP3* promoter has at least two CArG boxes, to which AP3/PI heterodimers can bind, whereas the *PI* promoter does not contain any CArG boxes (HILL *et al.* 1998; RIECHMANN *et al.* 1996; TILLY *et al.* 1998). Heterodimers of B-class orthologs are observed also in basal eudicots and monocots, but the presence of an autoregulation loop in these lineages still needs to be investigated (DREA *et al.* 2007; KANNO *et al.* 2003; KRAMER *et al.* 2007a; MOON *et al.* 1999a; TZENG and YANG 2001; WHIPPLE *et al.* 2004; WINTER *et al.* 2002b). In core eudicots, B protein heterodimers are required for (i) autoregulation of their own expression via binding to CArG boxes in the promoter region, and (ii) formation of multimeric protein complexes (EGEA-CORTINES *et al.* 1999; HONMA and GOTO 2000; HONMA and GOTO 2001; IMMINK *et al.* 2009; LESEBERG *et al.* 2008).

In gymnosperms, B genes were characterized in *Gnetum gnemon (G. gnemon)* and *Picea abies (P. abies)*. They are expressed in the male, but not in the female cone (BECKER and THEIßEN 2003; MOURADOV *et al.* 1999; SUNDSTROM *et al.* 1999; SUNDSTROM and ENGSTROM 2002; WINTER *et al.* 1999). *GGM2* and *GGM15* are B-class genes in *G. gnemon,* whereas *DAL12* is the DEF/GLO-like gene ortholog in *P. abies* (BECKER and THEIßEN 2003; SUNDSTROM *et al.* 1999; WINTER *et al.* 2002a). Within gymnosperms, there are three B-class gene clades, which are sister clades to the angiosperm *DEF/GLO* clade and are found only in gymnosperms, *GGM2*-like, *DAL12*-like and *CJMADS1*-like clades. None of the three gymnosperm B gene clades is a direct sister group of *DEF* or *GLO* genes (WINTER *et al.* 2002). The function of *GLO* homologs in specifying the identity of male reproductive organs in angiosperms is possibly derived from the ancestral roles of B-like genes in the common

ancestor of angiosperms and gymnosperms, where they might have had a similar function in controlling male organ identity (WINTER *et al.* 2002).

It was predicted by the 'floral quartet' model that in *A. thaliana,* different combinations of floral homeotic proteins encoded by the main classes floral genes specify the identities of the floral organs (THEISSEN and SAEDLER 2001). According to this model, petal identity in the second whorl is conferred by protein tetramers composed of the A-class protein AP1, the B-class proteins AP3 and PI, and the E protein SEP, whereas stamen identity is specified by quartets made up of the B-class proteins, the C-class proteins AG and one SEP protein.

2. Aims of the thesis

Angiosperms represent the most progressive and dominant plant group nowadays. The flower is the most characteristic feature of angiosperms, which despite the enormous diversity in flower shape, colour and size, shares a basic common architecture and usually consists of four floral organ types. The evolutionary developmental (evo-devo) genetics of flower development tries to elucidate the origin of the flower and its subsequent diversification. Within the flower, the origin of the carpel was undoubtedly a main prerequisite of the enormous evolutionary success of angiosperms.

A main aim of this work was to obtain the expression patterns of the orthologs of key floral organ identity and carpel developmental genes in *E. californica. E. californica* is an emerging model plant within basal eudicots and is considered to be an early diverging genus within Ranunculales. We investigated the expression of the *E. californica* carpel developmental genes *EcCRC* and *EcSPT,* and of the organ identity genes *EScaAG1/2* and *SIR* using RT-PCR and in *situ* hybridisation on vegetative and flower organs through developmental stages. The examination of expression patterns of a particular gene is a general starting strategy to get a first hint about a gene function. Most genes function in the tissues, where they are expressed. A main focus of my work was the comparison of the expression patterns of *EcCRC*, *EcSPT*, *EScaAG1/2* and *SIR* to their related orthologous genes in representative species from distinct angiosperm lineages. The conservation and diversity between expression patterns allow suggesting, how the function within a particular gene family has evolved in angiosperms. For full characterization of gene function, functional analyses are required. The gathered functional data on *EcCRC*, *EcSPT*, *EScaAG1/2* and *SIR* was compared to the available data from other species. The function of *EcCRC*, *EcSPT* and *EScaAG1/*2 genes was examined via

transient down regulation of their expression. The characterization of *SIR* function was based on the mutant phenotype of the stable *sirene (sir)* mutants.

Another main focus of this work was to elucidate the evolutionary path, with the help of the methods mentioned above, that carpel developmental genes as well as homeotic gene function have undergone in flowering plants. Such results contribute to our understanding of the genetic programs underling flower and in particular carpel development in *E. californica* and how these programs have evolved.

3. Results and Discussion

Table 2 Methods, done by the author in publications I and II, and manuscripts I and II:

Methods	Publications and Manuscripts
Total RNA isolation	Publication I, Manuscript I
cDNA synthesis	Publication I, Manuscript I
RT-PCR primer design	Publication I, Manuscript I
RT-PCR	Publication I, Manuscript I
PCR amplification	Publication I, Manuscript I
Gene cloning	Manuscript I
In situ hybridization	Publications I and II, Manuscripts I and II
VIGS	Manuscript I

3.1 Conservation and novelty in expression and function of carpel developmental genes in *E. californica*

In this chapter, the expression patterns and the function of orthologs of carpel developmental genes in *E. californica* will be discussed by comparing them to ones of functional orthologs from representative species of angiosperm lineages. The expression of the putative orthologs of carpel developmental genes in *E. californica* was examined via RT-PCR and *in situ* hybridization. Their function was revealed using Virus-induced gene silencing (VIGS), which is a method for transient down regulation of gene expression. In addition, suggestions for

future investigations will be made, necessary to elucidate the specific roles of the *E. californica* homologs in the common genetic pathways underling carpel development in *E. californica*.

3.1.1 *EcCRC*

EcCRC is the single ortholog of the *A. thaliana* gene *CRC,* a member of the small plant-specific YABBY family of transcription factors. *EcCRC* is the only reported *CRC*-like gene within the *Papaveraceae* family, and together with the *CRC* orthologs in the early diverged species *A. formosa* and *Grevillea robusta (G. robusta)* is a member of the *CRC*-like gene clade of the basal eudicots (Figure 1, Publication I).

3.1.1.1 *EcCRC* expression is confined to carpels and mature seeds

The RT-PCR experiments I performed on vegetative and reproductive organs of *E. californica* revealed that *EcCRC* expression is confined to carpels, but is excluded from all other floral organs, leaves and green seeds (Figure 2A, C). Additionally, *EcCRC* expression is detected in mature seeds. Moreover, *EcCRC* is continuously expressed throughout developmental stages as the expression starts in stage 1-5 in floral buds with 0-1 mm in diameter and decreases in floral buds with 3mm in diameter, when female meiosis occurs (stages according to (BECKER *et al.* 2005).

To obtain more detailed information on the spatial and temporal expression of *EcCRC*, I performed *in situ* hybridization. In stage 5, *EcCRC* is expressed in the entire gynoecium, which has just initiated (Figure 2B, Publication I). During stage 6, the *EcCRC* expression changes dynamically. Longitudinal section through floral buds shows that at the beginning of stage 6, the expression of *EcCRC* is confined to abaxial domains embracing two-thirds of the carpel walls, but is excluded from the most apical and basal carpel regions (Figure 2C, Publication I). Additionally, *EcCRC* expression domain is present at the centre of the gynoecium base, where the cell division of the floral meristem was terminated just after gynoecium inception in the previous stage. In a cross section, *EcCRC* expression occurs in two wide strips surrounding the presumptive replum regions of the gynoecium, but without being expressed inside (Figure 2E, Publication I). In addition, *EcCRC* transcripts are distributed uniformly in the carpel walls. Longitudinal sections through flowers of stage 6 show that the *EcCRC* expression in the carpel walls loses its abaxial character and expands

into the entire gynoecium, while remaining further excluded from its apical part (Figure 2D, Publication I). In longitudinal view of the gynoecium at stage 7, *EcCRC* expression is apparent as abaxial slender streaks along the carpel walls, enclosing the presumptive replum and placenta, but without being expressed in there (Figure 2F, Publication I). Moreover, the domain of expression at the gynoecium base is maintained in a small group of cells. After ovule initiation, the *EcCRC* hybridization signal is detected in abaxial domains along the carpel walls enveloping the placenta and presumptive replum (Figure 2G, H, I, Publication I). *EcCRC* expression was not detected in the carpel margins, placenta, replum and ovules at any of the examined developmental stages.

The early carpel expression seems to be characteristic for *CRC* orthologs, suggesting that *CRC*-like genes control the establishment of carpel features since carpel inception (Figure 4). I detected initial expression of *EcCRC* in the just initiated gynoecium at stage 5. Similar to *E. californica,* in *A. thaliana,* the gynoecium also develops at around stage 5 (stages according to (BOWMAN and SMYTH 1999b; SMYTH *et al.* 1990). *CRC* expression is firstly detected at stage 6, showing that conceivably *EcCRC* is required earlier in the carpel development than *CRC* (Figure 4). Expression of *CRC* orthologs in the centre of the floral meristem before carpel inception has been reported for *AfCRC,* the *CRC* ortholog in the Ranunculales species *A. formosa* (LEE *et al.* 2005d). Besides *EcCRC*, *AfCRC* is the only other basal eudicot *CRC* orthologous gene, on which expression data, although incomplete, is available (Figure 4). The expression in the floral meristem seems to be characteristic also for *CRC*-like genes in monocot grasses. Such expression is reported for *DL* in *O. sativa,* the first identified monocot *CRC* ortholog, and recently also for *DL*-like genes within three further grass species, *Z. mays*, *Triticum aestivum (T. aestivum*, wheat) and *Sorghum bicolor (S. bicolor,* sorghum) (ISHIKAWA *et al.* 2009; YAMAGUCHI *et al.* 2004) (Figure 4).

Abaxial carpel expression

The abaxial expression of *EcCRC* in the gynoecium wall of *E. californica* resembles the expression of *CRC*-like genes across eudicots (Figure 4). Such expression pattern has been reported for *CRC* orthologs in the core eudicot species *A. thaliana* and *P. hybrida* (BOWMAN and SMYTH 1999b; LEE *et al.* 2005a). Abaxial carpel expression has been demonstrated also for *AfCRC* (Figure 4). In mature flowers, *AfCRC* is expressed abaxially around the central vascular bundle of the carpel (LEE *et al.* 2005b). Abaxial expression in the gynoecium is also reported for *A. trichopoda*, considered to be the earliest diverged angiosperm species,

indicating that very likely, the abaxial expression of *CRC*-like genes has developed already in the lineage leading to *A. trichopoda* (FOURQUIN *et al.* 2005). Furthermore, the abaxial pattern of expression seems to be characteristic for the ancestral *CRC* gene and suggests an ancestral function of *CRC*-like genes in elaboration of abaxial cell fate in the gynoecium wall. The characteristic abaxial expression is independently lost only in grasses, where the *DL* genes are expressed uniformly in the entire carpel (ISHIKAWA *et al.* 2009; YAMAGUCHI *et al.* 2004). In contrast, the expression of the *CRC* ortholog in the non-grass monocot *A. asparagoides*, *AaDL,* resembles rather the expression of *CRC*-like genes in eudicots than the ones in monocot grasses as this persists only in the abaxial gynoecium wall (Figure 4). This indicates that *CR*C orthologs acquired ubiquitous carpel expression only within grasses after their split from non-grasses (NAKAYAMA *et al.* 2010). Another possibility is that the abaxial expression has been remained only within the *Asparagus* lineage, which branched off earlier than *Poaceae* within monocots, but has been lost in grass monocots. The differential expression of *CRC*-like genes in grasses shows that they might have acquired an additional function in establishment of the adaxial carpel wall in difference to the eudicot *CRC* orthologs, which function only in the abaxial tissue differentiation.

Apical carpel expression

In contrast to *CRC* homologs in core eudicots, monocots and even in *A. formosa*, which are expressed continuously in the apical region of the gynoecium, *EcCRC* is not expressed there (BOWMAN and SMYTH 1999b; ISHIKAWA *et al.* 2009; LEE *et al.* 2005c; NAKAYAMA *et al.* 2010; YAMAGUCHI *et al.* 2004) (Figure 4). Therefore, *EcCRC* possibly does not control carpel fusion in *E. californica* pointing out a functional diversification of the *E. californica CRC* ortholog from the other *CRC*-like genes in carpel fusion. It is possible that the apical domain of expression has been lost in the members of *Papaveraceae* or only in the lineage leading to *Eschscholzia*. Due to the lack of expression data on *CRC*-like genes outside of *E. californica* and *A. formosa*, both scenarios seems to be plausible.

Adaxial carpel expression

EcCRC is expressed uniformly in the carpel walls, comprising also the adaxial regions, in stage 6 (Figure 2E, Publication I). Also *CRC* is expressed in adaxial domains at stage 7, but these comprise only the outermost adaxial cell layer of the carpels (BOWMAN and SMYTH 1999a) (Figure 4). Adaxial internal domains of *AaDL* expression within the carpel walls, similar to the ones reported for *CRC*, persists in later developmental stages of the monocot *A.*

asparagoides (NAKAYAMA *et al.* 2010) (Figure 4). This puts forward that such temporal adaxial expression might be acquired independently in some eudicot and monocot species.

Placenta expression

We did not observed *EcCRC* expression in the placenta at any of the developmental stages analysed with *in situ* hybridization. Placental expression is reported for *PhCRC* in *P. hybrida,* but not for *CRC* (BOWMAN and SMYTH 1999b; LEE *et al.* 2005a) (Figure 4). It was hypothesized that the pattern of placentation determines the timing of meristem termination (COLOMBO *et al.* 2008). In *E. californica* and *A. thaliana*, the placenta develops from the inner ovary wall. In contrast, in *P. hybrida,* the placenta originates from the central part of the floral meristem, which in difference to *E. californica* and *A. thaliana* is not terminated after gynoecium inception (ANGENENT *et al.* 1995; COLOMBO *et al.* 2008). This might explain the absence of placenta expression of *CRC* orthologs in *E. californica* and *A. thaliana* in comparison to *P. hybrida*.

Replum expression

We also did not observe *EcCRC* expression in the replum (Figure 4). Replum expression has been reported only for *CRC* and it might have been acquired independently in the lineage leading to *A. thaliana* (BOWMAN and SMYTH 1999a).

Carpel margin expression

In the gynoecium at stage 6, *EcCRC* expression occurs in two distinct stripes along the lateral carpel margins (Figure 4). Similar expression has been reported for *CRC* in flowers at stage 6, suggesting that both *EcCRC* and *CRC* function in the establishment of the lateral carpel margins (BOWMAN and SMYTH 1999b).

Ovule expression

EcCRC, similar to other reported *CRC*-like genes across eudicots and monocots, is not expressed in the ovules (BOWMAN and SMYTH 1999a; LEE *et al.* 2005b; NAKAYAMA *et al.* 2010; YAMAGUCHI *et al.* 2004). In *E. californica, A. thaliana* and *A. asparagoides*, the ovules develop from the placenta, whereas in *O. sativa*, the ovules arise directly from the floral meristem (BOWMAN and SMYTH 1999a; ITOH *et al.* 2005; NAKAYAMA *et al.* 2010).

Mature seeds' expression

The *EcCRC* expression in mature seeds may hint to a function of *EcCRC* in late embryogenesis or seed maturation, but such expression has not been reported for any other *CRC*-like gene.

Nectary expression

Expression in the nectary seems to be restricted to core eudicots, because such expression has been reported only for *CRC* orthologs in core eudicots (BOWMAN and SMYTH 1999a; LEE *et al.* 2005b) (Figure 4). *CRC* homologs are not expressed in the nectaries either in the basal eudicot *A. formosa* or in the monocot *A. asparagoides*. *E. californica* does not develop nectaries. This let assuming that the nectary expression arose independently only in the core eudicot lineage after it diverged from basal eudicots and monocots (FOURQUIN *et al.* 2005; LEE *et al.* 2005b).

Leaf expression

Our RT-PCR experiments did not reveal expression of *EcCRC* in leaves of *E. californica*. Such expression is reported only for *DL* genes in monocots (ISHIKAWA *et al.* 2009; NAKAYAMA *et al.* 2010; YAMAGUCHI *et al.* 2004) (Figure 4). This indicates that the *DL* genes might have acquired additional expression in leaves independently of eudicots.

In summary, the *EcCRC* expression patterns change dynamically throughout developmental stages. *EcCRC* exhibit the conserved expression of *CRC*-like genes outside of grasses in the abaxial gynoecium wall, but in addition shows a unique expression domain at the base of the gynoecium, which is not reported for any other *CRC* ortholog. Furthermore, *EcCRC* expression is excluded from the apical region of the gynoecium, in contrast to the rest of the eudicot *CRC* orthologs, suggesting that *EcCRC* does not function there. The *EcCRC* expression patterns put forward that *EcCRC* shares the conserved function of core eudicot *CRC* orthologs in establishment the abaxial polarity of the gynoecium, and may function in floral meristem. Furthermore, the *EcCRC* expression patterns illustrate the dynamic nature of *CRC*-like gene expression across angiosperm lineages, and particularly in *E. californica*.

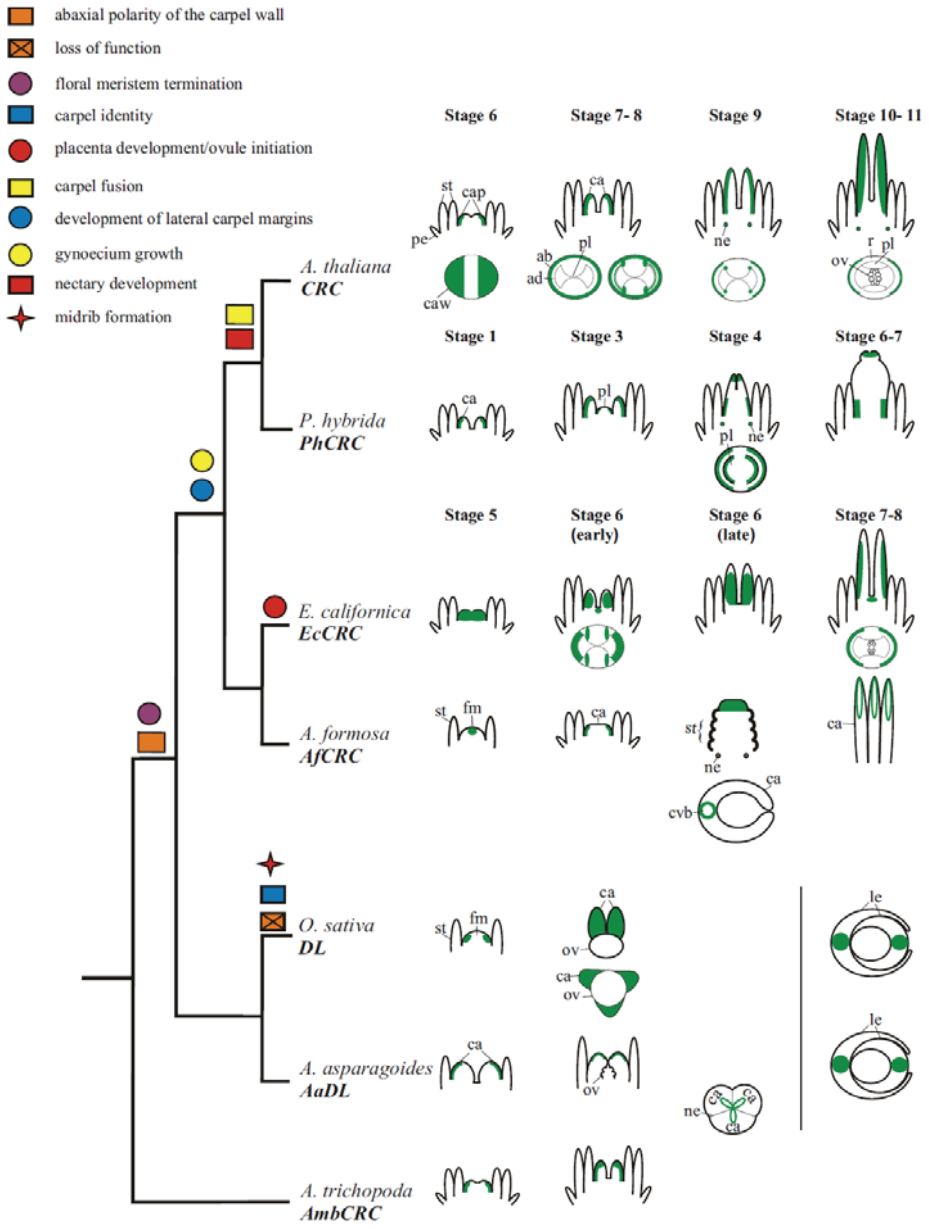

Figure 4 Schematic diagram showing a simplified phylogeny of the major angiosperm lineages (left side) and summarizing the expression patterns of *CRC* orthologs (right side) as well as the gain and loss in *CRC*-like gene function (left side) across angiosperms. Symbols represent a gain and a loss of function of *CRC* orthologs in different angiosperm lineages. Mapping of *CRC*-like gene function in the angiosperm phylogeny tree is restricted to those *CRC*-like genes, for which functional data is available. Flower developmental stages are described only for *A. thaliana*, *E. californica* and *P. hybrida*

(ANGENENT *et al.* 1995; BECKER *et al.* 2005; SMYTH *et al.* 1990).

Abbreviations: ab, abaxial; ad, adaxial; ca, carpel; cap, carpel primordium; caw, carpel wall; cvb, central vascular bundle; fm, floral meristem; gp, gynoecia primordium le, leaf; ne, nectary; ov, ovule; r, replum; pe, petal; pl, placenta; st, stamen.

3.1.1.2 *EcCRC* functions in floral meristem determinacy, gynoecium differentiation and ovule initiation

In the *EcCRC*-VIGS plants, the reduction of *EcCRC* expression affects the gynoecium and fruit development. *EcCRC*-silenced plants develop fruits, which are strongly reduced in length compared to wild-type fruits, and form less seeds (Figure 3c, d, Publication I). In the most severely affected *EcCRC*-VIGS plants, the fruits even form only a single seed (Figure 3c, d, Publication I). Mildly affected *EcCRC*-VIGS plants have normally developed fruits, enclosing a second fruit as both fruits are attached to each other via their ovary walls (Figure 3a, b, Publication I). Sections through floral buds of *EcCRC*-VIGS plants with mild phenotype revealed that at least one ectopic gynoecium arise inside the fourth whorl (Figure 3g, h, Publication I). This ectopic gynoecium is detectable firstly at stage 7 (Figure 3g, h, Publication I). In some flowers of *EcCRC*-silenced plants, even two or three additional gynoecia develop. Transverse sections through severely affected fruits of *EcCRC*-VIGS plants show two slender ectopic tissue layers arranged in concentric whorls, and enclosed into a wider one (Figure 3e, f). In all these concentric layers, abaxial/adaxial tissue differentiation, ridge and replum formation are completely abolished. Section of fruits with mild *EcCRC*-VIGS phenotype, revealed that the replum region narrower and extended compared to wild-type fruit (Figure 3m, n). Also, the characteristic bulge shape of the abaxial replum side is missing and the replum region appears much thinner than that in wild-type fruits. But the subepidermal and epidermal cell layers, suiting abaxially the carpel walls, are not affected (Figure 3m, n). The strongly lignified cells, located between the replum region and the valves, and along the carpel margins of wild-type fruits are completely absent in the *EcCRC*-VIGS fruits. Moreover, placenta development is reduced and placental outgrowths fail to develop. In fruits of severely affected *EcCRC*-VIGS plants, the ovules are absent (Figure 3j). Carpel fusion is not affected in *EcCRC*-silenced plants.

Abaxial tissue differentiation

In wild-type gynoecium of *E. californica*, each carpel wall develops five abaxial ridges in stage 8 (BECKER *et al.* 2005). These are closely associated with the gynoecium vasculature and have characteristic cell structure (Figure 3e). The carpel ridges in *E. californica* consist of cellulose depositing parenchyma cells, called collenchymas cells, arranged in a circular manner, and surrounded by large parenchyma cells. The adaxial side of the collenchyma cells of each ridge is suited by one vascular bundle, whereas their abaxial side is occupied by epidermal and sub-epidermal cell layers (Figure 3i). The reduction in the *EcCRC* expression causes complete loss of the ridges in the gynoecia of *EcCRC*-VIGS plants. Moreover, in the gynoecium wall of *EcCRC*-silenced plants, the accumulating cellulose cells are replaced by extended patches of lignified cells, distributed irregularly in the gynoecium wall. These are enclosed by several vascular bundles (Figure 3i, j). This and the defects of the replum region indicate that *EcCRC* controls the establishment of abaxial/adaxial polarity and medial/lateral tissue formation in the gynoecium wall of *E. californica*. Although in *A. thaliana* the knockout of *CRC* does not lead to a noticeable loss of abaxial/adaxial polarity of the gynoecium wall, the vasculature differentiates prematurely, similarly to *EcCRC*-VIGS plants, where the lignification of the parenchyma cells occurs earlier and the distribution of the vascular bundles is altered (ALVAREZ and SMYTH 2002). This indicates that both *CRC* and *EcCRC* function in establishing the abaxial polarity of the gynoecium wall, which is important for subsequent abaxial tissue differentiation of bundles and vasculature, although the *EcCRC* role in this aspect seems to be more pronounced (Figure 4). Probably, the elaboration of abaxial/adaxial polarity of the gynoecium wall is a conserved aspect of *CRC*-like gene function across eudicots and even in basal angiosperms. *EcCRC* and *CRC* show abaxial expression in the gynoecium wall and both genes function in abaxial tissue differentiation, suggesting that all other angiosperm *CRC* orthologs, which display abaxial expression domain in the carpel walls, might share this function (Figure 4).

Adaxial tissue differentiation

In contrast to *EcCRC*-VIGS gynoecia, the replum region in *crc-1* mutants is only mildly affected as it matures earlier than in wild-type (ALVAREZ and SMYTH 2002). Also placenta and ovules initiate and develop normally in *crc-1*, but fewer ovules are formed than in wild-type. Alvarez and Smyth assumed that the reduced ovule number in the *crc-1* gynoecia is owing to the reduced gynoecium length and to the increased spacing between them (ALVAREZ and SMYTH 2002). In *E. californica* and *A. thaliana,* the wild-type gynoecia mature into dry

capsules, which dehiscence and subsequently break as the valves separate from the presumptive replum region (BECKER *et al.* 2005). In *EcCRC*-VIGS plants, the severe defects in the entire replum region and the absence of lignified cell layers marking the region of fruit rupture are probably accounting for the development of fruits that failed to dehiscence and needed to be opened manually (Figure 3 m, n). In contrast, the fruits of *crc-1* mutants are able to open normally. The reduced placenta development indicates that *EcCRC* controls not only the abaxial tissue differentiation of the gynoecium wall, but also the elaboration of the adaxial margins tissues. Due to a lack of placenta outgrowths, *EcCRC*-silenced plants produce only few ovules as the few ovules that have initiated are also able to develop fully. Aborted ovules were not observed, which shows that *EcCRC* possibly controls ovule initiation in *E. californica*. To estimate, if *EcCRC* is important for ovule initiation or whether the defects in placenta development account for the reduced ovule number, stable *eccrc* mutants are required. It can be concluded that *EcCRC* plays a more prominent role in elaboration of carpel margin tissues than *CRC*. This might be due to the recruitment of redundantly acting genes in the differentiation of the gynoecium wall in *A. thaliana*.

Function in the replum development is not reported for any other *CRC*-like gene. But *EcCRC* is not expressed in the replum, placenta and ovules. This shows that *EcCRC* control the establishment of the medial gynoecium tissues non-cell autonomously, probably from the regions adjacent to the carpel margins. *EcCRC* is expressed there in a gynoecium at stage 6, when the placenta regions swell inside of the ovary. Non-cell autonomous action has been reported for *YAB1*, another transcription factor of the *YAB* family in *A. thaliana* (GOLDSHMIDT *et al.* 2008). *YAB1* contributes non-autonomously to boundary establishment in the periphery of the shoot apical meristem (SAM) and to the process of primordia initiation in the floral meristem (GOLDSHMIDT *et al.* 2008). In these, *YAB1* functions non-cell autonomously and this is mediated by the organ-meristem boundary factor *LATERAL SUPPRESSOR (LAS).* Logically, *EcCRC* might control placenta and replum formation via direct or indirect activation or repression of other genes, associated with the development of these margin tissues. Similar to *EcCRC, PhCRC* also seems to control placenta development. In *N. benthamiana* plants silenced for *PhCRC,* the placenta tissue is replaced by an ectopic flower (LEE *et al.* 2005b). This phenotype is in conformity with the placental expression of *PhCRC* and points out that *PhCRC,* in difference to *EcCRC,* may control placenta development cell autonomously. It might be that the different type of placentation between *E. californica* and *P. hybrida* accounted for the different manner of *CRC*-like gene action in these species.

CRC and *EcCRC* might share a conserved function in controlling gynoecium growth (Figure 4). In *crc-1* mutants, the gynoecium is composed of fewer, but larger cells. In *E. californica*, the strong reduction in the length of *EcCRC*-VIGS fruits might be due to (i) the shorter gynoecia or (ii) the reduced ovule number. Also the seed set formed in the *EcCRC*-silenced fruits is strongly reduced, similar to the *crc-1* fruits (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002). In *A. thaliana*, the reduced growth of the pollen tubes was accounted for the reduced seed number in the *crc-1* fruits, whereas the reduced seed number in *EcCRC*-VIGS plants is probably a consequence of the strongly reduced placenta development and impaired ovule initiation (ALVAREZ and SMYTH 2002; BOWMAN and SMYTH 1999a)

Meristem termination

The development of ectopic gynoecia in *EcCRC*-VIGS plants indicates that the meristem fails to terminate when *EcCRC* is silenced. In *E. californica*, similarly to *A. thaliana*, the central floral meristem is terminated after the gynoecium initiates at stage 5. In *crc-1* mutants, the floral meristem determinacy is only slightly affected (ALVAREZ and SMYTH 2002). Occasionally, single carpel or parts of carpel arise inside of the normal gynoecium in *crc-1* mutants. In *E. californica* the loss of meristem determinacy is much severe, when *EcCRC* is silenced. The function of *EcCRC* in floral termination of *E. californica* flowers is in accordance with its expression at the gynoecium base. This pattern of *EcCRC* expression is maintained in *E. californica* during developmental stages, assuming that *EcCRC* might be required throughout development for maintenance the terminated state of the central portion of the floral meristem. In contrast, *CRC* is not expressed in the meristem, but functions there, probably in non-cell autonomous manner. Although both *EcCRC* and *CRC* share the function in controlling floral meristem determinacy, *EcCRC* contributes stronger to this functional aspect than *CRC,* as this might due to the cell autonomous action of *EcCRC* (Figure 4) (BOWMAN and SMYTH 1999a; PRUNET *et al.* 2008). In *A. thaliana*, due to the recruitment of many redundantly functioning genes also in floral meristem determinacy, severely undetermined floral meristem can be often observed only in multiple mutants. Meristem determinacy is more severely affected when *crc-1* is combined with *rbl*, *sqn* and *ulp1* single mutants, showing that *CRC* may function redundantly with *REBELOTE (RBL), SQUINT (SQN), ULTRAPETALA1 (ULT1)* in meristem termination (PRUNET *et al.* 2008). Also the combination of *crc-1* with heterozygote *ag* mutants lead to indeterminate floral meristem suggesting that *CRC* functions redundantly also with *AG* in meristem termination (ALVAREZ and SMYTH 1999). In *A. thaliana*, *AG* is the main determinant of floral meristem termination.

Additionally, VIGS of the *PhCRC* expression in *N. benthamiana* plants also causes loss of meristem termination (LEE *et al.* 2005d). The meristem fails to terminate also in *dl* mutants of *O. sativa*, demonstrating that the termination of meristem activity is characteristic for *CRC*-like genes at least across monocots and eudicots.

Carpel fusion

Apparently, *EcCRC* does not control carpel fusion in *E. californica* as we did not observe any defects in the apical region of the *EcCRC*-VIGS gynoecia. In this aspect, *EcCRC* differs from *CRC* orthologs from core eudicots. Carpel fusion is abolished not only in *crc-1* gynoecium, but also in a small number of *N. benthamiana* and *P. hybrida* plants, silenced for *PhCRC* via VIGS*,* carpel fusion is impaired, suggesting that this function is conserved within core eudicot *CRC*-like genes (Figure 4) (LEE *et al.* 2005d). *EcCRC* was not expressed in the apical gynoecium region, whereas all *CRC*-like genes across core eudicots display expression there. Such expression is reported also for the basal eudicot gene *AfCRC*, but there is no functional information on *AfCRC* on hand (LEE *et al.* 2005d).

In *O. sativa*, mutation of *DL* results into complete loss of carpel identity, as the strong *dl* mutants exhibit a complete homeotic conversion of carpels into stamens (YAMAGUCHI *et al.* 2004). Similar defects as observed in *dl* mutants have been reported for the grass species *Penissetum americanum and Panicum aestivum* (YAMAGUCHI et al. 2004). This shows that *DL* is a main determinant of carpel identity in *O. sativa* and this function might be conserved across grasses (Figure 4). It also put forward a gain of lineage-specific function of the grass *CRC* orthologs in the overall carpel development. This function is in accordance with the ubiquitous expression of the grass *DL* genes in carpels. Furthermore, this additional function possibly arose in grasses after they diverged from the common last ancestor of monocots and eudicots (Figure 4).

Leaf development

EcCRC, similarly to core eudicot *CRC* orthologs, is not expressed in leaves and does not function in leaf development. In difference, *DL* is involved in leaf development as it controls mid-rib formation in *O. sativa*, which correlates with the leaf expression of *DL*. *dl* mutants develop cylinder-like formed leaves (YAMAGUCHI *et al.* 2004). Hence, at least in some monocot species, *CRC*-like genes have acquired a unique function in leaf development, which is absent in the rest of angiosperms. Also, *DL* genes in other monocots are expressed in the

leaf and therefore the function of *CRC*-like genes in leaf development might be monocot-specific (Figure 4).

Nectary development

Another functional aspect of *CRC*-like genes is the control of nectary development, which seems to be restricted to core eudicots. Mutation of *CRC* and knock-down of *PhCRC* completely abolish the development of the nectaries (HEISLER *et al.* 2001; LEE *et al.* 2005b) (Figure 4). *E. californica* does not develop nectaries. Also *A. trichopoda* does not have nectaries and *CRC* was not expressed even in the highly secretory tissues of female flowers (FOURQUIN *et al.* 2005). But *AfCRC* and *AaDL* are not expressed in the nectaries of the basal eudicot *A. formosa* and the monocot non-grass *A. asparagoides,* respectively, demonstarting that this functional aspect of *CRC*-like gene function is conserved only between core eudicot *CRC* orthologs (LEE *et al.* 2005b; NAKAYAMA *et al.* 2010).

Fourquin and colleagues hypothesized that the function of *CRC*-like genes in carpel development and fusion was characteristic for the ancestral *CRC* gene. They demonstrated that the coding sequence of *AmbCRC* is capable of partially restoring the wild-type phenotype in *crc-1* mutants (FOURQUIN *et al.* 2007). *AmbCRC* complemented the defects in carpel fusion, and increased carpel and silique length to some degree in *crc-1* mutants*,* but could not restore the nectaries. This implies that the ancestral *CRC* gene controlled carpel fusion as well as carpel development, and these functions have been conserved since the common ancestor of the angiosperms. In contrary, the coding sequence of *DL* was sufficient to complement both, the carpel and nectary defects in *crc-1* mutants (FOURQUIN *et al.* 2007). The observation that *DL* is able to restore wild-type nectaries in *A. thaliana* contradicts the statement that *CRC*-like function in the nectaries is restricted only to core eudicots. It seems that the function in of *CRC* orthologs in nectary development has evolved after the most basal angiosperm *A. trichopoda* diverged from the rest of the angiosperms, but this was obviously lost outside of core eudicots. Fourquin and colleagues assumed that the gain of the new function in nectaries is an outcome from (i) changes in the promoter region, affecting the *CRC*-like gene expression and its interaction with possible gene partners or (ii) changes in the coding sequences of *CRC*-like genes, which lead to the changes in the protein sequence and possible changes in its interacting partners (FOURQUIN *et al.* 2007). This could also be the situation in *E. californica,* where a novel function for *EcCRC* in placenta development/ovule initiation and a loss of the *EcCRC*-like gene function in carpel fusion were observed (Figure 4). At the

same time, the conserved function of *CRC*-like genes in establishing abaxial polarity of the gynoecium wall has been maintained in *E. californica*. Also the function of *CRC*-like genes in meristem termination, which seems to be highly conserved across eudicots and monocots, is characteristic for *EcCRC*. In contrast to *CRC*, *EcCRC* seems to operate cell autonomously in the termination of the central floral meristem, but non-cell autonomously in placenta development/ovule initiation and proper replum formation. This suggests that non-cell autonomous action might be characteristic for *CRC*-like genes as non-cell autonomous action is characteristic for *CRC* in the process of meristem termination in *A. thaliana*. The functional diversity in the *EcCRC* function demonstrates that conservation and novelty accompany the evolution of gene function.

3.1.2 *EScaAG1/2*

Multiple independent duplication events have occurred among the floral homeotic C-class genes of Ranunculales. The ranunculid *AG* subclade is a sister clade to the core eudicot *AG* clade, which includes both the *euAG* and *PLE* subclades. A major duplication event at the base of Ranunculales has led to two major paralogous *AG* sublineages within the ranunculids. One of them, placed at the base of Ranunculales, contains the *AG* orthologs from *E. californica* and *Sanguinaria, EScaAG1/2* and *ScAG*, respectively (ZAHN *et al.* 2006). The second paralogous sublineage within Ranunculales includes the rest of the basal *AG* orthologs, arisen by multiple duplication events within this sublineage. *AG* orthologs from *Thalictrum, Aquilegia, Berberis, Clematis, Akebia, Ranunculus* and *Helleborus* belong to this linegae (ZAHN *et al.* 2006). Probably further duplication events in the *Eschscholzia* lineage led to the recent paralogs *EScaAG1* and *EScaAG2* of *E. californica.* Thus, *EScaAG1* and *EScaAG2* are the orthologs of the single *A. thaliana AG* gene in the basal eudicot *E. californica.* Both *E. californica* paralogs share a high degree of similarity on the nucleotide and the protein level is including the 5'-UTR regions.

3.1.2.1 *EscaAG1/2* expression is localized in carpels and stamens throughout flower development

Real-time PCR experiments revealed that *EScaAG1* and *EScaAG2* are expressed at the highest level in male and female reproductive organs (Figure 2A, Publication II).

Additionally, both genes are expressed in young fruits and in floral buds through all examined developmental stages. Generally, *EScaAG1* is expressed at a higher level than *EScaAG2* with the exception of *EScaAG1* expression in the stamens, which is lower than that of *EScaAG2.* Zahn and colleagues showed by real-time RTq-PCR that EScaAG1 is expressed highest in carpels, stamens and fruits similar to our study whereas EScaAG2 is expressed in all analyzed floral (sepals, petals, stamens, carpels) and non-floral organs (fruits and leaves) at a similar level (ZAHN *et al.* 2010). The expression they revealed for EScaAG1 is in concordance with our RTq-PCR data whereas that of EScaAG2 differs significantly from our observations (YELLINA *et al.* 2010). Independent RTq-PCR analyses, recently conducted by Dr. Matthias Lange, confirmed the EScaAG2 expression data (Figure 6J).

In order to precisely investigate the spatial and temporal expression of *EScaAG1* and *EScaAG2* and to be able to distinguish between both paralogs, I conducted *in situ* hybridization experiments. Owing to the high degree of sequence similarity between both *AG* orthologs in *E. californica*, it was impossible to generate specific probes to discriminate between the two paralogs and the obtained expression patterns for *EScaAG1* and *EScaAG2* were almost identical. For that reason, in this work, the *EScaAG1* and *EScaAG2* expression data, obtained through *in situ* hybridization, will be referred to as *EScaAG1/2* expression.

The expression of *EScaAG1/2* is initially detected in the floral meristem of buds at stage 2, where it is confined to groups of cells positioned at the regions of the future stamen primordia (Figure 2B, Manuscript II). In the next stage, the transcripts of *EScaAG1/2* are present in the boundary regions between the stamen primordia and between the stamen and carpel primordia. Only weak expression of *EScaAG1/2* was detected in the central dome of the floral meristem, from which the gynoecium will develop (Figure 2C, Publication II). At stage 4, the *EScaAG1/2* expression expands uniformly in the floral meristem including the regions of the initiating stamen primordia, but is absent from the central dome of the floral meristem shortly before gynoecium inception (Figure 2D, Publication II). In late stage 6, expression of *EScaAG1/2* is then detected in carpels and stamens (Figure 2E, Publication II). During stage 7, the carpel expression of *EScaAG1/2* becomes restricted to the adaxial regions of the carpel walls and the stamen expression is further maintained (Figure 2F, Publication II). The expression of *EScaAG1/2* is present in the ovules since their inception in stage 7 (Figure 2G, H, I, Publication II). I did not observe any *EscaAG1/2* expression in the placenta.

The expression patterns of EScaAG1 and EScaAG2 have been previously published (ZAHN *et al.* 2006). Zahn and colleagues conducted radioactive *in situ* hybridization revealing uniform expression of EScaAG1 and EScaAG2 in the entire floral meristem of *E. californica* flowers

at stage 1 and 2 (ZAHN *et al.* 2006). The EScaAG1 and EScaAG2 expression in stage 2 differ from the expression I obtained for this stage (see above). This is probably due to the usage of radioactive in situ hybridization by Zahn and colleagues, which often shows weak background signal resulting from the long exposure time of the tissue with the probe (ZAHN *et al.* 2006). With exception of stage 2, the EScaAG1 and EScaAG2 expression during all following stages resemble the expression patterns I obtained, and are present in carpels, stamens and ovules (Zahn et al., 2005). They found also expression in the seed coats. I did not examine EScaAG1 and EScaAG2 expression in seeds.

Reproductive organ expression

Expression patterns, similar to those I obtained for *EScaAG1/2,* have been reported for many *AG* orthologs across angiosperms. These resemble the expression of the *AG* gene in *A. thaliana,* which is predominantly expressed in stamens and carpels (BRUNNER *et al.* 2000; KATER *et al.* 1998; PAN *et al.* 2010; YU *et al.* 1999). The paralogs *PLE* and *FAR* in *A. majus*, members of *PLE* and *euAG* clades of the core eudicot C-lineages, respectively, display similar expression in carpel and stamen primordia and in the developing carpels and stamens. Only in late stages of flower development, *PLE* and *FAR* exhibit distinct expression domains in the anther. In the gynoecium, both are constantly expressed in ovules, placenta and carpel walls (BRADLEY *et al.* 1993; DAVIES *et al.* 1999). Duplications within the C-lineage of core eudicots have resulted in multiple *AG* orthologs also in petunia*,* cucumber, gerbera and poplar (BRUNNER *et al.* 2000; KATER *et al.* 1998; YU *et al.* 1999). In *P. trichocarpa*, both *AG* orthologs are constantly expressed in the third and fourth whorls since inception (BRUNNER *et al.* 2000). In the *Thalictrum* sublineage of basal eudicots, a duplication event preceding the divergence of the *Thalictrum* species has led to multiple *AG* orthologs, *ThdAG1* and *ThdAG2* in *Thalictrum dioicum (T. dioicum),* and *ThtAG1* and *ThtAG2* in *T. thalictroides*, respectively. *ThdAG1* is the putative ortholog of *ThtAG1,* while *ThdAG2* that of *ThtAG2. ThdAG1* and *ThdAG2* display very distinct expression patterns throughout flower development (DI STILIO *et al.* 2005). The expression of *ThdAG1* resembles the expression pattern characteristic for *AG* orthologs in core eudicots, whereas the *ThdAG2* expression is present only in the ovules of the mature carpel. *In situ* hybridization expression patterns are not available for *ThtAG1* and *ThtAG2,* but RT-PCR experiments reveal similar expression to their putative orthologs in *T. dioicum* as *ThtAG1* is expressed in stamens and carpels, while *ThtAG2* expression is confined to carpels. The carpel-specific expression of the putative orthologs *ThdAG2* and *ThtAG2* is in contrast to their paralogs *ThdAG1* and *ThtAG1,* respectively, putting forward a significant

subfunctionalization between paralogs in specifying reproductive organ identities within the *Thalictrum* lineage (DI STILIO *et al.* 2005). This subfunctionalization is more pronounced than in *A. majus,* where the expression patterns of paralogs differ only in later developmental stages (BRADLEY *et al.* 1993; DAVIES *et al.* 1999). In contrast, we did not observe any differences in the spatial expressions of *EScaAG1/2* in Real-time RT-PCR and *in situ* hybridization experiments, which could hint to subfunctionalization of the paralogs. The two isoforms *PapsAG1* and *PapsAG2* in the basal eudicots *Papaver somniferum (P. somniferum, Papaveraceae),* are similarly expressed in carpels and stamens, and are additionally slightly expressed in sepals and petals in RT-PCR experiments (HANDS *et al.* 2011). Also in the grass monocots, C-class genes display expression in the reproductive organs, resembling the eudicot *AG* gene expression. However, paralogs in *O. sativa, OSMADS3* and *OSMADS58* show distinct expression patterns as only *OSMADS58* is constantly expressed in stamens, carpels and ovules since their inception, whereas *OSMADS3* expression disappears completely before these initiate (YAMAGUCHI *et al.* 2006b). In difference, *ZAG1* and *ZMM2* in maize exhibit identical spatial expression, similar to *EScaAG1/2* (MENA *et al.* 1996; YAMAGUCHI *et al.* 2006b).

The expression domains of the *AG* orthologs in the basal angiosperms *Amborella* and *Nuphar* are also confined to the reproductive organs (KIM *et al.* 2005). This implies that the expression patterns of *AG* orthologs in the male and female structures across angiosperm lineages represent the expression pattern of the ancestral angiosperm *AG* gene. The expression has been conserved across angiosperms, despite the multiple duplication events in the *AG* gene lineage. Gymnosperm C-class gene orthologs from all extant gymnosperm groups are also expressed in male and female reproductive structures, the male and female cones, respectively. These expression patterns are conserved between extant gymnosperm groups of gnetophytes *(Gnetum)*, cycads (Cycas), conifers (*Pinaceae*) and *Ginkgo* (JAGER *et al.* 2003; RUTLEDGE *et al.* 1998; TANDRE *et al.* 1995; WINTER *et al.* 1999). This shows that the *AG* subfamily might have originated in the last common ancestor of angiosperms and gymnosperms before the two lineages diverged around 300-400 million years ago (MYA) and might have been maintained for at least 300 MYA (BECKER and THEIßEN 2003; BECKER *et al.* 2000; JAGER *et al.* 2003; TANDRE *et al.* 1995; ZHANG *et al.* 2004). Zahn and colleagues suggested that the ancestor of the *AG* lineage probably controlled the identity and development of male and female reproductive structures (ZAHN *et al.* 2006).

Ovule expression

The adaxial expression domain of *EScaAG1/2* that we detected in the ovary wall of a mature gynoecium at stage 7 might be associated with the development of the ovule primordia, which initiate in this stage. The ovule expression of *EScaAG1/2* is characteristic for *AG* homologs in eudicots and monocots (DAVIES *et al.* 1999; DI STILIO *et al.* 2005; KATER *et al.* 1998; PAN *et al.* 2010; YAMAGUCHI *et al.* 2006b; YANOFSKY *et al.* 1990b). Expression in the ovules has been reported also for *AG* orthologs in the gymnosperms *Cycas edentate (C. edentata)* and *P. abies* (TANDRE *et al.* 1995; ZHANG *et al.* 2004). This illustrates that the ovule expression, similarly to the carpel and stamen expression, was probably characteristic for the ancestral *AG* gene in the last common ancestor of gymno- and angiosperms.

Placenta expression

The *EScaAG1/2* genes*,* similarly to *AG,* are not expressed in the placenta. Placenta expression is also not observed for the two isoforms *PapsAG1* and *PapsAG2* in the basal eudicot *P. somniferum* (HANDS *et al.* 2011). In contrast, such expression was reported for *AG* orthologs in the core eudicots *P. trichocarpa* and *A. majus,* and in the basal eudicot *T. dioicum* (BRUNNER *et al.* 2000; DAVIES *et al.* 1999; DI STILIO *et al.* 2005). It could be that the placenta expression of *AG* orthologs has been independently acquired in some eudicots.

Meristematic expression

Additionally to carpel and stamen expression, *EScaAG1/2* transcripts are present in the floral meristem of *E. californica* flowers. Zahn and colleagues showed that unlike *AG,* which is absent in stages 1 and 2, *EScaAG1/2* are expressed uniformly in the entire floral meristem of *E. californica* flowers at stage 1 and 2 (ZAHN *et al.* 2006). This indicates that *EscaAG1/2* are required earlier in flower development than *AG* and probably function in the floral meristem. The early expression in the floral meristem is characteristic for the angiosperm *AG* orthologs and suggests a conserved function in meristem determinacy. In late stage 2, the *EScaAG1/2* expression in the floral meristem becomes confined to the regions of the future stamen primordia (Figure 2C, Publication II). Due to the absence of *EScaAG1/2* expression in the central dome of the floral meristem, shortly before carpel primordium inception, it can be assumed that in *E. californica*, similarly to *O. sativa,* additional genes are required to set up carpel identity at very early stages. In *O. sativa,* besides *OSMADS58*, another key gene required for establishment of carpel identity at early stages is *DL* (YAMAGUCHI *et al.* 2004). *DL* is specifically expressed in the carpel-like organs of *osmads58-s1* and *osmads3-*

2/osmads58-s1 plants illustrating that *DL* is probably able to confer carpel identity independently of both C-class genes in *O. sativa*. In contrast, in *A. thaliana, AG* is the prime determinant of carpel identity, but the existence of a genetic pathway controlling some aspects of carpel identity independently of *AG* was hypothesized, as carpeloid organs instead of sepals are formed in the first whorl of *ag ap2* double mutants (BOWMAN *et al.* 1991b). These organs have stigmatic papillae, style, replum and placenta with ovules, indicating that in *A. thaliana* other genes function in concert with *AG* in the margin tissue development, but also in absence of *AG* are able to determine some carpel characteristics. Recently, it has been demonstrated that the *SHP1/2* as well as the *STY* genes, *STY1* and *2* function redundantly with *CRC* in apical tissue development of *A. thaliana* (COLOMBO *et al.* 2010). It seems that the *SHP1/2* and *STY1/2* genes, also members of the *AG* clade in core eudicots, although functionally diverged from *AG*, have apparently maintained some functional redundancy with *AG*.

The meristematic expression of *EScaAG1/2* at the boundaries between the stamen anlagen in the third whorl and between the third and fourth whorls at stage 3 was not reported for any other *AG* ortholog. In *A. thaliana*, the *SUPERMAN (SUP)* gene is expressed in the floral meristem between the third and fourth whorls in late stage 3 flower primordia and controls the establishment of the boundaries between these two whorls (BOWMAN *et al.* 1992; SAKAI *et al.* 1995). Furthermore, *SUP* prevents the expansion of B gene expression into the central whorl of the *A. thaliana* flower, but does not require C-gene expression to do so (SAKAI *et al.* 1995). In contrast, the putative *SUP* ortholog in *A. majus OCTANDRA (OCT)* requires both C-class genes to restrict the B-gene expression to the third whorl (DAVIES *et al.* 1999). It seems likely that a putative *SUP* ortholog is more similar to *OCT* than to *SUP* in *E. californica,* and requires C-class gene expression for its function in (i) establishing boundaries between third and fourth whorls or/and (ii) preventing B gene expression from the flower centre.
We showed that both *E. californica AG* orthologs display overlapping expression patterns, suggesting that, if some subfunctionalization between *EScaAG1* and *EScaAG2* has occurred, it is at an early state and cannot be detected by *in situ* hybridization. A similar situation might be also present in *P. trichocarpa* and *Z. mays,* where the paralogs are almost identically expressed, suggesting no significant subfunctionalization of the paralogs in these species. In contrast, in *A. majus*, *T. dioicum* and *O. sativa,* the duplication events obviously have introduced a significant divergence in expression patterns of paralogs as these overlap only partially. This puts forward that each of the paralogs has been specialized in particular

aspect(s) of the original *AG* function in these species, but some functional redundancy between paralogs has been also preserved (DI STILIO *et al.* 2005; YAMAGUCHI *et al.* 2006b).

In summary, the *AG* orthologs in *E. californica* share similar expression patterns in the floral meristem, carpels and stamens, which are reminiscent of the expression patterns of *AG*-like genes across core eudicots and monocots, indicating high conservation in *AG*-like gene expression. In addition, the specific lateral domains of expression of *EScaAG1/2* in the stamen anlagen of the floral meristem at stage 2 suggest that the *AG* orthologs in *E. californica* may function in establishing stamen identity very early in the development, but possibly other genes are also required to set up early carpel identity. Furthermore, *EScaAG1/2* could be required for the expression or function of a putative *SUP* ortholog in *E. californica*. Our *in situ* hybridization revealed similar expression for *EScaAG1* and *EScaAG2* and do not hint to a subfunctionalization of the paralogs.

3.1.2.2 *EscaAG1/2* genes confer stamen and carpel identity and control floral meristem determinacy

In order to study the functions of *EScaAG1* and *EScaAG2* and to discriminate between both paralogs, transient knock downs of *EscaAG1, EscaAG2* and *EscaAG1/2* were performed by employing VIGS.

The defects caused by the reduction of *EscaAG1* and *EscaAG2* expression were constrained only to the flower. The silencing of *EscaAG1* and *EscaAG2* resulted in different degrees of homeotic conversion of stamens into petals and loss of carpel features (Figure 3A and C, Publication II). Only a low percentage of *EScaAG1*-, *EScaAG2*- and *EScaAG1/2*-VIGS plants exhibit a full homeotic conversion of all stamens into petals, whereas most of the flowers show only a partial conversion of stamens into petaloid organs (Table 1, Figure 3B-D, Publication II). Interestingly, in most flowers of *EScaAG1*-VIGS treated plants, only the outermost whorls of stamens are homeotically transformed into petaloid organs. In contrast, in flowers of *EScaAG2*-VIGS plants, preferentially the innermost stamen whorls are converted into petaloid organs. In flowers of *EScaAG1/2*-VIGS plants, a different degree of homeotic conversion of the outer- and innermost stamen whorls into petaloid organs occurs, whereas the middle stamen whorls have wild-type appearance. Furthermore, the morphology of the gynoecia in *EScaAG1-, EScaAG2-* and *EScaAG1/2*-VIGS plants is altered. These are transformed into green flattened structures without ovules in some cases (Figure 4A,

Publication II) or into orange coloured flattened gynoecium with petal characteristics (Figure 4B, Publication II). Strongly silenced plants develop structures with petaloid/carpeloid features enclosing an ectopic carpel, a gynoecium or even a whole new flower in the flower center (Figure 4F). Additionally, the organ number is elevated in the third and fourth whorls of *EScaAG1*-, *EScaAG2*- and *EScaAG1/2*-VIGS plants (Figure 3F, I, J and K, Publication II). Mildly silenced plants show only an increased stamen number, but not homeotic conversions of any organ type.

Reproductive organ identity

EScaAG1 and *EScaAG2* are the first functionally characterized C-class genes in basal eudicots. The development of green flat gynoecia or flattened gynoecia with acquired petal characteristics by down regulation of *EscaAG1* and *EscaAG2* indicates that carpel identity is strongly impaired in the VIGS plants, and shows that *EscaAG1* and *EscaAG2* probably redundantly control carpel identity. The observation that some carpel characteristics are still present in most silenced plants shows that (i) the remaining expression of *EscaAG1* and *EscaAG2* in the silenced plants is still sufficient to confer residual carpel identity or (ii) in *E. californica* additional genes are involved in establishing carpel identity.

The different degree of homeotic conversion within the stamen whorls of *EscaAG1* and *2*-VIGS indicates that also stamen identity is severely affected and that both paralogs might also control redundantly stamen identity. Also in the closely related basal eudicot *P. somniferum* (opium poppy), similar silencing phenotype in the floral centre was reported, when the two alternative splicing variants of the single *PapsAG* gene, *PapsAG1* and *PapsAG2* were simultaneously silenced via VIGS (HANDS *et al.* 2011). *PapsAG1/2*-VIGS plants also show a homeotic conversion of carpels into petals. Similarly, the double mutants *ple-1/far* of *A. majus* develop petal-like structures and an additional flower in the fourth whorl (DAVIES *et al.* 1999). The development of petal characteristics in the *ple-1/far* mutants was attributed to the absence of C gene expression, leading to ectopic B expression in the gynoecium. In contrast, the *ag* mutants of *A. thaliana* display loss of carpel and stamen identity as stamens are homeotically transformed into petals, but the carpels are transformed not into not petals, but into sepals (BOWMAN *et al.* 1989). It seems that in *E. californica*, similarly to *A. majus* and possibly also to *P. somniferum*, a C-dependent B gene regulation exists in the central whorl. In contrast, the homeotic transformation of carpels into sepals in the *A. thaliana* flower supposes ectopic A gene expression, but irrespective of B and C gene expression.

In contrast to *E. californica*, where the VIGS-silencing of the single paralogs result in overlapping phenotypes, mutations in *PLE* and *FAR* in *A. majus* result in distinct phenotypes, showing that a significant subfunctionalization of each of the paralogs has occurred. *PLE* controls stamen and carpel identity, as stamens and carpels are homeotically transformed into petals in *ple-1* mutants, whereas *FAR* is required only for pollen development due to the variable degree of male sterility in *far* mutants (BRADLEY *et al.* 1993; DAVIES *et al.* 1999). Thus, in *A. majus*, not only subfunctionalization of the paralogs, but also neofunctionalization of *FAR* is evident. Also in *O. sativa*, both C-class genes have undergone subfunctionalization. *OSMADS3* confer stamen identity, while *OSMADS58* control carpel morphogenesis, but only slightly contribute to stamen identity (YAMAGUCHI *et al.* 2006b).

Ovule identity

The silencing of *EscaAG1* and *EscaAG2* further shows that they control ovule identity. Almost all VIGS plants exhibit a reduced ovule number and in the strong silenced plants, the ovary wall fails to differentiate, and placenta and ovules are completely missing (Figure 3L, M, Publication II). *EscaAG1* and *EscaAG2* possibly control ovule development in combination with ovule identity genes such as *EScaAGL11*, the ortholog of the *A. thaliana* D-class gene *STK*. *EScaAGL11* is the only gene within Ranunculales, which has been classified as a member of the basal D-class gene lineage (ZAHN *et al.* 2006). In *A. thaliana, AG* controls ovule development redundantly with the ovule identity genes *STK*, *SHP1* and *2* and similar functional redundancy has been suggested for the D- and C-class genes in *P. hybrida, FBP 11* and *FBP7*, and *pMADS3* and/or *FBP6,* respectively (RIJPKEMA *et al.* 2006).

Meristem termination

The elevated number of organs in the third and fourth whorls of *EScaAG1-, EscaAG2-* and *EscaAG1/2*-VIGS plants indicates that *EscaAG1* and *EscaAG2* confer floral meristem determinacy, where they possibly function redundantly. Also *PLE* and *FAR* function redundantly in the control of meristem determinacy, as it is severely impaired in *ple-1/far* double mutants (DAVIES *et al.* 1999). Meristem activity is prolonged also in *O. sativa*, when each of the C-class genes is mutated, showing that they also redundantly confer meristem determinacy (YAMAGUCHI *et al.* 2006a).

The mild reduction in the *EscaAG1* and *EscaAG2* expression significantly increases the stamen number in *EScaAG1, EscaAG2* and *EScaAG1/2* plants, but without causing any loss of organ identities (Table 1, Publication II). It was postulated that *EScaAG1/2* control the stamen number via controlling the activity of the ring-like meristem, after the central portion of the floral meristem has been terminated. In contrast to *A. thaliana*, where the gynoecium is the last floral organ initiated from the floral meristem, in *E. californica,* stamens still initiate adjacent to the fourth whorl after the carpel primordia developed, possibly from a ring-like meristem, which remains active after termination of the central floral meristem (BECKER *et al.* 2005). A mild reduction of *EScaAG1/2* is sufficient to bring the ring-like meristem into an undetermined state, but only a strong reduction in the *EScaAG1/2* expression causes a loss of meristem termination in the centre of the flower, suggesting that *EScaAG1/2* controls meristem determinacy differentially in the ring-like and the central floral meristem. It seems likely that a low amount of *EScaAG1/2* proteins is required in the central meristem, while a higher amount is needed in the ring-like meristem, probably indicating a dosage-dependent regulation of meristem determinacy by *EScaAG1/2*. In wild-type *E. californica* flower, up to seven stamen whorls can develop and the number of stamens can vary between 34 and 40. It was assumed that the stamen number in wild-type *E. californica* flowers is dependent on the amount of *EscaAG1/2* transcripts, and coincides with the plant's stature. Such role has not been reported previously for any *AG* ortholog.

In addition to the almost identical expression patterns of *EscaAG1* and *EscaAG2,* which did not indicate a clear subfunctionalization for any of the paralogs, also their individual transient down regulation did not result in clearly distinguishable phenotypes. As the silencing of *EscaAG1* and *EscaAG2* resulted in overlapping phenotypes, two different explanations are plausible. Firstly, both paralogs might redundantly confer stamen and carpel identity, and meristem determinacy. This is less probable according to the model proposed by Force and Lynch, which postulates that gene paralogs cannot exist for long time without undergoing sub- or neofunctionalization (FORCE *et al.* 1999; LYNCH and CONERY 2000; MOORE and PURUGGANAN 2003). Furthermore, subfunctionalization in the long term might result in some divergences as the paralogs specialize and eventually even gain a function, referred to as neofunctionalization. Despite this, functional redundancy can be maintained for astonishing long time (HUGHES and HUGHES 1993). The slight differences between the expression levels of *EscaAG1* and *EscaAG2* observed in Real-Time RT-PCR experiments (Figure 2A, Publication II), and the preferential homeotic conversion of the outer and inner stamen whorls

into petals in plants silenced only for *EscaAG1* and *EscaAG2,* respectively, might suggest some degree of subfunctionalization between the paralogs. But the individual silencing of each of the paralogs causes simultaneous reduction of the expression of both genes, although always with higher residual expression of *EscaAG1.* This could also explain the overlapping phenotypes of the *EScaAG1* and *EScaAG2*-VIGS plants, and indicates that the VIGS method may not be able to silence specifically only one of the paralogs, because of their high sequence similarity. Obviously, to functionally discriminate between *EscaAG1* and *EscaAG2*, stable mutants of each of the paralogs are required. As demonstrated, subfunctionalization is characteristic for C-gene paralogs within core eudicots and grass monocots as observed for *PLE/FAR* and *OSMADS3/OSMADS58*, respectively. Examples of neofunctionalization within the AG clade are evident for *FAR* in *A. majus* and for the recent duplicates *SHP1/2* in *A. thaliana,* the orthologs of *PLE*, which acquired a new function in fruit development, but also maintained the function in carpel and stamen development characteristic for *AG* (PINYOPICH *et al.* 2003).

In summary, the *EscaAG1/2* genes control the same developmental aspects as the single *AG* gene in *A. thaliana*, carpel and stamen identity, and floral meristem termination. Furthermore, *EScaAG1/2* might regulate the central and the ring-like floral meristem in a dosage-dependent manner. Although it is supposed that two redundant genes cannot be maintained for long time without undergoing sub- or neofunctionalization or even becoming pseudogenic, partial or facultative functional redundancy seems to be maintained as long as the paralogs have some non-overlapping function, which results in selective preservation of partially redundant genes (FORCE *et al.* 1999; MOORE and PURUGGANAN 2003; ZAHN *et al.* 2006). As described above, some redundancy in a function is evident for almost all C-class genes within core eudicot and monocot subclades, although not to such an extent as for *EScaAG1/2*. Furthermore, the function of C-class genes in specifying reproductive organ identity seems to be conserved not only across angiosperm lineages, but also in gymnosperms. *CyAG*, the *AG* ortholog in the primitive gymnosperm *Cycas edentate (C. edentata),* is capable of restoring the wild-type appearance in *ag* mutants in the core eudicot *A. thaliana* (ZHANG *et al.* 2004). This shows that the first C-class gene arose already in the last common ancestor of gymno- and angiosperms about 300 MYA and already specified reproductive organ identity and has been conserved since then (KIM *et al.* 2005; ZHANG *et al.* 2004).

3.2 Expression and function of *SIR* in *E. californica*

In the basal eudicot *E. californica,* three B-class genes have been identified *SIR (EScaGLO), EScaDEF1, EScaDEF2* and *EScaDEF3* (ZAHN *et al.* 2005) (Manuscript II). *EScaDEF1, EScaDEF2* and *EScaDEF3* are the orthologs of the *A. majus DEF* and *A. thaliana AP3* genes in *E. californica,* whereas *SIR* is the ortholog of *GLO* and *PI* of *A. majus* and *A. thaliana,* respectively.

3.2.1 *SIR* is expressed in petals and stamens throughout developmental stages

The examination of the *SIR* expression via in *situ* hybridization revealed initial expression at stage 3, restricted to the regions of the floral meristem, where petal and stamen primordia arise at stage 3 and 4, respectively (Figure 1I-K, Manuscript II) (stages according to (BECKER *et al.* 2005). *SIR* is expressed in the developing petal and stamen primordia since their inception (Figure 1I-K, Manuscript II). The expression of *SIR* in petals and stamens is maintained during stage 5 (Figure 1L and M, Manuscript II). *SIR* expression always remains excluded from the sepals, the region of the floral meristem, where carpel primordia develop, and from the carpels (Figure I-M).

Zahn and colleagues showed similar expression for SIR in petals and stamens with in situ hybridization. Additionally, they detected SIR expression in the ovules of later developmental stages (ZAHN *et al.* 2005). I did not analyze the expression of SIR late stage than stage 5 but real-time RTq-PCR experiments performed by Dr. Matthias Lange did not reveal carpel expression for SIR (Figure 1H-M, Manuscript II). Also previously published RTq-PCR data on SIR expression in *E. californica* buds, floral and non-floral organs, showed expression only in petals and stamens but not in carpels (ZAHN *et al.* 2010).

Meristematic expression

The early expression *SIR* in the floral meristem resembles the initial expression of most B-class genes across angiosperms. Generally, *GLO* orthologs start to be expressed in the floral meristem after sepal primordia inception. *GLO* is expressed at stage 1 of *A. majus* flowers (ANGENENT *et al.* 1995; TRÖBNER *et al.* 1992). In contrast, *PI* is expressed in the entire meristem of *A. thaliana* flower at stage 3, comprising also the cells giving rise to the carpel primordia, but this expression disappears before carpel primordia initiation (GOTO and

MEYEROWITZ 1994; TRÖBNER *et al.* 1992). Similar to *PI*, the initial expression of *AqvPI,* the *GLO* ortholog in the basal eudicot *A. vulgaris,* is uniformly distributed within the entire floral meristem (KRAMER *et al.* 2007a). Also in the monocot non-grass *A. officinalis,* the *GLO* orthologs *AOGLOA* and *AOGLOB* are firstly expressed only in the regions of the floral meristem, where second and third whorl organ primordia subsequently develop. It can be concluded that the initial expression of *GLO* orthologs of distinct angiosperm lineages in the early floral meristem is a common feature of *GLO*-like genes in monocots and eudicots and might suggests that *GLO*-like genes determine second and third whorl organ identities in eudicots and monocots, regardless to the organ type, which will develop in the second whorl.

Stamen expression

The *SIR* expression in the stamen primordia and subsequently in the developing stamens is conserved across angiosperm lineages. Within core and basal eudicots as well as across monocots, *GLO*-like genes are continually expressed in the stamens, which are positioned in all angiosperms in the third floral whorl (Figure 5). The conserved expression of *GLO*-like genes in stamens shows that they determine stamen identity. *AqvPI* is expressed also in the staminodium, a novel floral organ unique to the sister basal eudicot genera *Aquilegia* and *Semiaquilegia*, which develops between stamens and carpels, indicating that *GLO* orthologs probably confer also staminodium identity in these genera (KRAMER 2009; KRAMER *et al.* 2007a). Expression of *GLO* orthologs also in stamens of basal angiosperms puts forward that stamen expression was characteristic for the ancestral *GLO* gene in the precursor of the extant angiosperms (KIM *et al.* 2005; ZAHN *et al.* 2005).

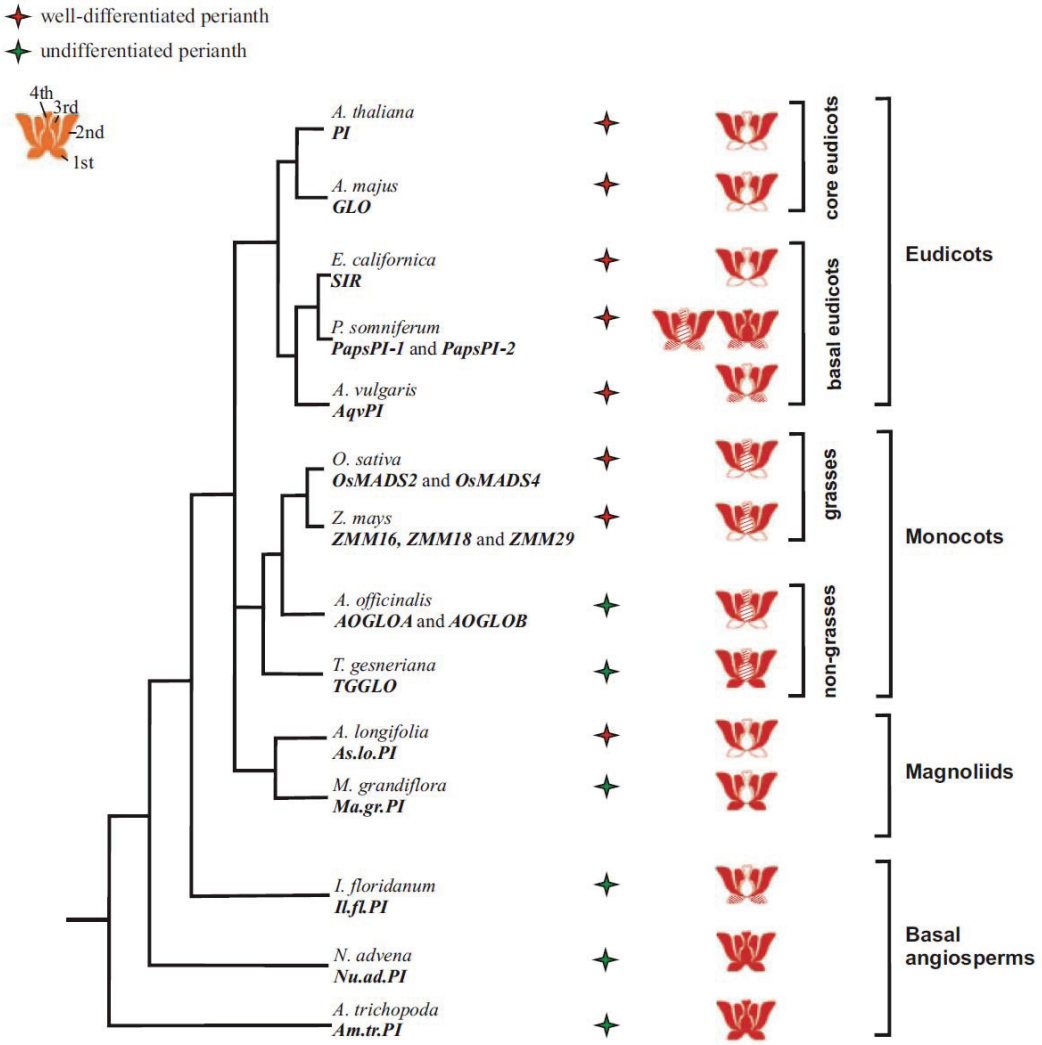

Figure 5 Expression patterns of *GLO*-like genes in representative species of angiosperm lineages (modified from (KIM *et al.* 2005). Red colour indicates consistent *GLO*-like gene expression throughout the flower developmental stages, white colour indicates no *GLO*-like gene expression and dashed colour indicates a weak *GLO*-like gene expression or *GLO*-like gene expression detected only in a particular developmental stage. In *P. somniferum*, the expression of both *GLO* orthologs is shown.

The expression of *GLO*-like genes in the male cone of gymnosperms illustrates that the expression in the male reproductive organs was very likely present already in the last common

ancestor of gymno- and angiosperms (WINTER *et al.* 2002). Furthermore, the highly conserved stamen expression may indicate a conserved function of *GLO*-like genes in specifying stamen identity and development across angiosperms, and that *GLO*-like genes might have controlled male organ identity already in the common ancestor of gymno- and angiosperms.

Petal expression

I obtained *SIR* expression also in the petal primordia and this was maintained in the developing petals throughout flower developmental stages. The *E. californica* flower, similarly to those of core eudicots, has second whorl composed of petals. Second whorl expression is characteristic for *GLO*-like genes across angiosperm lineages (ANGENENT *et al.* 1995; DREA *et al.* 2007; GOTO and MEYEROWITZ 1994; KANNO *et al.* 2003; KRAMER *et al.* 2003; KRAMER *et al.* 2007b; KRAMER and IRISH 2000; MOON *et al.* 1999a; MUNSTER *et al.* 2001; TRÖBNER *et al.* 1992; TZENG and YANG 2001; WHIPPLE *et al.* 2007). In many genera of the basal eudicot family *Ranunculaceae*, petaloid organs develop in the second whorl (KRAMER *et al.* 2003). Also across monocots, second whorl expression of *GLO*-like genes is continuously present, which in contrast to eudicots, do not develop petals there. In monocot grasses, the second whorl usually consists of lodicules. An exception is represented by the basal grass *Streptochaeta angustifolia (S. angustifolia),* which has bract-like organs instead of lodicules, developing outside of the stamens (WHIPPLE *et al.* 2007). In contrast, non-grass monocots such as *Lilium longiflorum* (*L. longiflorum,* lily), *Tulipa gesneriana* (*T. gesneriana*, tulip) and *A. officinalis* have a second whorl composed of petaloid tepals (KANNO *et al.* 2003; PARK *et al.* 2004; TZENG and YANG 2001). Also in the basal angiosperms *A. trichopoda* and *N. advena,* B gene expression is persisting in the second whorl, which is also constituted of tepals, similar to those of non-grass monocots (BUZGO *et al.* 2004; KIM *et al.* 2005).

The conserved expression of *GLO*-like genes in the second whorl of angiosperms, although the different floral organs developing there, demonstrates that *GLO* orthologs play a conserved role in determining second whorl identity across angiosperms. The expression patterns of *GLO*-like genes in eudicots and monocots show that *GLO*-like genes very likely control different second organ identity in these lineages. Within core and basal eudicots, *GLO*-like genes have possibly been independently recruited to specify petal identity and development, whereas across grasses, they probably specify lodicule identity and bract-like identity in *S. angustifolia* (WHIPPLE *et al.* 2007). In non-grasses, *GLO*-like genes are expressed in the inner tepals (KANG *et al.* 1998; PARK *et al.* 2004; TZENG and YANG 2001). It was assumed that the bract-like organs in the *Streptochaeta* lineage, which has diverged from

the rest of the grasses before the evolution of the lodicules, and the lodicules in grasses might have evolved by alteration of inner tepals of the monocot flower (WHIPPLE *et al.* 2007). It was proposed that a common mechanism underlies the specification of the eudicot petal and the monocot lodicule (WHIPPLE *et al.* 2007). Probably, *GLO*-like genes have been independently recruited to determine different second whorl identity across angiosperm lineages. In eudicots, they specify petal identity, whereas in most grasses, *GLO* orthologs confer lodicule identity (WHIPPLE *et al.* 2007). It was hypothesized that the lodicules of grasses and the second whorl bract-like organs in *S. angustifolia* have evolved by modification of inner tepals, whereas the petals possibly evolved many times independently in different clades during angiosperm evolution and therefore the petals of core eudicots are not homologous to the petals of basal eudicots (DREA *et al.* 2007; WHIPPLE *et al.* 2007; ZANIS *et al.* 2003). Furthermore, it was proposed that the establishment of second whorl identity and the specification of petal identity are separable functions of *GLO*-like genes across eudicots, as are the determining of second whorl identity and lodicule identity in most monocot grasses.

Carpel expression

SIR is not expressed in carpels of *E. californica* flowers at any of the analyzed developmental stages. *GLO*-like genes across basal eudicots are variably expressed in carpels, whereas among core eudicots, carpel expression has not been reported (DREA *et al.* 2007; GOTO and MEYEROWITZ 1994; KRAMER *et al.* 2007b; TRÖBNER *et al.* 1992) (Figure 5). Also across monocots, carpel expression has been reported. In monocots such as *O. sativa*, *Z. mays* and *A. officinalis, GLO*-like genes are also expressed in carpels, whereas in others like *Joinvillea ascendens (J. ascendens)* and *Elegia elephas (E. elephas)* and carpel expression has not been found (KYOZUKA *et al.* 2000; MOON *et al.* 1999b; MUNSTER *et al.* 2001; PARK *et al.* 2004; WHIPPLE *et al.* 2007). In the monocots, which exhibit carpel expression, this is weaker than the petal and stamen expression and/or is present only shortly in the development.

The carpel expression was probably specific to the ancestral *GLO* gene, due to *GLO*-like gene expression in the carpels of the basal angiosperms *A. trichopoda* and *N. advena* (KIM *et al.* 2005) (Figure 5). After these diverged from the rest of the angiosperms, the expression in the carpels was possibly reduced to 'weak/temporal expression' or 'no expression' in all other angiosperms (KIM *et al.* 2005) (Figure 5). The only known exception is the constantly carpel expression of *PapsPI-2* in *P. somniferum*, which has apparently been maintained in this species at ancestral state (DREA *et al.* 2007) (Figure 5). In the lineages leading to *E.*

californica and *A. vulgaris*, respectively, the carpel expression might have been loss independently.

Sepal expression

SIR is not expressed in sepals at any of the examined developmental stages. The *E. californica* flower has a well-differentiated perianth with first whorl sepals and second whorl petals, resembling the perianth of core eudicots. Similar to *SIR, GLO* orthologs are not expressed in the sepals of core eudicots (ANGENENT *et al.* 1992; GOTO and MEYEROWITZ 1994; TRÖBNER *et al.* 1992) (Figure 5). In difference, most basal eudicot *GLO*-like genes show variable expression in the first floral whorl, despite of the diverse organs developing there, although this expression is present only at particular developmental stages (DREA *et al.* 2007; KRAMER *et al.* 2003; KRAMER *et al.* 2007a; KRAMER and IRISH 2000) (Figure 5). Within *Ranunculaceae*, the first whorl organs are usually petaloid, but are referred to as sepals due to their position, development and morphology (KRAMER *et al.* 2003). In *Aquilegia,* petaloid sepals and petals occupy the first and second whorl, respectively, whereas in others such as *Clematis*, the entire perianth consists of petaloid sepals (KRAMER *et al.* 2003). In contrast, *GLO* orthologs are not expressed in the first whorl of monocot grasses. Grasses are characterized by a well-differentiated perianth with first whorl usually composed of palea/lemma (KYOZUKA *et al.* 2000; MOON *et al.* 1999a; MUNSTER *et al.* 2001; WHIPPLE *et al.* 2007) (Figure 5). In the basal grass genus in *S. angustifolia,* the perianth is made up of morphologically distinct bract-like organs in the first and second whorl. A higher diversity in the first whorl organs is characteristic for non-grasses. In *Joinvillea* and *Elegia,* the closest extant relatives to the grasses, the entire perianth consists of tepals, which are morphologically distinct in both whorls (WHIPPLE *et al.* 2007). The members of other non-grass genera such as *Lilium, Tulipa* and *Asparagales* have an undifferentiated perianth, composed of identical, spirally arranged inner and outer tepals in both outer whorls. *GLO*-like genes are expressed in both outer and inner tepals of *Lilium longiflorum (L. longiflorum)* and *Tulipa gesneriana (T. gesneriana),* but only in the inner tepals of *A. officinalis* (KANNO *et al.* 2003; PARK *et al.* 2004; TZENG and YANG 2001). The perianth of basal angiosperms and some magnoliids is also undifferentiated and also consists of spirally arranged identical tepals. In *Magnolia grandiflora (M. grandiflora)* and *Persea,* which have undifferentiated perianth, *GLO*-like genes are expressed in the entire perianth, whereas in magnoliids with well-differentiated perianth like *Asimina longifolia (A. longifolia),* the expression of the *GLO* ortholog *As.lo.PI* is present in the inner, but not in the outer tepals (KIM *et al.* 2005) (Figure

5). Also in the basal angiosperms *A. trichopoda* and *N. advena, GLO* orthologs are expressed in the whole perianth (KIM *et al.* 2005).

Obviously, the expression of *GLO*-like gene in the first whorl was characteristic for the *GLO*-like gene in the ancestor of angiosperms, but has been independently lost several times across angiosperms (KIM *et al.* 2005) (Figure 5). The exclusion of *GLO*-like gene expression from the first whorl in most genera with a well-differentiated perianth seems to have occurred during angiosperm evolution. In contrast, angiosperm species with an undifferentiated perianth generally display almost always first whorl expression. It was assumed that the elimination of *GLO*-like gene expression from the first whorl is possibly related to the process of perianth differentiation during the angiosperm evolution. It was hypothesized that an inward shift of B-class gene expression from the entire perianth to the second whorl has led to the transition between entirely petaloid perianth to those composed of distinct sepals and petals in core eudicots (BOWMAN 1997; KRAMER *et al.* 2003).The presence of first whorl expression has obviously been maintained only in particularly developmental stages of basal eudicots, which possibly represent an intermediate state in the *GLO*-like gene expression. Additionally, *GLO*-like gene expression has been maintained in the entire perianth of most non-grasses, suggesting an ancestral state of *GLO*-like gene expression, whereas in grasses, the first whorl expression has been lost (KANNO *et al.* 2003). The broader expression of *GLO*-like genes in basal eudicots than in core eudicots might indicate that the sepal expression has been lost at the base of core eudicots (KRAMER *et al.* 2003). With respect to sepal expression, *SIR* resembles rather the core eudicot *GLO* homologs than the ones from basal eudicots, proposing that sepal expression might also have been independently lost in the lineage leading to *Eschscholzia*.

Due to the broad expression of *GLO*-like genes in basal angiosperms present in all floral organs, it was presumed that the *GLO* ancestor gene was expressed in all floral organs of the common ancestor of angiosperms (KIM *et al.* 2005). According to the expression patterns described, two major tendencies become visible throughout the evolution of *GLO*-like gene expression, (i) the establishment of the expression in petals and stamens (ii) reduction of the expression in carpels and sepals to 'weak/temporally' expressed or 'not' expressed.

In summary, the expression analyses of *SIR* revealed that it is expressed in petal and stamen primordia and subsequently in petals and stamens, similar to *GLO*-like genes across

angiosperms, but is excluded from carpels and sepals. Apparently, the expression pattern of *SIR* resembles rather the expression of *GLO* orthologs in core eudicots than the expression of *GLO*-like genes in other basal eudicots, where *GLO* orthologs are variably expressed in carpels and sepals. Due to the reported carpel and sepal expression of the *GLO*-like genes in *P. somniferum*, also a member of the *Papaveraceae*, it is possible that the carpel and sepal expression of *GLO*-like genes have been loss independently in the lineage leading to *Eschscholzia.*

3.2.2 *SIR* determines petal and stamen identities and development

In the *sirene (sir)* mutants of *E. californica,* the second whorl organs, the petals, are homeotically transformed into sepals (Figure 1 A-E, Manuscript II). Along the stamen whorls of *sir-1*, intermediate organs combining sepal and carpel features develop. Only the most outer stamen whorls, positioned adjacent to the second sepal whorl, are homeotically transformed into sepal-like organs, whereas only the innermost stamen whorls nearby the gynoecium are ectopically replaced by carpel-like structures with stigmatic tissues on the apex.

Stamen identity

The homeotic transformation in the stamen whorls clearly demonstrates that *SIR* confers stamen identity in *E. californica.* But the appearance of organs with mixed features has not been reported for any other *GLO* ortholog. Within core eudicots, the loss-of-function of *GLO*-like genes causes homeotic conversion of stamens only into carpels (BOWMAN *et al.* 1989; BOWMAN *et al.* 1991b; TRÖBNER *et al.* 1992; VANDENBUSSCHE *et al.* 2004). Also in basal eudicots, the reduction in the *GLO*-like gene expression coverts stamens into carpels, but sepal features do not appear in the third whorl (DREA *et al.* 2007; KRAMER *et al.* 2007a). This is in correspondence to the ABC model, which postulates that stamen identity is determined by the combinatorial action of B and C class genes. When B function is absent, C function specifies carpel identity in the third whorl (COEN and MEYEROWITZ 1991). Furthermore, the loss of stamen identity indicates that the *GLO*-like gene function in specifying stamen identity is conserved across eudicots. In *O. sativa,* the down regulation of *OSMADS4,* but not that of *OSMADS2,* causes a loss of stamen identity as the stamens are homeotically transformed into

carpels, indicating that also in monocots, *GLO* orthologs control stamen identity (PRASAD and VIJAYRAGHAVAN 2003; YADAV *et al.* 2007).

Furthermore, the constitutive expression of *GMM2,* the *GLO/DEF* ortholog in the gymnosperm *G. gnemon*, partially complements the stamens defects in the in *pi-1* mutants of *A. thaliana* (WINTER *et al.* 2002). It was suggested that the function of *GLO*-like genes in establishing male reproductive organ identity might have originated already in the last common ancestor of gymno- and angiosperms about 300 MYA (THEISSEN and BECKER 2004). This is in accordance with the stamina expression observed across angiosperms. Furthermore, in *Aquilegia*, the *GLO* genes have recently acquired a novel function in specifying staminodium identity (KRAMER 2009; KRAMER *et al.* 2007a). The staminodia are transformed into carpeloid organs when *AquPI* is silenced (KRAMER *et al.* 2007a). This kind of neofunctionalization of *GLO*-like genes in *Aquilegia* determines a new organ identity, and it is possibly derived from the already existing function of *GLO*-like genes in the stamen identity program.

Petal identity

The homeotic transformation of petals into sepals in *sir-1* indicates that petal identity is lost, when *SIR* expression is absent. Similar second whorl mutant phenotype has been reported for *GLO*-like genes across eudicots. Within core eudicots such as *A. thaliana, A. majus* and *P. hybrida,* mutation in *GLO* orthologs cause the same homeotic conversion of petals into sepals (BOWMAN *et al.* 1989; TRÖBNER *et al.* 1992; VANDENBUSSCHE *et al.* 2004). Also in the basal eudicots *A. vulgaris* and *P. somniferum*, VIGS down regulation of *GLO*-like gene expression causes the replacement of petals with sepals or the appearance of sepal features in the petals, respectively (DREA *et al.* 2007; KRAMER *et al.* 2007a). This indicates that *GLO*-like genes confer petal identity across eudicots. The replacement of petals by sepals is in accordance with the ABC model, which postulates that petals are determined by the combinatorial action of A and B class genes. In the absence of B function, the A function specifies sepal identity. In *O. sativa*, the down regulation of *OsMADS*2 and *OsMADS4* expression causes a loss of lodicule identity as lodicules are homeotically replaced by palea/lemma-like structures (KANG *et al.* 1998; PRASAD and VIJAYRAGHAVAN 2003; YADAV *et al.* 2007). That demonstrates that the role of *GLO*-like genes in determining second whorl identity is conserved among mono- and eudicots, independently of the high diversity of floral organs developing. Within these lineages, *GLO* orthologs have been recruited in specifying second whorl identity, assuming a common origin of second whorl organ identity program. Subsequently, *GLO* orthologs have

become specialized in conferring petal and lodicule identity within eudicots and grasses, respectively. This assumes a common origin of second whorl petaloidy across angiosperms (RONSE DE CRAENE 2007). In contrary, the petaloidy in the sepals has arisen independently many times during angiosperm evolution and is linked to the shifting of B gene expression towards the first floral whorl (RONSE DE CRAENE 2007; THEISSEN and MELZER 2007).

Although the broader expression of basal eudicot *GLO* orthologs outside of *E. californica*, any defects in sepal and carpel development have been reported. Neither the down regulation of *AquPI* nor those of *PapsPI-1/2* in *A. vulgaris* and *P. somniferum*, respectively, causes obvious defects in sepal and carpel development indicating that *GLO*-like genes do not confer first and fourth whorl organ identity (DREA *et al.* 2007; KRAMER *et al.* 2007a). Furthermore, *AquPI* is not responsible for determining the petaloid sepals in *A. vulgaris* (KRAMER 2009). It was suggested that the development of two different petaloid organ types in *Aquilegia* occurred by recruiting *GLO*-like genes in petal identity program, whereas sepal identity is specified by a separated developmental program.

In summary, *SIR* confers petal and stamen identity in *E. californica*, similar to *GLO* orthologs across mono- and eudicots. The discrepancy between the mutant phenotype in *sir-1* and those in mono- and eudicot *GLO*-like genes mutants demonstrates that very likely, in specifying stamen identity, different interactions between organ identity genes compared to core eudicots and monocots have been evolved in *E. californica*. It could be that in *E. californica*, the genetic program behind stamen identity is also conserved, but has been somehow modified across stamen whorls. This might due to the formation of numerous stamen whorls in *E. californica* varying between four and eight (BECKER *et al.* 2005). In contrast, mono- and core eudicots develop much less stamens usually organized in one or few whorls.

3.3 Detailed protocol for *in situ* hybridization in floral tissues of *E. californica*

3.3.1 Fixation and embedding of plant material

Fresh buds were collected in falcons filled with ice cold, freshly prepared fixation solution containing ethanol, acetic acid and formaldehyde (FAA solution) and placed in a beaker filled with ice. A drop of Tween20 was added to the collected plant material before starting with

vacuum infiltration. Vacuum infiltration was performed for 1 hour and during this, the vacuum was slowly released each 15 minutes. After vacuum infiltration, the buds were placed in fresh FAA and incubated overnight at 4°C, at a maximum of 16 hours with shaking.
For subsequent dehydration, the plant material was subjected to the following ethanol series at 4°C. The material was incubated for 30 min in 50% ethanol, 70% ethanol, 85% ethanol, 90% ethanol and finally in 100% ethanol containing 0.1% eosin. Tissue can be stored for long time in 70% ethanol at 4°C. After finishing the ethanol series, the 100% ethanol is replaced by fresh 100% ethanol, containing 0.1% eosin, and left overnight at 4°C. All exchanging steps on the next day were made at room temperature (RT). Firstly, the 100% ethanol is exchanged by fresh 100% ethanol with 0.1% eosin and left for 1 hour. Subsequently, the tissues were incubated in a 50% ethanol / 50% limonene solution for 2 hours, then in 100% limonene also for 2 hours. After the 2 hours, the limonene was replaced by a small amount of fresh 100% limonene, which was just enough to cover the tissues, and chips of Paraplast Plus were added. This was incubated for three days at 60°C and the Paraplast was exchanged with fresh molten Paraplast two to three times every day.

3.3.2 Preparation of an anti-sense DIG-labelled RNA probe

For preparation of an anti-sense DIG-labelled *in situ* probe, a 150- 230 bp fragment was amplified by a standard RT-PCR on cDNA using sequence-specific primers. The resulted fragment was subsequently cloned into a pDrive vector (Quiagen) and subsequently verified by sequencing. 15 µg of the plasmid were digested for at least 2 hours at 37 °C with an appropriate restriction enzyme that was identified using the BioEdit program to create a restriction map of the probe sequence. After restriction, 2µl of the restriction mix were tested on an agarose gel to verify that the restriction was completed. After verifying, the restriction mixture was precipitated overnight in 2.5 volumes of 100% ethanol and 1/10 volume of NaAc at -20°C. On the next day, the mixture was centrifuged for 10 min. at 14 000 rpm, washed with 70% ethanol, centrifuged for 5 min. at 14 000. The rest ethanol was carefully removed with a pipette without touching the probe. The probe was left to dry out for 15 sec. and finally cautiously dissolved in 0.1% DEPC (Diethylpyrocarbonate) water by pipetting. Thereafter, *in-vitro* transcription was performed using SP6/T7 RNA polymerase. The *in vitro* transcription was made for 2 hours at 37°C. The reaction was stopped by applying 75 µl TMS puffer, 2 µl tRNA (100 mg/ml) and 1 µl RNase free DNase I Recombinant I, and further incubated for 10 min at 37°C. After this, 100 µl of ice cold 3.8 M NH_4Ac and 600 µl of ice cold 100 % ethanol

were added, and the mixture was incubated for 1 hour at -20°C. After incubation, the pellet was centrifuged, and subsequently washed with ice cold 70% ethanol: 0.15 M NaCl and centrifuged again. Finally, the pellet was dissolved in 50 µl 0.1% DEPC-H_2O. In case, the size of the probe was over 150 bp, hydrolysis of the probe was subsequently performed.

Hydrolysis

The whole amount of the DNA probe (50 µl) was mixed with 50 µl carbonate buffer and hydrolyzed at 60°C in water bath for the accounted time. The hydrolysis time was accounted using the following formula:

$$t = \frac{L_0 - L_f}{K \times L_0 \times L_f}$$

t = time in minutes

K = 0.11 cuts/kb/min

L_0 = initial length in kb

L_f = final length in kb

Subsequently, the probe was transferred on ice. 10 µl of 10% CH_3COOH and 12 µl 3 M NaAc were added to the probe, and mixed carefully. 312 µl of 100% ethanol were added. This mixture was than incubated for 1 hour at -20°C. Subsequently, the mixture was centrifuged at 14 000 rpm for 10 min., followed by washing of the pellet with 70% ethanol / 0.15 M NaCl and again centrifuged at 14 000 rpm for 5 min. Finally, the probe was resuspended in 50 µl 0.1% DEPC water and stored at -80°C.

Dot blot of the DIG-labelled RNA probe

To test the capacity of the DIG-labelled RNA probe, dot blot was conducted. Different concentrations of the DIG-labelled RNA probe, 30 ng, 100 ng and 300 ng, were transferred to a nitrocellulose membrane and fixed on the membrane under UV-light for 2 min. All next steps take place at by a slow shaking on a shaker. Subsequently, the nitrocellulose membrane with the fixed probe was washed in buffer 1 for 1 min. Follows incubation in buffer 1 containing 0.5% Roche blocking reagent for 30 min. Subsequently, the membrane was washed in buffer 1 for 1 min., followed by incubation in 5 ml buffer 1 containing 1 µl Anti-DIG Alkaline Phosphatase–conjugated Antibody for 30 min. After that, the membrane was washed twice in buffer 2 for 5 min. each, and subsequently in puffer 2 for 1 min. Next, the

membrane was incubated in 5 ml puffer 2, containing 5 µl NBT (Nitro-blue tetrazolium chloride) and BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) for 10 min. On the membrane, colour signals become visible. Finally, the membrane was washed in water and dried out.

3.3.3 Sectioning

The embedded tissues were cut on a microtome (Zeiss) with a thickness of the sections 8 µm. The made sections were dried for 24-48 hours on a hot plate at 42°C. On the next day, the dried sections were stored at 4°C.

3.3.4 Cleaning of the cover slips

The cover slips were washed in acetone for 15 min. After drying, the cover slips were packed in an aluminium foil and heat-sterilized at 180°C for at least 2 hours.

3.3.5 Pre-hybridization (Day I *in situ*)

On day I in *situ*, all treatment steps take place in black boxes with 200 µl volume. The pre-hybridization starts with treating the tissue sections with limonene for 15 min., followed by treatment with 50% limonene: 50% ethanol for 5 min. The sections were then transferred to 100% ethanol for 10 min. and immersed subsequently for 2 min each in 95% ethanol : 0.85% NaCl; 85% ethanol : 0.85% NaCl; 70% ethanol : 0.85% NaCl, 50% ethanol : 0.85% NaCl, 30% ethanol: 0.85% NaCl. After that, the slides were immersed in 0, 2 M HCl for 20 min, washed shortly twice with sterile water for 10 sec each, and transferred to pre-warmed 2XSSPE for 20 min at 70°C. In the next step, the tissue sections were digested with Proteinase K (with final concentration 5 µg /µl) in 200 µl Proteinase K puffer for 20 min, at 37°C. The digesting reaction was stopped by washing the slides twice in 2XSSPE for 5 min each, followed by their transfer into 100 mM Triethanolamin, pH= 8 containing 0, 5% acetic anhydride. After that, the sections were washed twice with 2XSSPE for 5 min. and subsequently rinsed for 10 sec. each in 0.85% NaCl, 30%, 50%, 70%, 85%, 95% ethanol and finally in 100% ethanol for 20 sec. The slides can be stored at 4°C in a box filled with small amount of 100% ethanol for up to several hours during preparation of the probe.

3.3.6 Hybridization

For hybridization, 30 ng, 100 ng and 300 ng of the probe were used per slide. The amount of the probe needed has to be accounted for the number of sections. The probes were heated at 80°C for 2 min. and left on ice for at least 5 min. Subsequently, 100 µl of the hybridization puffer was added to each probe and carefully mixed by pipetting. The accounted amount of

the probe was transferred to the slides. The sections were than incubated in big boxes. A paper soaked with 4XSSPE was put on the box bottom. The hybridization took place overnight at 55°C.

3.3.7 Washing (Day II *in situ*)

On the next day, the hybridization was stopped by washing the sections 3 times with pre-warmed 3X SSPE with each of the washing steps lasting 30 min. at 45°C. After the washing steps, the slides were transferred in the pre-warmed NTE buffer and left for 20 min. at 37°C. Subsequently, the plant tissues were incubated for 30 min. at 37°C in pre-warmed NTE buffer containing 20 µg/ml RNase A. The slides were than washed two times with NTE buffer for 5 min each at 37°C and subsequently with 1.5X SSPE, 1X SSPE and 0.4X SSPE for 30 min. each at 52°C.

3.3.8 Antibody incubation

The slides were immersed in buffer1 for 5 min and afterwards incubated for 30 min with slowly shaking in buffer1 containing 0.5% Roche blocking reagent. Next, the slides were incubated for 30 min. in buffer1 with 0.5% bovine serum albumin (BSA) and 0.1% Tween20. After that, the slides were taken out of buffer 1 and each section was incubated with 300 µl buffer 1+1% BSA, containing diluted 1:3000 Anti-DIG Alkaline Phosphatase–conjugated Antibody for 1 hour. After incubation, the slides were washed four times in puffer1 containing 0.1% Tween20 and stored overnight at 4°C.

3.3.9 Detection

The slides were washed in buffer 2 containing 0.1% Tween20 for 5 min., followed by incubation of the slides in buffer 2 containing 10% polyvinyl alcohol. This solution was made on a heat plate at maximal 60°C and after cooling on ice, 1.5 µl/ml NBT and 1.5 µl/ml BCIP were added and the slides were incubated for at least 3-4 days in darkness.

3.3.10 Inactivation

To inactivate the reaction, the slides were washed with water, 70% ethanol and 95% ethanol for 5 min. each and dried at RT. The dried slides were mounted with Entellan and covered with a glass cover slip.

3.3.11 Buffers and solutions

Fixation solution (FAA, formalin/acetic acid/alcohol):

10 % formaldehyde

5 % acetic acid

50 % ethanol

***In vitro* transcription mixture**:

10X Transcription puffer	2 µl
DIG RNA Labelling Mix	2 µl
RNase Inhibitor	2 µl
DNA Template	1 µg
SP6/T7 polymerase	2 µl
DEPC- H_2O	X
End volume	20 µl

TMS buffer:

0.01M Tris-HCl, pH= 7.5

0.01 M MgCl2

0.05 M NaCl

Hybridization solution

Formamide (deionised)	4 ml
50X Denhardts Reagent	200 µl
50 % Dextran sulphate	2 ml
10X Salts	1 ml
tRNA (10 mg/ml)	100 µl
DEPC-H_2O	700 µl
End volume	8 ml

10X Salts (RNase free):

3 M NaCl

0.1 M Tris-HCl, pH= 6.8

0.1 M $NaHPO_4$

0.05 m EDTA

2x Carbonate buffer, (pH= 10.2):

0.08 M $NaHCO_3$

0.12 M Na_2CO_3

20XSSPE:

3 M NaCl

20 mM EDTA

200 mM $NaH_2PO_4.2H_2O$, pH=7.4

Proteinase K buffer:

20 mM Tris-HCl, pH=7.0

2 mM $CaCl_2$

NTE:

0.5 M NaCl

10 mM Tris-HCl

1 mM EDTA, pH=8.0

Buffer 1:

100 mM Tris-HCl

150 mM NaCl, pH=7.5

Buffer 2:

100 mM Tris-HCl

100 mM NaCl

50 mM $MgCl_2$, pH=9.5

3.3.12 Chemicals and kits:

Acetic anhydride (Roth, Karlsruhe, Germany)
Anti-DIG Alkaline Phosphates–conjugated Antibody (Roche, Manheim, Germany)
BCIP (Roche, Manheim, Germany)
Bovine serum albumin (Sigma-Aldrich, Taufkirchen, Germany)
DEPC water (Roth, Karlsruhe, Germany)
DIG RNA Labelling Mix (Roche, Manheim, Germany)
Entellan (Sigma-Aldrich, Taufkirchen, Germany)
Eosin (Roth, Karlsruhe, Germany)
50x Denhardts Reagent (Roth, Karlsruhe, Germany)
50 % Dextran sulphate (Roth, Karlsruhe, Germany)
Deionised Formamide (Roth, Karlsruhe, Germany)
Limonene (Sigma-Aldrich, Taufkirchen, Germany)
NBT (Roche, Manheim, Germany)
Paraplast Plus (Sigma-Aldrich, Taufkirchen, Germany)
pDrive vector (Quiagen, Hilden, Germany)
Polyvinyl alcohol (Sigma-Aldrich, Taufkirchen, Germany)
Proteinase K (Roth, Karlsruhe, Germany)
RNase A (Roth, Karlsruhe, Germany)
RNase free DNase I Recombinant (Roche, Manheim, Germany)
RNase Inhibitor (Roche, Manheim, Germany)
10x Transcription buffer (Roche, Manheim, Germany)
SP6/T7 RNA polymerase (Roche, Manheim, Germany)
tRNA (100 mg/ml) (Roche, Manheim, Germany)
Triethanolamin (Roth, Karlsruhe, Germany)
Tween20 (Sigma-Aldrich, Taufkirchen, Germany)

4. Synopses

AG is a central gene, which is not only a main regulator of meristem determinacy, and stamen and carpel organ identity, but also coordinates the action of most key players in the process of flower development by integrating different developmental programs (LIU and MARA 2010).

Due to the fundamental role of *AG* in *A. thaliana*, it is important to gain insight into the function of *AG* orthologs in other species. Owing to its important phylogenetic position, *E. californica* is a suitable model plant for studying the genetics and evolution of flower development. In order to investigate the degree of conservation in the *AG*-like gene function in *E. californica* and to gain insight into the genetic programs underling flower development, we investigated the expression and function of *EScaAG1* and *EScaAG2*. The genetic analyses of *EScaAG1* and *EScaAG2* demonstrate that they are similarly expressed and generally, control the same functional aspects of flower development as *AG* in *A. thaliana.* Additionally, both *E. californica AG* orthologs seems to have gained a specific role in the termination of the ring-like meristematic region, separable from their function in the cessation of the floral meristem in the centre of the flower. The specific regulation of the ring-like meristem by *EScaAG1/2* could account for the variation in stamen number in wild-type *E. californica* flowers, suggesting a stature-dependent regulation of both C-class genes there. Furthermore, the establishment of carpel and stamen identities in *E. californica* is under the precise control of C and B class gene expression, as a C-dependent B gene repression occurs in the fourth floral whorl, whereas a B-dependent (*EScaAG2)* and a B-independent (*EScaAG1)* regulation of C-gene expression is evident in the *E. californica* stamens (Publication II, Manuscript II).

Floral meristem termination

The expression and functional analyses of *EScaAG1* and *EScaAG2* clearly demonstrate that they control floral meristem termination in the *E. californica* flower (Publication II). The silencing of *EScaAG1/2* cause a significant elevation in floral organ number of *EScaAG1/2*-VIGS plants (Table 2, Manuscript II). In *A. thaliana*, *AG* is the only gene that is absolutely required for floral meristem termination and even the partial loss-of-function of *AG* results in a complete loss of floral meristem termination (MIZUKAMI and MA 1993; MIZUKAMI and MA 1995; MIZUKAMI and MA 1997; SIEBURTH *et al.* 1995a). In contrast, in *E. californica,* the mild reduction in the *EScaAG1/2* expression is sufficient to impair the termination only of the ring-like meristem, whereas only a strong reduction in the *EScaAG1/2* expression is required to lose meristem determinacy in the central floral meristem. This puts forward a dosage-dependent regulation of the activity in the central and ring-like meristem by *EScaAG1/2* in *E. californica*, which has not been reported previously. In *A. thaliana*, the gynoecium is the last floral organ initiated from the floral meristem and the floral meristem termination coincides with female organ initiation (PRUNET *et al.* 2009; SMYTH *et al.* 1990). In contrast, in *E.*

californica, a ring-like division of the floral meristem remains active after the gynoecium develops and continually produces inner stamen whorls (BECKER *et al.* 2005). This is interesting in relation to the variation in stamen number of wild-type *E. californica* flowers, which range from 18 to 34 (BECKER *et al.* 2005). It seems likely that the stamen number in *E. californica* is dependent on the amount of EScaAG1 and EScaAG2 proteins and is related to the plant's stature. Hence, a differential dosage-dependent regulation of *EScaAG1/2* in (i) the central floral meristem, and (ii) in the ring-like meristem, can be proposed in *E. californica* (Publication II).

In *A. thaliana*, *AG* terminates the meristem activity by switching off the expression of the meristem identity gene *WUS* in the centre of the floral meristem (LAUX *et al.* 1996a; LENHARD *et al.* 2001; LOHMANN *et al.* 2001). Moreover, *RBL, SQN* and *ULT1* redundantly promote the *AG* expression in the centre of the floral meristem and this is dependent on *WUS* (PRUNET *et al.* 2008). In *E. californica,* besides *EScaAG1/ 2,* also *EcCRC* plays a significant role in the termination of the central floral meristem. Even only a reduction of *EcCRC* expression causes a more severe loss of meristem termination than observed in the stable *crc-1* mutants of *A. thaliana* (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; BOWMAN and SMYTH 1999a). In *crc-1* mutants, occasionally a single ectopic carpel develops medially between the two lateral carpels, while in *E. californica,* even the reduction in *EcCRC* expression results in prolonged activity of the central meristem, generating a whole ectopic gynoecium, and in some instances even two, three or four, enclosed in the central gynoecium. This indicates that *EcCRC* plays a more prominent role in meristem termination than *CRC,* and that might be due to the recruitment of many redundantly acting genes in the control of meristem determinacy in *A. thaliana*, such as *RBL, SQN, ULT1* (PRUNET *et al.* 2008). Meristem determinacy is severely affected, when *crc-1* is combined with *rbl*, *sqn* and *ulp1* single mutants. Furthermore, *CRC* seems to contribute, direct or indirect, to the meristem determinacy function of *AG* to some degree (ALVAREZ and SMYTH 1999). In *crc-1 ag+/-* mutants, the number of ectopic carpels in the fourth whorl is increased compared to *crc-1*alone and also alternating groups of stamens and carpels develop in the fourth whorl (Alvarez and Smyth, 1999, 2002). The way how *CRC* influences floral meristem termination and the relationship between *AG* and *CRC* in meristem termination still remains obscure. With respect to meristem termination, *CRC* possibly functions downstream of *AG* and this occurs via (i) modifying the *AG* mediated *WUS* repression or (ii) direct repression of *WUS* in the centre of the floral meristem (PRUNET *et al.* 2008). Additionally, it has been demonstrated

by microarray experiments that AG activates *CRC* expression (GOMEZ-MENA *et al.* 2005). This possibly occurs via binding of AG containing protein complexes to a CArG-box within the *CRC* promoter (Lee et al., 2005). Also in the promoter region of *EcCRC*, similarly to the *CRC* promoter, several CArG-boxes have been identified (S. Nintemann, personal communication). It can be speculated that also in *E. californica*, *EcCRC* expression might be dependent on the expression of *EscaAG1* and *EScaAG2*. It is very likely that the functions of *EcCRC* and *ESCaAG1/2* in the central floral meristem are dependent on each other, whereas in contrast to *EScaAG1/2*, *EcCRC* does not function in the ring-like meristem. This suggests that *EScaAG1/2* control the meristem determinacy of the entire floral meristem, whereas *EcCRC* has subfunctionalized only in the termination of the central floral meristem, but not in the ring-like meristem.

Also in *O. sativa*, the determinacy of the floral meristem is regulated by the *CRC* ortholog *DL* and the C-class gene (YAMAGUCHI *et al.* 2006b; YAMAGUCHI *et al.* 2004). The expression of *DL* and *OsMADS58* expression occur independently from each other, but their functional dependence in the process of meristem termination is still unclear.

B and C gene interactions in the establishment of floral organ identities

Another key function of the *EScaAG1/2* genes, conserved across angiosperms, is to specify carpel organ identity. The homeotic transformation of carpels into petals in the *EScaAG1/2*-silenced plants differs from the phenotype of *ag* mutants, where carpels are homeotically converted into sepals. In contrast, in the double mutant *ple-1/ far* of *A. majus,* the carpel is converted into a petal, similar to that observed in the *EScaAG1/2*-VIGS plants (DAVIES *et al.* 1999). In *A. majus,* the development of petal characteristics in the central whorl of *ple-1/ far* is due to an expansion of *DEF/GLO* gene expression into the fourth whorl (DAVIES *et al.* 1999). In contrast, in the *ag* mutants of *A. thaliana*, the expansion of *AP3/PI* expression in the fourth whorl is prevented by *SUP. SUP* maintains the boundary between the two inner whorls of *A. thaliana* by regulating cell proliferation between them. Furthermore, *SUP* mediates C-independent B gene repression in the central whorl (SAKAI *et al.* 1995). It was assumed that in *A. majus*, the putative *SUP* ortholog *OCT* also limits the B gene expression to the third whorl, but requires *PLE* and *FAR* to do so, demonstrating that in difference to *A. thaliana*, a C-dependent B-gene repression in the central whorl (BOWMAN *et al.* 1992; DAVIES *et al.* 1999). The acquisition of petal features in the central whorl organs of *EScaAG1/2*–VIGS plants also suggests extended B gene expression into the fourth whorl, when *EScaAG1/2* expression is reduced, suggesting a similar C-dependent-B-gene regulation in the fourth whorl of *E.*

californica. Indeed, the B genes *EScaDEF2* and *SIR,* but not *EScaDEF1*, are ectopically expressed in the centre of the *E. californica* flower, when the *EScaAG1/2* expression is silenced, determining ectopic petal features (Figure 4H, Manuscript II). *EScaDEF2* and *SIR* were hardly detectable in carpels of untreated plants. This shows that *EScaDEF2* and *SIR* expression is dependent, whereas that of *EScaDEF1* is independent on the *EScaAG1/2* expression. In stamens, where B and C genes are both expressed*, SIR* and *EScaDEF2* expression is independent of *EScaAG1/2*. It could be that also in *E. californica*, a putative *SUP* ortholog might function, dependent on *EScaAG1/2,* in preventing B gene expression from the central whorl. Furthermore, it was presumed that *OCT* functions dependently on the B genes, whereas in *A. thaliana*, *SUP* functions independently on B genes, in repressing B gene expression in the flower centre (DAVIES *et al.* 1999). The regulation of B function genes in the fourth whorl of *E. californica* seems to be more similar to that in *A. majus* as the absence of C gene expression causes an expansion of B genes beyond the third whorl, indicating a C-dependent B gene repression. It can be hypothesized that the putative *SUP* ortholog in *E. californica*, if it exists, requires both C class genes to prevent B gene expression from the flower centre, but its dependence on B gene function still needs to be investigated (Publication II). We observed expression of *EScaAG1/2* in the boundary between whorls 3 and 4 of buds in stage 3 and 4 via *in situ* hybridization, supporting the hypothesis that a putative *SUP/OCT* ortholog in *E. californica* might require *EScaAG1/2* expression in the boundary regions to prevent B gene expression from the central whorl (Figure 2C and D, Publication II). Similar domains of expression are reported for *SUP* in *A. thaliana* (SAKAI *et al.* 1995). In *sup* mutants, extra stamens develop instead of carpels due to expansion of B gene expression into the fourth whorl (BOWMAN *et al.* 1992). In *E. californica*, the reduction in *EScaAG1/2* expression also leads to an increased stamen number. It cannot be ruled out that *SUP*-like genes might have acquired the function of preventing B gene expression from the central whorl only within the core eudicot lineage, as *SUP* orthologs have not been found outside of core eudicots (BERETERBIDE *et al.* 2001; HIRATSU *et al.* 2002; NAKAGAWA *et al.* 2005; NANDI *et al.* 2000; NIBAU *et al.* 2011; YUN *et al.* 2002).

EScaAG1/2 also confers stamen identity in *E. californica*, similarly to *AG*-like genes in monocots and eudicots. According to the ABC model, stamen identity is determined by the combinatorial action of B, C and E class genes. In *E. californica*, the B class gene *SIR* controls stamen identity together with the *DEF* orthologs *EScaDEF1/2*. In contrast to the *pi* mutants of *A. thaliana,* where all stamens are homeotically transformed into carpels

(BOWMAN *et al.* 1991b), in the *sir-1* mutants, the stamen whorls display different mutant phenotypes. The stamen whorls next to the gynoecium are homeotically converted into carpels, whereas those adjacent to the second whorl sepals are transformed into sepals (Figure 1A-E, Manuscript II). In the middle stamen whorls, mixed organs with sepal and carpel features develop.

The hypothesis was tested that the absence of *SIR* expression leads to the generation of an expression gradient of the C-class genes *EScaAG1/2* in the transformed organs of *sir-1*. This expression gradient declines from the carpel in the centre of the flower toward the stamen whorls and determines a gradual manifestation of ectopic carpel characteristics. The investigation of the *EScaAG1/2* expression in both wild-type and *sir-1* flowers by *in situ* hybridization revealed differences (Figure 6). In wild-type flowers at stage 3, *EScaAG1/2* are expressed in the floral meristem (Figure 6A). During stage 4, C gene expression persists in the carpel and stamen primordia of wild-type flowers. Also in *sir-1* mutants, *EScaAG1/2* are expressed in the floral meristem of a bud at a stage corresponding to wild-type stage 2, similar as in wild- type (Figure 6A, E). The *EScaAG1/2* expression is strong in the central carpel primordia and the surrounding organs of *sir-1* flowers, and resembles the wild-type *EScaAG1/2* expression (Figure 6B, F). Differences in the *EScaAG1/2* expression between wild-type and *sir-1* flowers are firstly evident in stage 6 (Figure 6C, G). In wild-type flowers at this stage, the transcripts of *EScaAG1/2* are equally distributed in carpels and stamens, whereas in *sir-1,* their expression is present in the central carpels, the adjacent ectopic carpels and the intermediate organs, but seems to decline toward these mixed organs (Figure 6C, G). This gradient is maintained in *sir-1* also during the next stage 7, in contrast to wild-type, where *EScaAG1/2* are expressed also equally in stamens and carpels (Figure 6D, H). Furthermore, the two outermost organ whorls in *sir-1*, adjacent to the first whorl sepals, are occupied by an ectopic second sepal whorl and a sepaloid-like organ, and both do not express *EScaAG1/2* (Figure 6G, H, J). In both wild-type and *sir-1*, *EScaAG1/2* are not expressed in sepals at any developmental stage. The detected differential expression of *EScaAG1/2* in wild-type and *sir-1* supports the hypothesis that a declining gradient in the C gene expression is generated, when *SIR* expression is absent.

Additional to the *in situ* hybridization results, Real-Time PCR data obtained by Dr. Matthias Lange further confirmed the hypothesis that a gradient in the C gene expression exists in *sir-1* flowers (Figure 6J). A declining gradient of the *EScaAG1* expression was observed in both *sir-1* and surprisingly also wild-type flowers, whereas the *EScaAG2* expression was strongly

reduced only in *sir-1*. Differently to the *in situ* hybridization, the Real-Time PCR experiments could differentiate clearly between *EScaAG1* and *EScaAG2* expression.

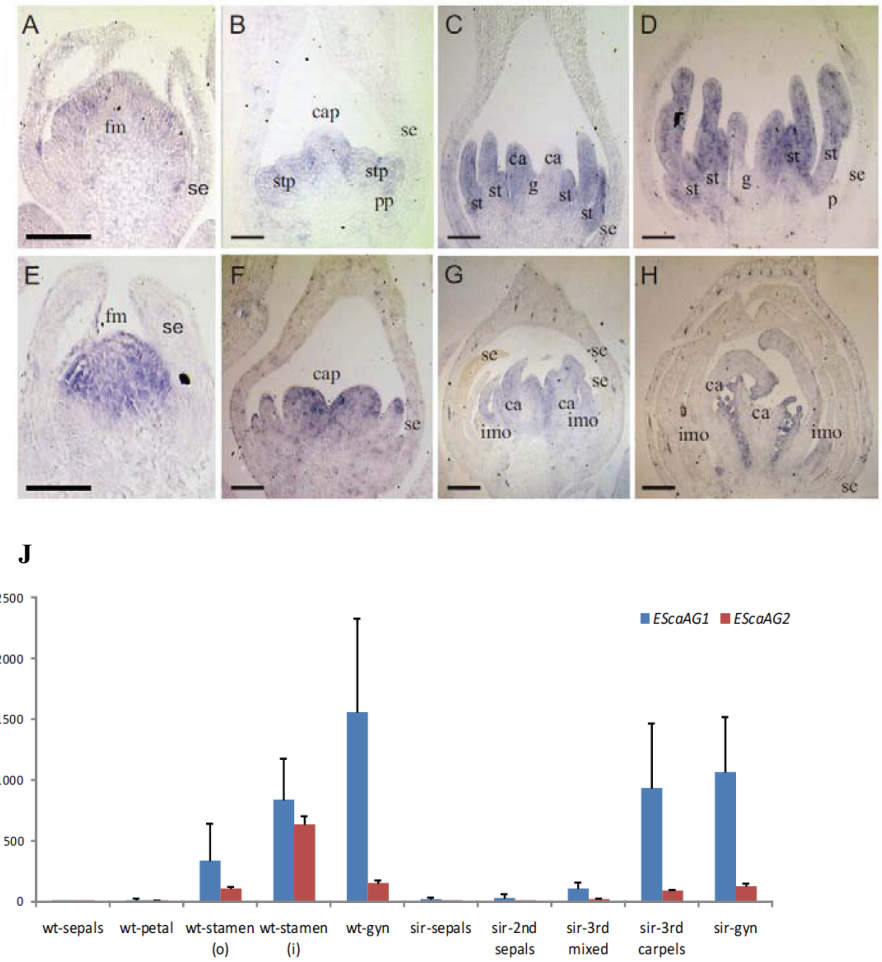

Figure 6 Expression analysis of *EScaAG1 and 2* in floral organs of wild-type and *sir-1* flowers. **(A-D)** Expression patterns of *EScaAG1/2* in wild-type. Longitudinal sections of buds in stage 3 **(A)** and 4 **(B)**. **(C-D)** Longitudinal sections of buds in early **(C)** and in late **(D)** stage 6. **(E-H)** Expression patterns of *EScaAG1/2* in *sir*. **(E)** Longitudinal section of a bud in stage corresponding to stage 2 in wild-type. **(F)** Longitudinal sections of a bud in stages, corresponding to early stage 5 in wild-type. **(G)** Longitudinal section of a bud in stage corresponding to late stage 6 in wild-type. **(H)** Longitudinal section of a bud in stage corresponding to the wild-type stages 7. **(J)** Real time PCR-based analyses of *EScaAG1* and *EScaAG2* expression in floral organs of wild-type and *sir-1* plants. Shown are two biological replicas.

Abbreviations: ca, carpel; cap, carpel primordium; g, gynoecium; fm, floral meristem; imo, intermediate organs; p, petal; pp, petal primordium; se, sepal; st, stamen; stp, stamen primordium. Scale bars: 100 µm.

We were not able to detect an *EScaAG1* expression gradient in wild-type flowers by *in situ* hybridization, almost certainly due to the identical spatial expression patterns of both *EScaAG1* and *EScaAG2*, demonstrated previously (Publication II). Furthermore, as *EScaAG2* expression in *sir-1* flowers is hardly detectable, the expression gradient, detected in our *in situ* hybridization experiments, probably reflects the gradient of *EScaAG1*expression (Figure 6J). Presumably, in wild-type, the gradient of *EScaAG1* expression is masked by the presence of *EScaAG2* transcripts.

When the *EScaAG2* expression was strongly reduced as a consequence of the missing *SIR* expression, we were able to detect the declining gradient in the *EScaAG1* expression from the centre of the flower through the intermediate organs in the *sir-1* flower. The Real-Time PCR results suggest that the absence of *SIR* causes a strong reduction of *EScaAG2* expression in *sir-1* flowers, but does not affect the *EScaAG1* expression. Thus, *SIR* is required for proper *EScaAG2* expression in *E. californica* flowers, but apparently does not influence the *EScaAG1* expression. It seems likely that in *E. californica,* a concomitant B-dependent (*EScaAG2*) and B-independent (*EScaAG1*) regulation of C gene expression occurs, which has not been reported elsewhere and might hint to a subfunctionalization of both C-class genes. Furthermore, the presence of C-dependent regulation of B gene expression in the floral centre has been recently reported in *E. californica* (Publication II). Therefore, genetic interactions between B and C genes in the *E. californica* flower are evident. These interactions might underlie the well-restricted B and C gene expression in *E. californica,* more similar to core eudicots than to the basal ones, where B and C genes are often broader expressed. In many basal eudicots, B genes are expressed in the whole perianth, in contrast to core eudicots, where their perianth expression is restricted to the petals (DREA *et al.* 2007; GOTO and MEYEROWITZ 1994; KRAMER *et al.* 2003; KRAMER *et al.* 2007b; KRAMER and IRISH 2000; TRÖBNER *et al.* 1992; ZAHN *et al.* 2005). In contrast to *E. californica*, many basal eudicot species of Ranunculales have a perianth constituted of distinct petaloid sepals and true petals (KRAMER *et al.* 2003; KRAMER *et al.* 2007b). Similar to basal eudicots, broader expression of B class homeotic genes is characteristic for the basal angiosperms *Amborella, Nuphar* and *Illicium* (CHANDERBALI *et al.* 2006; KIM *et al.* 2005; KRAMER *et al.* 2003; KRAMER and IRISH 2000). In these species, floral organs are arranged in a spiral phyllotaxy with a gradual

transition between floral organ types, and the undifferentiated perianth consists of identical petaloid organs referred to as tepals (BUZGO *et al.* 2004; SOLTIS *et al.* 2007). This gradual transition results in organs combining morphological characteristics of more than one floral organ type. In contrast, the typical eudicot flower has distinct floral organs, organized in well-defined concentric whorls. It was hypothesized that the gradual transition between organ identities in the basal angiosperm flower is a consequence of a gradient in the expression level of B class genes throughout the entire flower (BUZGO *et al.* 2004; SOLTIS *et al.* 2007). Also C class genes are expressed in the perianth of some basal angiosperms and magnoliids such as *Illicium* and *Persea,* respectively (CHANDERBALI *et al.* 2006; KIM *et al.* 2005). The broader patterns of organ identity gene expression in basal angiosperms support the 'fading border' model (BUZGO *et al.* 2004). This model postulates the existence of gradients in the expression levels of organ identity genes across the floral meristem of basal angiosperms. Weak expression of organ identity genes overlap at their margins and this leads to the formation of organs combining morphological features of two adjacent organ types (SOLTIS *et al.* 2007). Besides basal angiosperms, the 'fading border' model is applicable also to the monocots *Lilium* and *Tulipa,* which have a perianth composed of floral organs with mixed sepal/petal features, and basal eudicots with petaloid sepals, showing that in these species, the ancestral broad expression of B class genes has probably been preserved (KANNO *et al.* 2003; KRAMER *et al.* 2003; SOLTIS *et al.* 2007). The process of perianth differentiation is accompanied by restriction of the ubiquitous B-gene expression, characteristic for basal angiosperms, to the petals in the well-differentiated perianth of core eudicots (KIM *et al.* 2005; SOLTIS *et al.* 2007). It is plausible that the C-dependent B gene repression in the fourth whorl and the B-dependent as well as the B-independent regulation of C gene expression in the stamen whorl in the *E. californica* flower, resembles rather that observed in core eudicots than the broader expression typical for basal eudicots.

Carpel margin development

A central gene in carpel development of *E. californica* is *EcCRC*. It determines the abaxial ridge structure of the carpel wall and restricts the number and size of vascular bundles in the *E. californica* gynoecium (Publication I). In *A. thaliana*, the role of *CRC* in establishing abaxial polarity of the carpel wall is less pronounced than that of *EcCRC*. Loss-of-function mutants of *CRC* show only premature vascular differentiation of the carpel wall, but no obvious defects in the abaxial/adaxial polarity of the gynoecium wall. Moreover, *EcCRC* has gained a novel function in the differentiation of the carpel margin tissues in placenta

development and ovule initiation of *E. californica* gynoecium, without being expressed there. In this, *EcCRC* functions non-cell autonomously, possibly from the carpel margins, where it is constantly expressed throughout developmental stages (Publication I). The VIGS-mediated silencing of *EScaAG1/2* also causes a loss of tissue differentiation in the gynoecium wall, and placenta and ovules fail to develop, suggesting that also *EScaAG1/2* function in carpel margin differentiation. The VIGS-mediated down regulation of *EScaAG1/2* expression revealed the specific *EScaAG1/2* function in margin tissue differentiation of *E. californica* gynoecium. In *A. thaliana*, the loss-of-function *ag* mutants do not develop gynoecia at all, and the knockout of the *AG* expression masks the specific function of AG in of the carpel tissue development, which is apparent only when *AG* expression is down regulated. In *A. thaliana*, *CRC* expression seems to be independent of *AG* expression as *CRC* expression still occurs in absence of *AG*, but is weaker and its spatial expression pattern is modified compared to wild-type *CRC* expression. Therefore, *AG* expression might be required for proper *CRC* expression (BOWMAN and SMYTH 1999a). Also the *CRC* function in carpel development seems to be, at least partially, independent of *AG* as some carpel features are still present in *ag ap2-2* double mutants (BOWMAN and SMYTH 1999a). The appearance of residual carpel features, developing in absence of *AG* expression in *ap2 ag* double and *ap2 ag pi* triple mutants, were attributed to the action of *CRC* and *SPT* in carpel morphogenesis, indication that very likely neither *CRC* nor *SPT* lie directly downstream of *AG* (ALVAREZ and SMYTH 1999; BOWMAN and SMYTH 1999b). Furthermore, *SPT* and *CRC* expression domains do not overlap at any developmental stage, and although some functional redundancy between them seem to exist, they are expressed, and probably also function independently in the marginal tissues development (BOWMAN and SMYTH 1999b; HEISLER *et al.* 2001). In contrast, it could be hypothesized that in *E. californica, EcCRC* and *EcSPT* might directly interact in the process of margin differentiation, as both display overlapping expression along the carpel margins (Publication I, Manuscript I).

Ovule identity and development

EScaAG1 and *EScaAG2* are required for proper ovule development of *E. californica*, where they may function redundantly with *EcCRC*. In contrast to other functionally analyzed *CRC*-like genes, *EcCRC* obviously has gained a novel function in placenta development/ovule initiation of *E. californica* as it acts non-cell autonomously in this (Publication I). Unlike *EcCRC, CRC* apparently does not function in ovule initiation or development. The fewer ovules in the *crc-1* mutants were attributed to the reduced length of the gynoecium and the

increased spacing between them (ALVAREZ and SMYTH 2002). This functional diversity of *CRC*-like genes between both species might be due to the recruitment of many redundantly acting genes in controlling ovule development in *A. thaliana*. The carpel developmental gene *SPT* is expressed in ovules and is involved in ovule initiation, as in *spt-2* mutants, the ovule number developed is reduced relative to wild-type (ALVAREZ and SMYTH 2002). Also, due to the absence of the transmitting tract, only about a quarter of the ovules are pollinated. This results in a reduced seed set in fruits of the *spt-2* mutants (ALVAREZ and SMYTH 2002; HEISLER *et al.* 2001). We also suggested a possible function of *EcSPT* in ovule/seed development of *E. californica*, because of the ovule expression of *EcSPT* and the observed tendency in *EcSPT*-silenced plants to form a reduced seed set, but this still needs to be investigated in more detail (Manuscript I). It can be assumed that *EScaAG1/2*, *EcCRC* and *EcSPT* function redundantly in ovule development of *E. californica*.

In summary, the combinatorial action of organ identity genes and developmental genes is required for flower development in all angiosperms including *E. californica. EScaAG1/2* and *EcCRC* share a high degree of functional conservation with their angiosperm orthologs, but also have gained novel functions during evolution. *EScaAG1/2* exhibit the conserved function of *AG*-like genes in meristem termination, carpel and stamen identity, but probably have gained an additional function in controlling stamen number via the control of the ring-like meristem. Also *EcCRC* shares the conserved function of *CRC*-like genes in meristem termination and abaxial carpel wall differentiation, but gained a function in placenta and ovule development. Obviously, not gene functions alone, but gene interactions are determining proper flower development. Furthermore, the importance of genetic studies in species with a key phylogenetic position as *E. californica* has been demonstrated in this study, to gain insights into the evolution of the regulatory networks underling flower and carpel development across angiosperms. This evo-devo approach enables the identification of conserved gene functions required for floral organ development across broad phylogenetic distances, and others that are specific to individual phylogenetic clades.

5. Outlook

In order to investigate a possible functional redundancy of *EcCRC* and *EScaAG1/2*, the relationship between their expression and function needs to be further investigated. The *EcCRC* expression in *EScaAG1/2*-VIGS/stable mutant plants or the expression of *EScaAG1/2* in *EcCRC*-VIGS/stable mutants via *in situ* hybridization and Real-Time PCR should be examined. To explore the functional relationship between *EcCRC* and *EScaAG1/2* in the meristem determinacy, marginal tissue development, and ovule initiation, simultaneous knockout or knockdown plants defective in *EcCRC* and *EScaAG1/2* could be created. These might reveal the functional relationship between *EcCRC* and both *EScaAG1* and *EScaAG2,* and may help to discriminate functionally between both C class genes in *E. californica*. Although protein interactions between YABBY-like proteins and *AG* orthologs have not been demonstrated previously, the protein interactions between EcCRC and EScaAG1/2 proteins could be investigate via protein interaction studies in *E. californica*.

The identification and subsequent functional characterization of a putative *SUP* ortholog in *E. californica* could help to clear the question whether the *SUP* role in preventing B-class gene expression from the flower centre is conserved in *E. californica.* The Floral Genome Project (FGP) has provided a large number of expressed sequence tags (EST) of flower developmental genes in *E. californica* and also large datasets of flower-specific next generation sequencing data are available (CARLSON *et al.* 2006; WALL *et al.* 2009). If a putative *SUP*-like sequence is found, it can be used to design primers for direct amplification of the *SUP* ortholog in *E. californica* via RT-PCR. Another possibility could be the screening of the *E. californica* genomic BAC library for *SUP*-like sequences. If a putative *SUP* ortholog exists in *E. californica*, its role in the B and C mutual regulation should be investigated via VIGS experiments or stable knock-down lines alone or in combination with B or C genes.

To investigate *EcSPT* function in flower development in more detail, a high number of *EcSPT*-VIGS plants should be generated and histological sections on *EcSPT*-VIGS floral buds and fruits could reveal the defects caused by *EcSPT* silencing in details. The dependence between *EcCRC* and *EcSPT* expression can be explored by real time PCR and *in situ* hybridization of *EcSPT* and *EcCRC* in *EcCRC*-VIGS plants and *EcSPT*-VIGS plants, respectively. Possible functional redundancy of both genes could be revealed by generating simultaneous knockdowns of both genes and comparing the caused defects to the single knock-down lines of either gene.

6. Publications and Manuscripts

Publication I:

Svetlana Orashakova, Matthias Lange, Sabrina Lange, Stefanie Wege, Annette Becker (2009) "The *CRABS CLAW* ortholog from California poppy *(Eschscholzia californica,* Papaveraceae*), EcCRC,* is involved in floral meristem termination, gynoecium differentiation and ovule initiation" Plant Journal, **58**(4): 682-693

Publication II:

Aravinda L. Yellina, Svetlana Orashakova, Sabrina Lange, Robert Erdmann, Jim Leebens-Mack, Annette Becker (2010)
"Floral homeotic C function genes repress specific B function genes in the carpel whorl of the basal eudicot California poppy *(Eschscholzia californica)."* EvoDevo, **1**: 1-13

Manuscript I:

Svetlana Orashakova and Annette Becker:
"*EcSPT*, the ortholog of the *Arabidopsis SPATULA* gene in *Eschscholzia californica,* is possibly involved in ovule and seed formation"

Manuscript II:

Matthias Lange, Svetlana Orashakova, Rainer Melzer, Günter Theißen& Annette Becker:
"The California poppy (*Eschscholzia californica)* mutant *sirene* sheds light on the function of the C-terminal domain of class B floral homeotic MADS domain proteins"

7. References

ALVAREZ, J., and D. R. SMYTH, 1999 CRABS CLAW and SPATULA, two Arabidopsis genes that control carpel development in parallel with AGAMOUS. Development 126: 2377-2386.

ALVAREZ, J., and D. R. SMYTH, 2002 CRABS CLAW and SPATULA Genes Regulate Growth and Pattern Formation during Gynoecium Development in Arabidopsis thaliana. International Journal of Plant Sciences 163: 17-41.

AMBROSE, B. A., D. R. LERNER, P. CICERI, C. M. PADILLA, M. F. YANOFSKY *et al.*, 2000 Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. Molecular Cell 5: 569-579.

ANGENENT, G. C., M. BUSSCHER, J. FRANKEN, J. N. MOL and A. J. VAN TUNEN, 1992 Differential expression of two MADS box genes in wild-type and mutant petunia flowers. Plant Cell 4: 983-993.

ANGENENT, G. C., J. FRANKEN, M. BUSSCHER, A. VAN DIJKEN, J. L. VAN WENT *et al.*, 1995 A novel class of MADS box genes is involved in ovule development in petunia. Plant Cell 7: 1569-1582.

BAILEY, P. C., C. MARTIN, G. TOLEDO-ORTIZ, P. H. QUAIL, E. HUQ *et al.*, 2003 Update on the Basic Helix-Loop-Helix Transcription Factor Gene Family in Arabidopsis thaliana. The Plant Cell Online 15: 2497-2502.

BALANZA, V., M. NAVARRETE, M. TRIGUEROS and C. FERRANDIZ, 2006 Patterning the female side of Arabidopsis: the importance of hormones. J Exp Bot 57: 3457-3469.

BAO, X. Z., R. G. FRANKS, J. Z. LEVIN and Z. C. LIU, 2004 Repression of AGAMOUS by BELLRINGER in floral and inflorescence meristems. Plant Cell 16: 1478-1489.

BAUM, S. F., Y. ESHED and J. L. BOWMAN, 2001 The Arabidopsis nectary is an ABC-independent floral structure. Development 128: 4657-4667.

BAXEVANIS, A. D., and D. LANDSMAN, 1995 The Hmg-1 Box Protein Family - Classification and Functional-Relationships. Nucleic Acids Research 23: 1604-1613.

BECKER, A., S. GLEISSBERG and D. R. SMYTH, 2005 Floral and vegetative morphogenesis in California poppy (Eschscholzia californica Cham.). International Journal of Plant Sciences 166: 537-555.

BECKER, A., and G. THEIßEN, 2003 The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Molecular Phylogenetics and Evolution 29: 464-489.

BENNETT, M. D., P. BHANDOL and I. J. LEITCH, 2000 Nuclear DNA amounts in angiosperms and their modern uses - 807 new estimates. Annals of Botany 86: 859-909.

BERETERBIDE, A., M. HERNOULD, S. CASTERA and A. MOURAS, 2001 Inhibition of cell proliferation, cell expansion and differentiation by the Arabidopsis SUPERMAN gene in transgenic tobacco plants. Planta 214: 22-29.

BOMMERT, P., N. SATOH-NAGASAWA, D. JACKSON and H.-Y. HIRANO, 2005 Genetics and Evolution of Inflorescence and Flower Development in Grasses. Plant and Cell Physiology 46: 69-78.

BOWMAN, J., and D. SMYTH, 1999a CRABS CLAW, a gene that regulates carpel and nectary development in Arabidopsis, encodes a novel protein with zinc finger and helix-loop-helix domains. Development 126: 2387-2396.

BOWMAN, J. L., 1997 Evolutionary conservation of angiosperm flower development at the molecular and genetic levels. Journal of Biosciences 22: 515-527.

BOWMAN, J. L., S. F. BAUM, Y. ESHED, J. PUTTERILL and J. ALVAREZ, 1999 Molecular genetics of gynoecium development in Arabidopsis. Current Topics in Developmental Biology, Vol 45 45: 155-205.

BOWMAN, J. L., G. N. DREWS and E. M. MEYEROWITZ, 1991a Expression of the Arabidopsis Floral Homeotic Gene AGAMOUS Is Restricted to Specific Cell Types Late in Flower Development. Plant Cell 3: 749-758.

BOWMAN, J. L., H. SAKAI, T. JACK, D. WEIGEL, U. MAYER *et al.*, 1992 SUPERMAN, a regulator of floral homeotic genes in Arabidopsis. Development 114: 599-615.

BOWMAN, J. L., and D. R. SMYTH, 1999b CRABS CLAW, a gene that regulates carpel and nectary development in Arabidopsis, encodes a novel protein with zinc finger and helix-loop-helix domains. Development 126: 2387-2396.

BOWMAN, J. L., D. R. SMYTH and E. M. MEYEROWITZ, 1989 Genes Directing Flower Development in Arabidopsis. Plant Cell 1: 37-52.

BOWMAN, J. L., D. R. SMYTH and E. M. MEYEROWITZ, 1991b Genetic Interactions among Floral Homeotic Genes of Arabidopsis. Development 112: 1-20.

BRADLEY, D., R. CARPENTER, H. SOMMER, N. HARTLEY and E. COEN, 1993 Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum. Cell 72: 85-95.

BRUNNER, A. M., W. H. ROTTMANN, L. A. SHEPPARD, K. KRUTOVSKII, S. P. DIFAZIO *et al.*, 2000 Structure and expression of duplicate AGAMOUS orthologues in poplar. Plant Molecular Biology 44: 619-634.

BUCK, M. J., and W. R. ATCHLEY, 2003 Phylogenetic analysis of plant basic helix-loop-helix proteins. J Mol Evol 56: 742-750.

BUSCH, M. A., K. BOMBLIES and D. WEIGEL, 1999 Activation of a floral homeotic gene in Arabidopsis. Science 285: 585-587.

BUZGO, M., P. S. SOLTIS and D. E. SOLTIS, 2004 Floral Developmental Morphology of Amborella trichopoda (Amborellaceae). International Journal of Plant Sciences 165: 925-947.

CARLSON, J. E., J. H. LEEBENS-MACK, P. K. WALL, L. M. ZAHN, L. A. MUELLER *et al.*, 2006 EST database for early flower development in California poppy (Eschscholzia californica Cham., Papaveraceae) tags over 6000 genes from a basal eudicot. Plant Molecular Biology 62: 351-369.

CASTILLEJO, C., M. ROMERA-BRANCHAT and S. PELAZ, 2005 A new role of the Arabidopsis SEPALLATA3 gene revealed by its constitutive expression. The Plant Journal 43: 586-596.

CAUSIER, B., D. BRADLEY, H. COOK and B. DAVIES, 2009 Conserved intragenic elements were critical for the evolution of the floral C-function. The Plant Journal 58: 41-52.

CHAE, E., Q. K.-G. TAN, T. A. HILL and V. F. IRISH, 2008 An Arabidopsis F-box protein acts as a transcriptional co-factor to regulate floral development. Development 135: 1235-1245.

CHANDERBALI, A. S., S. KIM, M. BUZGO, Z. ZHENG, D. G. OPPENHEIMER *et al.*, 2006 Genetic Footprints of Stamen Ancestors Guide Perianth Evolution in Persea (Lauraceae). International Journal of Plant Sciences 167: 1075-1089.

CHUANG, C. F., M. P. RUNNING, R. W. WILLIAMS and E. M. MEYEROWITZ, 1999 The PERIANTHIA gene encodes a bZIP protein involved in the determination of floral organ number in Arabidopsis thaliana. Genes & Development 13: 334-344.

CHUNG, Y. Y., S. R. KIM, H. G. KANG, Y. S. NOH, M. C. PARK *et al.*, 1995 Characterization of 2 Rice Mads Box Genes Homologous to Globosa. Plant Science 109: 45-56.

COEN, E. S., and E. M. MEYEROWITZ, 1991 The war of the whorls: genetic interactions controlling flower development. Nature 353: 31-37.

COLOMBO, L., R. BATTAGLIA and M. M. KATER, 2008 Arabidopsis ovule development and its evolutionary conservation. Trends in Plant Science 13: 444-450.

COLOMBO, L., J. FRANKEN, E. KOETJE, J. VAN WENT, H. J. DONS *et al.*, 1995 The petunia MADS box gene FBP11 determines ovule identity. Plant Cell 7: 1859-1868.

COLOMBO, M., V. BRAMBILLA, R. MARCHESELLI, E. CAPORALI, M. M. KATER *et al.*, 2010 A new role for the SHATTERPROOF genes during Arabidopsis gynoecium development. Developmental Biology 337: 294-302.

COOK, S. A., 1962 Genetic System, Variation, and Adaptation in Eschscholzia californica. Evolution 16: 278-.

DAVIES, B., P. MOTTE, E. KECK, H. SAEDLER, H. SOMMER *et al.*, 1999 PLENA and FARINELLI: redundancy and regulatory interactions between two Antirrhinum MADS-box factors controlling flower development. EMBO J 18: 4023-4034.

DEYHOLOS, M. K., and L. E. SIEBURTH, 2000 Separable whorl-specific expression and negative regulation by enhancer elements within the AGAMOUS second intron. Plant Cell 12: 1799-1810.

DI STILIO, V. S., E. M. KRAMER and D. A. BAUM, 2005 Floral MADS box genes and homeotic gender dimorphism in Thalictrum dioicum (Ranunculaceae) – a new model for the study of dioecy. The Plant Journal 41: 755-766.

DINNENY, J. R., and M. F. YANOFSKY, 2005 Drawing lines and borders: how the dehiscent fruit of Arabidopsis is patterned. Bioessays 27: 42-49.

DITTA, G., A. PINYOPICH, P. ROBLES, S. PELAZ and M. F. YANOFSKY, 2004 The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Current Biology 14: 1935-1940.

DREA, S., L. C. HILEMAN, G. DE MARTINO and V. F. IRISH, 2007 Functional analyses of genetic pathways controlling petal specification in poppy. Development 134: 4157-4166.

DREWS, N. G., J. L. BOWMAN and E. M. MEYEROWITZ, 1991 Negative regulation of the Arabidopsis homeotic gene AGAMOUS by the APETALA2 product. Science 65: 991-1002.

EGEA-CORTINES, M., H. SAEDLER and H. SOMMER, 1999 Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. The EMBO Journal 18: 5370–5379.

ENDRESS, P. K., 2001 Origins of flower morphology. Journal of Experimental Zoology 291: 105-115.

FERRANDIZ, C., 2002 Regulation of fruit dehiscence in Arabidopsis. J Exp Bot 53: 2031-2038.

FERRANDIZ, C., S. PELAZ and M. F. YANOFSKY, 1999 Control of carpel and fruit development in Arabidopsis. Annu Rev Biochem 68: 321-354.

FOURQUIN, C., M. VINAUGER-DOUARD, P. CHAMBRIER, A. BERNE-DEDIEU and C. P. SCUTT, 2007 Functional Conservation between CRABS CLAW Orthologues from Widely Diverged Angiosperms. Ann Bot 100: 651-657.

FRANKS, R. G., Z. C. LIU and R. L. FISCHER, 2006 SEUSS and LEUNIG regulate cell proliferation, vascular development and organ polarity in Arabidopsis petals. Planta 224: 801-811.

FRANKS, R. G., C. WANG, J. Z. LEVIN and Z. LIU, 2002 SEUSS, a member of a novel family of plant regulatory proteins, represses floral homeotic gene expression with LEUNIG. Development 129: 253-263.

GARRIS, A., T. TAI, J. COBURN, S. KRESOVICH and S. R. MCCOUCH, 2005 Genetic structure and diversity in Oryza sativa L. Genetics.

GOETHE, J. W., 1790 *Versuch, die Metamorphose der Pflanzen zu erklären*. Ettinger, Gotha.

GOLDSHMIDT, A., J. P. ALVAREZ, J. L. BOWMAN and Y. ESHED, 2008 Signals Derived from YABBY Gene Activities in Organ Primordia Regulate Growth and Partitioning of Arabidopsis Shoot Apical Meristems. Plant Cell.

GOMEZ-MENA, C. N., S. DE FOLTER, M. M. R. COSTA, G. C. ANGENENT and R. SABLOWSKI, 2005 Transcriptional program controlled by the floral homeotic gene AGAMOUS during early organogenesis. Development 132: 429-438.

GOTO, K., and E. M. MEYEROWITZ, 1994 Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. Genes & Development 8: 1548-1560.

GREGIS, V., A. SESSA, L. COLOMBO and M. M. KATER, 2006 AGL24, SHORT VEGETATIVE PHASE, and APETALA1 Redundantly Control AGAMOUS during Early Stages of Flower Development in Arabidopsis. The Plant Cell Online 18: 1373-1382.

GREGIS, V., A. SESSA, L. COLOMBO and M. M. KATER, 2008 AGAMOUS-LIKE24 and SHORT VEGETATIVE PHASE determine floral meristem identity in Arabidopsis. Plant J 56: 891-902.

GROSZMANN, M., Y. BYLSTRA, E. R. LAMPUGNANI and D. R. SMYTH, 2010 Regulation of tissue-specific expression of SPATULA, a bHLH gene involved in carpel development, seedling germination, and lateral organ growth in Arabidopsis. Journal of Experimental Botany 61: 1495-1508.

GROSZMANN, M., T. PAICU and D. R. SMYTH, 2008 Functional domains of SPATULA, a bHLH transcription factor involved in carpel and fruit development in Arabidopsis. The Plant Journal 55: 40-52.

HANDS, P., N. VOSNAKIS, D. BETTS, V. F. IRISH and S. A. DREA, 2011 Alternate transcripts of a floral developmental regulator have both distinct and redundant functions in opium poppy. Annals of Botany.

HEIM, M. A., M. JAKOBY, M. WERBER, C. MARTIN, B. WEISSHAAR *et al.*, 2003 The Basic Helixâ€“Loopâ€“Helix Transcription Factor Family in Plants: A Genome-Wide Study of Protein Structure and Functional Diversity. Molecular Biology and Evolution 20: 735-747.

HEISLER, M. G., A. ATKINSON, Y. H. BYLSTRA, R. WALSH and D. R. SMYTH, 2001 SPATULA, a gene that controls development of carpel margin tissues in Arabidopsis, encodes a bHLH protein. Development 128: 1089-1098.

HIRATSU, K., M. OHTA, K. MATSUI and M. OHME-TAKAGI, 2002 The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flowers. Febs Letters 514: 351-354.

HONG, R. L., L. HAMAGUCHI, M. A. BUSCH and D. WEIGEL, 2003 Regulatory Elements of the Floral Homeotic Gene AGAMOUS Identified by Phylogenetic Footprinting and Shadowing. The Plant Cell 15: 1296-1309.

HONMA, T., and K. GOTO, 2000 The Arabidopsis floral homeotic gene PISTILLATA is regulated by discrete cis-elements responsive to induction and maintenance signals. Development 127: 2021-2030.

HONMA, T., and K. GOTO, 2001 Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature 409: 525-529.

HUGHES, M. K., and A. L. HUGHES, 1993 Evolution of duplicate genes in a tetraploid animal, Xenopus laevis. Molecular Biology and Evolution 10: 1360-1369.

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IMMINK, R. G., I. A. TONACO, S. DE FOLTER, A. SHCHENNIKOVA, A. D. VAN DIJK *et al.*, 2009 SEPALLATA3: the 'glue' for MADS box transcription factor complex formation. Genome Biol 10: R24.

IRISH, E. E., and T. NELSON, 1989 Sex Determination in Monoecious and Dioecious Plants. Plant Cell 1: 737-744.

IRISH, V. F., and A. LITT, 2005 Flower development and evolution: gene duplication, diversification and redeployment. Curr Opin Genet Dev 15: 454-460.

ISHIKAWA, M., Y. OHMORI, W. TANAKA, C. HIRABAYASHI, K. MURAI *et al.*, 2009 The spatial expression patterns of DROOPING LEAF orthologs suggest a conserved function in grasses. Genes Genet Syst 84: 137-146.

ITOH, J.-I., K.-I. NONOMURA, K. IKEDA, S. YAMAKI, Y. INUKAI *et al.*, 2005 Rice Plant Development: from Zygote to Spikelet. Plant Cell Physiol. 46: 23-47.

JACK, T., L. L. BROCKMAN and E. M. MEYEROWITZ, 1992 The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. Cell 68: 683-697.

KANG, H.-G., J.-S. JEON, S. LEE and G. AN, 1998 Identification of class B and class C floral organ identity genes from rice plants. Plant Molecular Biology 38: 1021-1029.

KANNO, A., H. SAEKI, T. KAMEYA, H. SAEDLER and G. THEISSEN, 2003 Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (Tulipa gesneriana). Plant Molecular Biology 52: 831-841.

KATER, M. M., L. COLOMBO, J. FRANKEN, M. BUSSCHER, S. MASIERO *et al.*, 1998 Multiple AGAMOUS homologs from cucumber and petunia differ in their ability to induce reproductive organ fate. Plant Cell 10: 171-182.

KIM, S., J. KOH, M.-J. YOO, H. KONG, Y. HU *et al.*, 2005 Expression of floral MADS-box genes in basal angiosperms: implications for the evolution of floral regulators. The Plant Journal 43: 724-744.

KRAMER, E., H.-J. SU, C.-C. WU and J.-M. HU, 2006 A simplified explanation for the frameshift mutation that created a novel C-terminal motif in the APETALA3 gene lineage. BMC Evolutionary Biology 6: 30.

KRAMER, E. M., 2009 Aquilegia: A New Model for Plant Development, Ecology, and Evolution. Annual Review of Plant Biology 60: 261-277.

KRAMER, E. M., V. S. DI STILIO and P. M. SCHLUTER, 2003 Complex Patterns of Gene Duplication in the APETALA3 and PISTILLATA Lineages of the Ranunculaceae. International Journal of Plant Sciences 164: 1-11.

KRAMER, E. M., R. L. DORIT and V. F. IRISH, 1998 Molecular evolution of genes controlling petal and stamen development: Duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages Genetics 149: 765-783.

KRAMER, E. M., and J. C. HALL, 2005 Evolutionary dynamics of genes controlling floral development. Curr Opin Plant Biol 8: 13-18.

KRAMER, E. M., L. HOLAPPA, B. GOULD, M. A. JARAMILLO, D. SETNIKOV *et al.*, 2007a Elaboration of B gene function to include the identity of novel floral organs in the lower eudicot Aquilegia (Ranunculaceae). Plant Cell Epub ahead of print.

KRAMER, E. M., L. HOLAPPA, B. GOULD, M. A. JARAMILLO, D. SETNIKOV *et al.*, 2007b Elaboration of B Gene Function to Include the Identity of Novel Floral Organs in the Lower Eudicot Aquilegia. Plant Cell.

KRAMER, E. M., and V. F. IRISH, 2000 Evolution of the petal and stamen developmental programs: Evidence from comparative studies of the lower eudicots and basal angiosperms. International Journal of Plant Sciences 161: S29-S40.

KRAMER, E. M., M. A. JARAMILLO and V. S. DI STILIO, 2004 Patterns of Gene Duplication and Functional Evolution During the Diversification of the

AGAMOUS Subfamily of MADS Box Genes in Angiosperms. Genetics 166: 1011-1023.

KUUSK, S., J. J. SOHLBERG, D. MAGNUS EKLUND and E. SUNDBERG, 2006 Functionally redundant SHI family genes regulate Arabidopsis gynoecium development in a dose-dependent manner. Plant J 47: 99-111.

KUZOFF, R. K., and C. S. GASSER, 2000 Recent progress in reconstructing angiosperm phylogeny. Trends Plant Sci 5: 330-336.

KYOZUKA, J., T. KOBAYASHI, M. MORITA and K. SHIMAMOTO, 2000 Spatially and temporally regulated expression of rice MADS box genes with similarity to Arabidopsis class A, B and C genes. Plant Cell Physiol 41: 710-718.

LAMB, R. S., T. A. HILL, Q. K.-G. TAN and V. F. IRISH, 2002 Regulation of APETALA3 floral homeotic gene expression by meristem identity genes. Development 129: 2079-2086.

LAUX, T., K. F. MAYER, J. BERGER and G. JURGENS, 1996a The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development 122: 87-96.

LAUX, T., K. F. X. MAYER, J. BERGER and G. JURGENS, 1996b The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development 122: 87-96.

LEE, J.-Y., S. F. BAUM, J. ALVAREZ, A. PATEL, D. H. CHITWOOD *et al.*, 2005a Activation of CRABS CLAW in the Nectaries and Carpels of Arabidopsis. Plant Cell 17: 25-36.

LEE, J.-Y., S. F. BAUM, S.-H. OH, C.-Z. JIANG, J.-C. CHEN *et al.*, 2005b Recruitment of CRABS CLAW to promote nectary development within the eudicot clade. Development 132: 5021-5032.

LEE, J. Y., S. F. BAUM, S. H. OH, C. Z. JIANG, J. C. CHEN *et al.*, 2005c Recruitment of CRABS CLAW to promote nectary development within the eudicot clade. Development 132: 5021-5032.

LEE, J. Y., S. F. BAUM, S. H. OH, C. Z. JIANG, J. C. CHEN *et al.*, 2005d Recruitment of CRABS CLAW to promote nectary development within the eudicot clade. Development 132: 5021-5032.

LENHARD, M., A. BOHNERT, G. JURGENS and T. LAUX, 2001 Termination of Stem Cell Maintenance in Arabidopsis Floral Meristems by Interactions between WUSCHEL and AGAMOUS. Cell 105: 805-814.

LESEBERG, C. H., C. L. EISSLER, X. WANG, M. A. JOHNS, M. R. DUVALL *et al.*, 2008 Interaction study of MADS-domain proteins in tomato. J Exp Bot 59: 2253-2265.

LEVIN, J. Z., and E. M. MEYEROWITZ, 1995 UFO: An Arabidopsis Gene Involved in Both Floral Meristem and Floral Organ Development. The Plant Cell Online 7: 529-548.

LI, X., X. DUAN, H. JIANG, Y. SUN, Y. TANG *et al.*, 2006 Genome-Wide Analysis of Basic/Helix-Loop-Helix Transcription Factor Family in Rice and Arabidopsis. Plant Physiology 141: 1167-1184.

LILJEGREN, S. J., G. S. DITTA, Y. ESHED, B. SAVIDGE, J. L. BOWMAN *et al.*, 2000 SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. Nature 404: 766-770.

LILJEGREN, S. J., A. H. K. ROEDER, S. A. KEMPIN, K. GREMSKI, L. OSTERGAARD *et al.*, 2004 Control of fruit patterning in Arabidopsis by INDEHISCENT. Cell 116: 843-853.

LIU, C., Z. THONG and H. YU, 2009 Coming into bloom: the specification of floral meristems. Development 136: 3379-3391.

LIU, C., J. ZHOU, K. BRACHA-DRORI, S. YALOVSKY, T. ITO *et al.*, 2007 Specification of Arabidopsis floral meristem identity by repression of flowering time genes. Development 134: 1901-1910.

LIU, Z., and C. MARA, 2010 Regulatory mechanisms for floral homeotic gene expression. Seminars in Cell & Developmental Biology 21: 80-86.

LIU, Z., and E. M. MEYEROWITZ, 1995 LEUNIG regulates AGAMOUS expression in Arabidopsis flowers. Development 121: 975-991.

LOHMANN, J. U., R. L. HONG, M. HOBE, M. A. BUSCH, F. PARCY *et al.*, 2001 A Molecular Link between Stem Cell Regulation and Floral Patterning in Arabidopsis. Cell 105: 793-803.

LOHMANN, J. U., A. T. MAIER, S. STEHLING-SUN, H. WOLLMANN, M. DEMAR *et al.*, 2009 Dual roles of the bZIP transcription factor PERIANTHIA in the control of floral architecture and homeotic gene expression. Development 136: 1613-1620.

MA, H., M. F. YANOFSKY and E. M. MEYEROWITZ, 1991 AGL1-AGL6, an Arabidopsis gene family with similarity to floral homeotic and transcription factor genes. Genes & Development 5: 484-495.

MACKAY, J. P., and M. CROSSLEY, 1998 Zinc fingers are sticking together. Trends Biochem Sci 23: 1-4.

MAYER, K. F. X., H. SCHOOF, A. HAECKER, M. LENHARD, G. JURGENS *et al.*, 1998 Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell 95: 805-815.

MCSTEEN, P., D. LAUDENCIA-CHINGCUANCO and J. COLASANTI, 2000 A floret by any other name: control of meristem identity in maize. Trends in Plant Science 5: 61-66.

MENA, M., B. A. AMBROSE, R. B. MEELEY, S. P. BRIGGS, M. F. YANOFSKY *et al.*, 1996 Diversification of C-function activity in maize flower development. Science 274: 1537-1540.

MITCHELL, P. J., and R. TJIAN, 1989 Transcriptional Regulation in Mammalian-Cells by Sequence-Specific DNA-Binding Proteins. Science 245: 371-378.

MIZUKAMI, Y., and H. MA, 1993 Functional-Analysis of the Arabidopsis Floral Homeotic Gene Agamous in Transgenic Plants. Journal of Cellular Biochemistry: 15-15.

MIZUKAMI, Y., and H. MA, 1995 Separation of Ag Function in Floral Meristem Determinacy from That in Reproductive Organ Identity by Expressing Antisense Ag Rna. Plant Molecular Biology 28: 767-784.

MIZUKAMI, Y., and H. MA, 1997 Determination of Arabidopsis Floral Meristem Identity by AGAMOUS. Plant Cell 9: 393-408.

MONDRAGON-PALOMINO, M., L. HIESE, A. HARTER, M. A. KOCH and G. THEISSEN, 2009 Positive selection and ancient duplications in the evolution of class B floral homeotic genes of orchids and grasses. BMC Evol Biol 9: 81.

MOON, Y. H., J. Y. JUNG, H. G. KANG and G. H. AN, 1999a Identification of a rice APETALA3 homologue by yeast two-hybrid screening. Plant Molecular Biology 40: 167-177.

MOON, Y. H., H. G. KANG, J. Y. JUNG, J. S. JEON, S. K. SUNG *et al.*, 1999b Determination of the motif responsible for interaction between the rice APETALA1/AGAMOUS-LIKE9 family proteins using a yeast two-hybrid system. Plant Physiology 120: 1193-1203.

MUNSTER, T., L. U. WINGEN, W. FAIGL, S. WERTH, H. SAEDLER *et al.*, 2001 Characterization of three GLOBOSA-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. Gene 262: 1-13.

NAKAGAWA, H., C. J. JIANG, H. SAKAKIBARA, M. KOJIMA, I. HONDA *et al.*, 2005 Overexpression of a petunia zinc-finger gene alters cytokinin metabolism and plant forms. Plant Journal 41: 512-523.

NAKAYAMA, H., T. YAMAGUCHI and H. TSUKAYA, 2010 Expression Patterns of Aadl, a Crabs Claw Ortholog in Asparagus Asparagoides (Asparagaceae), Demonstrate a Stepwise Evolution of Crc/Dl Subfamily of Yabby Genes. American Journal of Botany 97: 591-600.

NANDI, A. K., K. KUSHALAPPA, K. PRASAD and U. VIJAYRAGHAVAN, 2000 A conserved function for Arabidopsis SUPERMAN in regulating floral-whorl cell proliferation in rice, a monocotyledonous plant. Current Biology 10: 215-218.

NEMHAUSER, J. L., L. J. FELDMAN and P. C. ZAMBRYSKI, 2000 Auxin and ETTIN in Arabidopsis gynoecium morphogenesis. Development 127: 3877-3888.

NG, M., and M. F. YANOFSKY, 2001 Activation of the Arabidopsis B class homeotic genes by APETALA1. Plant Cell 13: 739-753.

NIBAU, C., V. N. S. DI STILIO, H.-M. WU and A. Y. CHEUNG, 2011 Arabidopsis and Tobacco SUPERMAN regulate hormone signalling and mediate cell proliferation and differentiation. Journal of Experimental Botany 62: 949-961.

OKAMURO, J. K., B. CASTER, R. VILLARROEL, M. VANMONTAGU and K. D. JOFUKU, 1997 The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 94: 7076-7081.

PAN, I. L., R. MCQUINN, J. J. GIOVANNONI and V. F. IRISH, 2010 Functional diversification of AGAMOUS lineage genes in regulating tomato flower and fruit development. Journal of Experimental Botany 61: 1795-1806.

PARK, J.-H., Y. ISHIKAWA, T. OCHIAI, A. KANNO and T. KAMEYA, 2004 Two GLOBOSA-Like Genes are Expressed in Second and Third Whorls of Homochlamydeous Flowers in Asparagus officinalis L. Plant and Cell Physiology 45: 325-332.

PARK, J. H., Y. ISHIKAWA, R. YOSHIDA, A. KANNO and T. KAMEYA, 2003a Expression of AODEF, a B-functional MADS-box gene, in stamens and inner tepals of the dioecious species Asparagus officinalis L. Plant Molecular Biology 51: 867-875.

PARK, J. H., A. KANNO and T. KAMEYA, 2003b Isolation and characterization of class B floral organ identity gene from garden asparagus. Plant and Cell Physiology 44: S183-S183.

PATTANAIK, S., C. H. XIE and L. YUAN, 2008 The interaction domains of the plant Myc-like bHLH transcription factors can regulate the transactivation strength. Planta 227: 707-715.

PENFIELD, S., E.-M. JOSSE, R. KANNANGARA, A. D. GILDAY, K. J. HALLIDAY *et al.*, 2005 Cold and Light Control Seed Germination through the bHLH Transcription Factor SPATULA. Current Biology 15: 1998-2006.

PINYOPICH, A., G. S. DITTA, B. SAVIDGE, S. J. LILJEGREN, E. BAUMANN *et al.*, 2003 Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature 424: 85-88.

PNUELI, L., M. ABU-ABEID, D. ZAMIR, W. NACKEN, Z. SCHWARZ-SOMMER *et al.*, 1991 The MADS box gene family in tomato: temporal expression during floral development, conserved secondary structures and homology with homeotic genes from Antirrhinum and Arabidopsis. Plant J 1: 255-266.

PRASAD, K., and U. VIJAYRAGHAVAN, 2003 Double-stranded RNA interference of a rice PI/GLO paralog, OsMADS2, uncovers its second-whorl-specific function in floral organ patterning. Genetics 165: 2301-2305.

PRUNET, N., P. MOREL, I. NEGRUTIU and C. TREHIN, 2009 Time to stop: flower meristem termination. Plant Physiol 150: 1764-1772.

PRUNET, N., P. MOREL, A.-M. THIERRY, Y. ESHED, J. L. BOWMAN *et al.*, 2008 REBELOTE, SQUINT, and ULTRAPETALA1 Function Redundantly in the Temporal Regulation of Floral Meristem Termination in Arabidopsis thaliana. Plant Cell 20: 901-919.

RAJANI, S., and V. SUNDARESAN, 2001 The Arabidopsis myc/bHLH gene ALCATRAZ enables cell separation in fruit dehiscence. Current biology : CB 11: 1914-1922.

RIECHMANN, J. L., M. WANG and E. M. MEYEROWITZ, 1996 DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. Nucleic Acids Res 24: 3134-3141.

RIJPKEMA, A. S., S. ROYAERT, J. ZETHOF, G. VAN DER WEERDEN, T. GERATS *et al.*, 2006 Analysis of the Petunia TM6 MADS Box Gene Reveals Functional Divergence within the DEF/AP3 Lineage. The Plant Cell Online 18: 1819-1832.

ROBLES, P., and S. PELAZ, 2005 Flower and fruit development in Arabidopsis thaliana. Int J Dev Biol 49: 633-643.

RONSE DE CRAENE, L. P., 2007 Are Petals Sterile Stamens or Bracts? The Origin and Evolution of Petals in the Core Eudicots. Annals of Botany 100: 621-630.

ROUNSLEY, S. D., G. S. DITTA and M. F. YANOFSKY, 1995 Diverse roles for MADS box genes in Arabidopsis development. Plant Cell 7: 1259-1269.

SAKAI, H., L. J. MEDRANO and E. M. MEYEROWITZ, 1995 Role of Superman in Maintaining Arabidopsis Floral Whorl Boundaries. Nature 378: 199-203.

SCHWARZ-SOMMER, Z., P. HUIJSER, W. NACKEN, H. SAEDLER and H. SOMMER, 1990 Genetic Control of Flower Development by Homeotic Genes in Antirrhinum majus. Science 250: 931-936.

SCUTT, C. P., M. VINAUGER-DOUARD, C. FOURQUIN, C. FINET and C. DUMAS, 2006 An evolutionary perspective on the regulation of carpel development. J. Exp. Bot. 57: 2143-2152.

SESSIONS, A., J. L. NEMHAUSER, A. MCCOLL, J. L. ROE, K. A. FELDMANN *et al.*, 1997 ETTIN patterns the Arabidopsis floral meristem and reproductive organs. Development 124: 4481-4491.

SIEBURTH, L. E., and E. M. MEYEROWITZ, 1997 Molecular dissection of the AGAMOUS control region shows that cis elements for spatial regulation are located intragenically. Plant Cell 9: 355-365.

SIEBURTH, L. E., M. P. RUNNING and E. M. MEYEROWITZ, 1995a Genetic Separation of 3rd and 4th Whorl Functions of Agamous. Plant Cell 7: 1249-1258.

SIEBURTH, L. E., M. P. RUNNING and E. M. MEYEROWITZ, 1995b Genetic Separation of Third and Fourth Whorl Functions of AGAMOUS. Plant Cell 7: 1249-1258.

SMYTH, D. R., J. L. BOWMAN and E. M. MEYEROWITZ, 1990 Early Flower Development in Arabidopsis. The Plant Cell Online 2: 755-767.

SOLTIS, D. E., A. S. CHANDERBALI, S. KIM, M. BUZGO and P. S. SOLTIS, 2007 The ABC Model and its Applicability to Basal Angiosperms. Ann Bot 100: 155-163.

SOREFAN, K., and L. OSTERGAARD, 2007 Auxin homeostasis and Arabidopsis fruit development. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 146: S239-S239.

SRIDHAR, V. V., A. SURENDRARAO, D. GONZALEZ, R. S. CONLAN and Z. LIU, 2004 Transcriptional repression of target genes by LEUNIG and SEUSS, two interacting regulatory proteins for Arabidopsis flower development. Proceedings of the National Academy of Sciences of the United States of America 101: 11494-11499.

SRIDHAR, V. V., A. SURENDRARAO and Z. LIU, 2006 APETALA1 and SEPALLATA3 interact with SEUSS to mediate transcription repression during flower development. Development 133: 3159-3166.

STALDAL, V., and E. SUNDBERG, 2009 The role of auxin in style development and apical-basal patterning of the Arabidopsis thaliana gynoecium. Plant Signal Behav 4: 83-85.

SUNDSTROM, J. F., N. NAKAYAMA, K. GLIMELIUS and V. F. IRISH, 2006 Direct regulation of the floral homeotic APETALA1 gene by APETALA3 and PISTILLATA in Arabidopsis. Plant J 46: 593-600.

TANDRE, K., V. A. ALBERT, A. SUNDÅS and P. ENGSTRÖM, 1995 Conifer homologues to genes that control floral development in angiosperms. Plant Molecular Biology 27: 69-78.

THEISSEN, G., and A. BECKER, 2004 Gymnosperm orthologues of class B floral homeotic genes and their impact on understanding flower origin. Critical Reviews in Plant Sciences 23: 129-148.

THEISSEN, G., and R. MELZER, 2007 Molecular mechanisms underlying origin and diversification of the angiosperm flower. Ann Bot 100: 603-619.

THEISSEN, G., and H. SAEDLER, 2001 Plant biology. Floral quartets. Nature 409: 469-471.

TROBNER, W., L. RAMIREZ, P. MOTTE, I. HUE, P. HUIJSER *et al.*, 1992 Globosa - a homeotic gene which interacts with deficiens in the control of antirrhinum floral organogenesis. EMBO J 11: 4693 - 4704.

TRÖBNER, W., L. RAMIREZ, P. MOTTE, I. HUE, P. HUIJSER *et al.*, 1992 GLOBOSA: a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. EMBO J 11: 4693-4704.

TSAI, W.-C., C.-S. KUOH, M.-H. CHUANG, W.-H. CHEN and H.-H. CHEN, 2004 Four DEF-Like MADS Box Genes Displayed Distinct Floral Morphogenetic Roles in Phalaenopsis Orchid. Plant Cell Physiol. 45: 831-844.

TZENG, T. Y., and C. H. YANG, 2001 A MADS box gene from lily (Lilium Longiflorum) is sufficient to generate dominant negative mutation by interacting with PISTILLATA (PI) in Arabidopsis thaliana. Plant Cell Physiol 42: 1156-1168.

VANDENBUSSCHE, M., G. THEISSEN, Y. VAN DE PEER and T. GERATS, 2003 Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations. Nucl. Acids Res. 31: 4401-4409.

VANDENBUSSCHE, M., J. ZETHOF, S. ROYAERT, K. WETERINGS and T. GERATS, 2004 The Duplicated B-Class Heterodimer Model: Whorl-Specific Effects and Complex Genetic Interactions in Petunia hybrida Flower Development. Plant Cell 16: 741-754.

WALL, P. K., J. LEEBENS-MACK, A. S. CHANDERBALI, A. BARAKAT, E. WOLCOTT *et al.*, 2009 Comparison of next generation sequencing technologies for transcriptome characterization. BMC Genomics 10: -.

WELLMER, F., J. L. RIECHMANN, M. ALVES-FERREIRA and E. M. MEYEROWITZ, 2004 Genome-wide analysis of spatial gene expression in Arabidopsis flowers. Plant Cell 16: 1314-1326.

WHIPPLE, C. J., M. J. ZANIS, E. A. KELLOGG and R. J. SCHMIDT, 2007 Conservation of B class gene expression in the second whorl of a basal grass and outgroups links the origin of lodicules and petals. Proceedings of the National Academy of Sciences 104: 1081-1086.

WILKINSON, M. D., and G. W. HAUGHN, 1995 UNUSUAL FLORAL ORGANS Controls Meristem Identity and Organ Primordia Fate in Arabidopsis. The Plant Cell Online 7: 1485-1499.

WINTER, K. U., H. SAEDLER and G. THEISSEN, 2002 On the origin of class B floral homeotic genes: functional substitution and dominant inhibition in Arabidopsis by expression of an orthologue from the gymnosperm Gnetum. Plant Journal 31: 457-475.

YADAV, S. R., K. PRASAD and U. VIJAYRAGHAVAN, 2007 Divergent Regulatory OsMADS2 Functions Control Size, Shape and Differentiation of the Highly Derived Rice Floret Second-Whorl Organ. Genetics 176: 283-294.

YAMAGUCHI, T., D. Y. LEE, A. MIYAO, H. HIROCHIKA, G. AN *et al.*, 2006a Functional Diversification of the Two C-Class MADS Box Genes OSMADS3 and OSMADS58 in Oryza sativa. Plant Cell 18: 15-28.

YAMAGUCHI, T., D. Y. LEE, A. MIYAO, H. HIROCHIKA, G. AN *et al.*, 2006b Functional diversification of the two C-class MADS box genes OSMADS3 and OSMADS58 in Oryza sativa. Plant Cell 18: 15-28.

YAMAGUCHI, T., N. NAGASAWA, S. KAWASAKI, M. MATSUOKA, Y. NAGATO *et al.*, 2004 The YABBY Gene DROOPING LEAF Regulates Carpel Specification and Midrib Development in Oryza sativa. Plant Cell 16: 500-509.

YANG, Y. Z., and T. JACK, 2004 Defining subdomains of the K domain important for protein-protein interactions of plant MADS proteins. Plant Molecular Biology 55: 45-59.

YANOFSKY, M. F., H. MA, J. L. BOWMAN, G. N. DREWS, K. A. FELDMANN *et al.*, 1990a The protein encoded by the Arabidopsis homeotic gene *agamous* rersembles transcription factors. Naure 346: 35-40.

YANOFSKY, M. F., H. MA, J. L. BOWMAN, G. N. DREWS, K. A. FELDMANN *et al.*, 1990b The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. Nature 346: 35-39.

YELLINA, A., S. ORASHAKOVA, S. LANGE, R. ERDMANN, J. LEEBENS-MACK *et al.*, 2010 Floral homeotic C function genes repress specific B function genes in the carpel whorl of the basal eudicot California poppy (*Eschscholzia californica*). BMC Evo-Devo in press.

YU, D. Y., M. KOTILAINEN, E. POLLANEN, M. MEHTO, P. ELOMAA *et al.*, 1999 Organ identity genes and modified patterns of flower development in Gerbera hybrida (Asteraceae). Plant Journal 17: 51-62.

YUN, J. Y., D. WEIGEL and I. LEE, 2002 Ectopic expression of SUPERMAN suppresses development of petals and stamens. Plant and Cell Physiology 43: 52-57.

ZAHN, L. M., J. LEEBENS-MACK, C. W. DEPAMPHILIS, H. MA and G. THEISSEN, 2005 To B or Not to B a Flower: The Role of DEFICIENS and GLOBOSA Orthologs in the Evolution of the Angiosperms. J Hered 96: 225-240.

ZAHN, L. M., J. H. LEEBENS-MACK, J. M. ARRINGTON, Y. HU, L. L. LANDHERR *et al.*, 2006 Conservation and divergence in the AGAMOUS subfamily of MADS-box genes: evidence of independent sub- and neofunctionalization events. Evolution & Development 8: 30-45.

ZAHN, L. M., X. MA, N. S. ALTMAN, Q. ZHANG, P. K. WALL *et al.*, 2010 Comparative transcriptomics among floral organs of the basal eudicot Eschscholzia californica as reference for floral evolutionary developmental studies. Genome Biol 11: R101.

ZANIS, M. J., D. E. SOLTIS, P. S. SOLTIS, S. MATHEWS and M. J. DONOGHUE, 2002 The root of the angiosperms revisited. Proc Natl Acad Sci U S A 99: 6848-6853.

ZANIS, M. J., P. S. SOLTIS, Y. L. QIU, E. ZIMMER and D. E. SOLTIS, 2003 Phylogenetic Analyses and Perianth Evolution in Basal Angiosperms. Annals of the Missouri Botanical Garden 90: 129-150.

ZHANG, P., H. T. W. TAN, K.-H. PWEE and P. P. KUMAR, 2004 Conservation of class C function of floral organ development during 300 million years of evolution from gymnosperms to angiosperms. The Plant Journal 37: 566-577.

ZHANG, Q., J. XU, Y. LI, P. XU, H. ZHANG *et al.*, 2007 Morphological, Anatomical and Genetic Analysis for a Rice Mutant with Abnormal Hull. Journal of Genetics and Genomics 34: 519-526.

ZIK, M., and V. F. IRISH, 2003 Flower development: initiation, differentiation, and diversification. Annu Rev Cell Dev Biol 19: 119-140.

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The Plant Journal (2009) **58**, 682–693 doi: 10.1111/j.1365-313X.2009.03807.x

The *CRABS CLAW* ortholog from California poppy (*Eschscholzia californica,* Papaveraceae)*, EcCRC,* is involved in floral meristem termination, gynoecium differentiation and ovule initiation

Svetlana Orashakova[†], Matthias Lange[†], Sabrina Lange, Stefanie Wege[‡] and Annette Becker[*]

Evolutionary Developmental Genetics Group, Department of Biology and Chemistry, University of Bremen, Leobener Str., UFT, 28359 Bremen, Germany

Received 3 December 2008; revised 12 January 2009; accepted 16 January 2009; published online 25 February 2009.
*For correspondence (fax + 49 421 218 3240; e-mail annette.becker@uni-bremen.de).
[†]Svetlana Orashakova and Matthias Lange have contributed equally to this manuscript.
[‡]Stefanie Wege has recently moved to the Institut des Sciences du Végétal, UPR 2355, CNRS, 1 Avenue de la Terrasse, 91198 Gif sur Yvette Cedex, France.

SUMMARY

The Arabidopsis transcription factor *CRABS CLAW* (*CRC*) is a major determinant of carpel growth and fusion, and, in concert with other redundantly acting genes, of floral meristem termination. Its rice ortholog, however, has additional functions in specifying carpel organ identity. We were interested in understanding the history of gene function modulation of *CRC*-like genes during angiosperm evolution. Here, we report the identification and functional characterization of *EcCRC*, the Californica poppy (*Eschscholzia californica*) *CRC* ortholog. The downregulation of *EcCRC* by virus-induced gene silencing (VIGS) produces additional organ whorls that develop exclusively into gynoecia, resulting in a reiteration of the fourth whorl. Additionally, defects in carpel polarity and ovule initiation are apparent, and the observed phenotype is restricted to the gynoecium. Our results further show that the history of *CRC*-like genes during angiosperm evolution is characterized by gains of function, independent of duplication processes in this gene subfamily. Moreover, our data indicate that the ancestral angiosperm *CRC*-like gene was involved in floral meristem termination and the promotion of abaxial cell fate in the gynoecium, and that in the lineage leading to Arabidopsis, additional genes have been recruited to adopt some of these functions, resulting in a high degree of redundancy.

Keywords: evolutionary developmental genetics, carpel development, YABBY transcription factor, *CRABS CLAW*, *Eschscholzia californica*, California poppy.

INTRODUCTION

The most important specific character common to all flowering plants is the carpel, which is located in the centre of the flower, and protectively surrounds the ovules (Crane *et al.*, 1995). Most angiosperms develop carpels that are differentiated into the following structures: the ovary, where the seeds develop; the style; and the stigma, which is a specialized region were pollen germination takes place. The carpel may also provide a system for preventing self-fertilization, as a mechanical barrier and through a molecular self-incompatibility system (Dilcher, 2000).The carpel is also generally the last organ to be formed by the floral meristem, which is consumed in the process of carpel development. When fertilization of the ovules has commenced, the carpel differentiates into the fruit that protects the seeds and ensures their dispersal by a vast variety of mechanisms.

The female reproductive structures of the sister group of the angiosperms, the gymnosperms, are comparatively simple, as the seeds develop on a scale, and pollen germination takes place close to, or at, the ovule surface.

One possible reason for the general success of angiosperms, which dominate the terrestrial ecosystems of our planet, is the evolution of the morphological innovation of the carpel. To learn more about the evolution of the carpel will thus help to better understand the emergence and

effective radiation of angiosperms. As the fossil record has not yielded any carpel precursors from non-angiosperms, an alternative approach needs to be considered. Functional comparisons of gene networks directing carpel development in widely diverged angiosperm species could eventually unravel a basic set of gene functions necessary to orchestrate carpel development in all angiosperms. Carpel development control genes are being identified in the core eudicot *Arabidopsis thaliana* and in the monocot rice. However, the morphological differences between rice and Arabidopsis are vast, e.g. the ovules in Arabidopsis develop from secondary meristems within the carpel, whereas the rice ovule develops directly from the floral meristem (Itoh *et al.*, 2005), and additional reference species are required.

The YABBY gene *CRABS CLAW* (*CRC*) encodes a putative transcription factor regulating several important aspects of carpel development in the rosid *A. thaliana*. The YABBY proteins are a small family of plant-specific transcription factors, and are generally expressed abaxially in developing lateral organs. Phylogenetic analysis of YABBY genes suggest that the *CRC* subfamily represents a single orthologous lineage, without ancient duplications (Lee *et al.*, 2005b). Several mutant alleles have been identified in Arabidopsis and rice, yielding a wealth of functional data from these two highly divergent plant species (Bowman and Smyth, 1999; Yamaguchi *et al.*, 2004; Lee *et al.*, 2005b). *CRC* is involved in the control of radial and longitudinal growth of the Arabidopsis gynoecium, and also regulates carpel fusion, in part. *crc* mutants have gynoecia that are shorter and wider than the wild type, and show defects in carpel fusion. *CRC* is also essential for nectar gland formation in rosids and asterids, and in *crc* mutants of Arabidopsis, nectary formation is abolished completely (Alvarez and Smyth, 1999, 2002; Bowman and Smyth, 1999; Lee *et al.*, 2005a,b). *DROOPING LEAF* (*DL*) is the *CRC* ortholog from rice, and is necessary for midrib formation in the rice leaf, floral meristem determinacy and carpel organ identity (Yamaguchi *et al.*, 2004). Functional studies have also been carried out in petunia and tobacco via a small set of virus-induced gene silencing (VIGS)-treated plants (Lee *et al.*, 2005b). In addition to these functional studies, information on expression patterns of *CRC*-like genes is available for additional species, e.g. carpel expression for *Aquilegia formosa*, *Petunia hybrida* and *Amborella trichopoda*, and additional nectary expression for *Cleome sparsifolia*, *Lepidium africanum* and *Capparis flexuosa* (Fourquin *et al.*, 2005; Lee *et al.*, 2005a).

The Arabidopsis *CRC* gene is also involved in the termination of the floral meristem in the latest stage during gynoecium initiation. A small number of mutant *crc* gynoecia show more than two carpels, indicating a mild effect of *CRC* on floral meristem termination. Recently, it was shown that *CRC* acts in concert with three genes, *REBELOTE* (*RBL*), *SQUINT* (*SQN*) and *ULTRAPETALA1* (*ULT1*), to control the early and late phase of floral meristem termination. Interestingly, *CRC* is not expressed in the centre of the flower, where the activity of the floral meristem will cease, which hints at the possibility that *CRC* itself might not act in a cell-autonomous way (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Prunet *et al.*, 2008). Several studies so far have shown that the various *CRC*-like genes from a diverse set of angiosperm species appear to be involved in many important aspects of plant development, including functions in carpel, nectar gland and leaf-blade development, carpel organ identity, and floral meristem determination.

The plant analyzed in this study, *Eschscholzia californica* Cham. (California poppy) is a representative of a lineage that derived prior to the eudicots, and belongs to the family of Papaveraceae within the Ranunculales. The Ranunculales clade is the earliest diverging eudicot lineage according to recent phylogenies employing molecular markers (Angiosperm Phylogeny Group, 2003), and within the Papaveraceae, *Eschscholzia* is a rather early diverging genus (Hoot *et al.*, 1997).

In this study, the function of the *Eschscholzia* ortholog of *CRC*, *EcCRC*, was examined in order to deduce the evolutionary ancestral role of the *CRC*-like genes, and to understand the complex history of neofunctionalization in this gene subfamily. We determined *EcCRC* expression patterns and used VIGS to transiently knock-down *EcCRC* function. Based on these observations relative to what is known from other species, we propose that the ancestral functions of *CRC*-like genes included: (i) the establishment and maintenance of floral meristem determinacy, (ii) specifying abaxial cell fate within the carpel, and (iii) promoting differentiation of carpel marginal tissue. Mapping functional traits of *CRC*-like genes along phylogenetic trees, we can also infer that the *CRC*-like genes underwent a series of neofunctionalization events, leading to several divergent gene functions in the monocot and dicot lineages.

RESULTS

Cloning of the *Eschscholzia CRC* ortholog

3′ and subsequently 5′ RACE PCR cloning was used to amplify sequences homologous with the Arabidopsis and rice *CRC* and *DL* genes. Thorough Bayesian phylogenetic analysis was performed based on the nucleotide sequences of high overall quality present in the NCBI database. The potential *Eschscholzia CRC* ortholog, *EcCRC*, shows a domain structure typical for YABBY transcription factors. The phylogeny reconstruction presented in Figure 1a shows that *EcCRC* is the *CRC* ortholog, and non-stringent Southern blot hybridization (data not shown) demonstrates that it is a single-copy gene. To date, *CRC*-like YABBY transcription factors have not been identified outside the angiosperms. *EcCRC* is the only Papaveraceae *CRC*-like sequence so far, and it clusters robustly within the sequences of two other

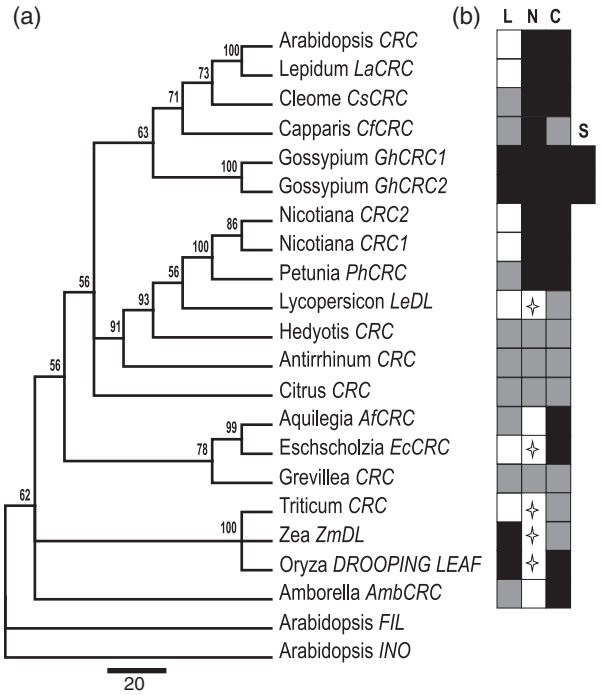


Figure 1. Bayesian phylogenetic tree of angiosperm orthologous *CRC*-like sequences.
(a) Phylogeny reconstruction of all available *CRC*-like sequences: the Arabidopsis YABBY genes *FIL* and *INNER NO OUTER* (*INO*) were used as the outgroups. The values above the branches denote posterior probabilities, and indicate clade support.
(b) Graphic representation of published expression patterns of *CRC*-like genes (covered in the Results and Discussion sections). Black boxes indicate that *CRC*-like expression has been experimentally detected. White boxes indicate no expression, whereas the gray boxes indicate that expression patterns have not been recorded. White stars show species lacking nectaries. Abbreviations: L, leaves; N, nectaries; C, carpels; S, seedling apices.

early diverging eudicot species, *A. formosa* and *Grevillea robusta*, being more closely related to the *A. formosa* sequence. Generally, the topology of our limited sample of *CRC* sequences is consistent with recent dicot species phylogenies (Soltis *et al.*, 2000), and with the YABBY gene phylogeny of Lee *et al.* (2005b).

EcCRC is expressed in floral and flower-derived tissues

RT-PCR experiments with cDNA amplified from diverse tissues were carried out to analyze the presence or absence of detectable *EcCRC* expression in *Eschscholzia* (Figure 2a). Within flowers at anthesis, *EcCRC* expression is restricted to the gynoecium, and no transcripts could be detected in sepals, petals, stamens or developing fruits. *EcCRC* expression was also absent in leaves and green seeds. However, mature seeds expressed *EcCRC*, which could hint to a function of *EcCRC* in late embryogenesis or seed maturation. *EcCRC* is consistently expressed from the earliest stages of flower development, i.e. stages 1–5, from the initiation of the floral meristem formation, when buds are 0–1 mm in diameter, until the buds are 3 mm in diameter, when male meiosis occurs. The expression level of *EcCRC* decreases in stage 9 (when female meiosis occurs), and is lower in gynoecia at anthesis than in developing buds (Figure 2, staging according to Becker *et al.*, 2005).

For a more detailed analysis of the expression pattern of *EcCRC*, *in situ* hybridization was performed. The *Eschscholzia* wild-type gynoecium consists of two fused carpels, which later differentiate into valves connected with a replum that will subsequently allow for fruit opening and seed dispersal (Becker *et al.*, 2005). The strong expression of *EcCRC* is first detected in stage 5, when the gynoecium initiates, and is observed in all subsequent flower development stages, although it remains restricted to the gynoecium (Figure 2b–i). In stage 6, when the gynoecium starts to elongate, *EcCRC* expression is found in two distinct domains: (i) in an abaxial domain covering about two-thirds of the gynoecium wall, but not in the most apical and basal regions of the elongating gynoecium wall; (ii) in the centre of the gynoecium base, where the floral meristem cell division has ceased (Figure 2c). In late stage 6, as the gynoecium elongates further, *EcCRC* expression is no longer confined to the abaxial side, or to the base of the gynoecium, but is present more widely in the region adjacent to the placenta. However, the apicalmost part of the gynoecium still does not show a hybridization signal (Figure 2d). In transverse orientation, a more complex expression pattern is revealed: (i) an even distribution of *EcCRC* expression over the medial domain of each carpel, (ii) strong expression in two broad strips enclosing the entire placenta region, but no expression can be detected in the central domain of the placenta (Figure 2e). In stage 7 prior to ovule formation, the expression can be found in three distinct domains: (i) the presumptive replum region, where a narrow strip of *EcCRC* expression can be detected in the abaxial domain; (ii) the region that will later form the ovary wall, which shows weak and uniform expression; and (iii) a few cells in the centre of the gynoecium that continue to express *EcCRC* (Figure 2f). The horizontal view into an older gynoecium shows that the expression domain of *EcCRC* is reduced to the central and abaxial domain of the ovary wall. No expression was detected in the developing ovules, and in the few cell layers of the adaxial ovary wall surface. Additionally, the presumptive replum region shows no *EcCRC* expression (Figure 2g). In a stage-8 bud, *EcCRC* expression is found exclusively on the abaxial side of the gynoecium. Strong domains of expression occur in the medial and lateral ridges of the ovary wall. However, the *EcCRC* expression domain continues to exclude the replum regions, placentae and ovules, creating a sharp border between the presumptive replum and the adaxial part of the ovary wall (Figure 2h,i).

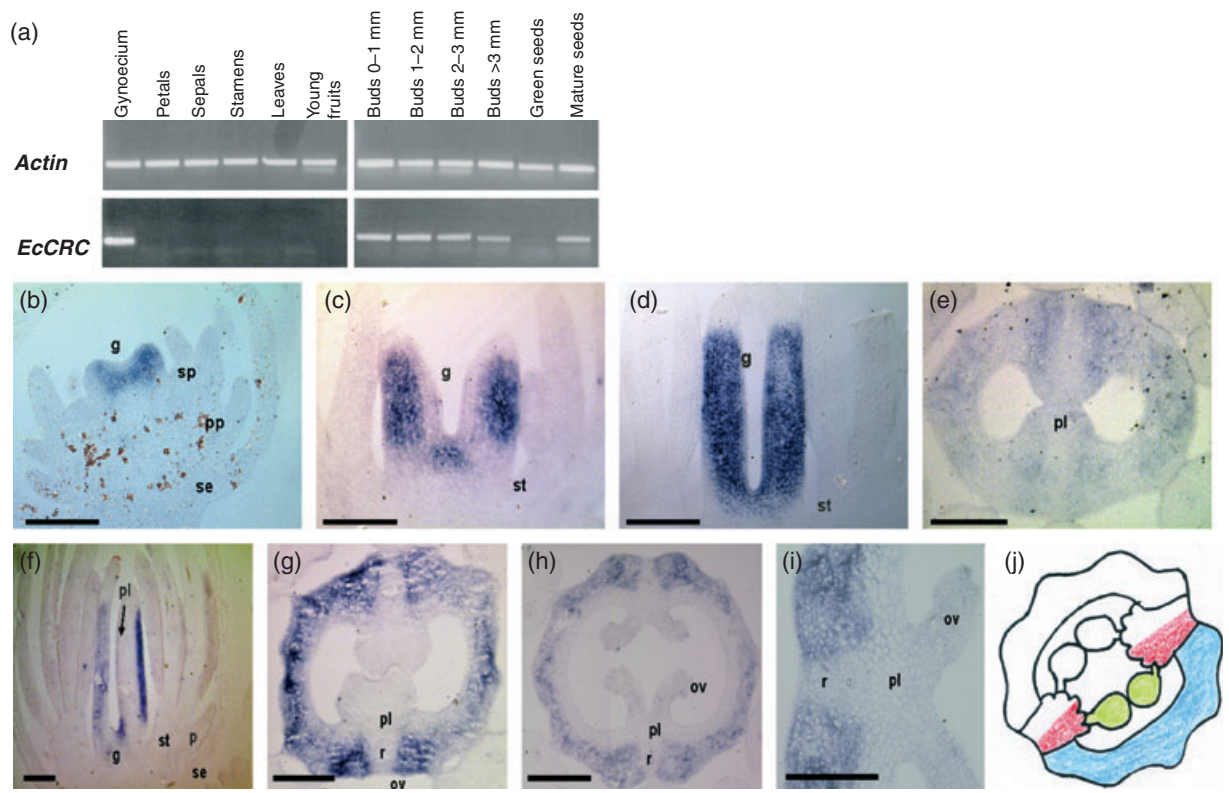


Figure 2. Expression of *EcCRC* in wild-type flowers shown by semi-quantitative RT-PCR and *in situ* hybridization.
(a) RT-PCR-based expression analysis of *EcCRC*, with *Actin* analyzed as an endogenous control. Tissues from which the RNA samples were collected are listed above.
(b–i) *In situ* hybridization pattern of *EcCRC*. (b) Longitudinal section of a bud in stage 5, when all floral organs are initiated. (c, d) Longitudinal sections of a bud in early (c) and late (d) stage 6. (d) shows the region directly adjacent to the placenta. (e) Transverse section of the gynoecium of a stage-6 bud. (f) Longitudinal section of a bud at stage 7. An arrow shows the presumptive placenta region, to the left, and the section shows part of the ovary wall. (g) Transverse section of a stage-7 gynoecium. (h) Transverse section of the gynoecium of a stage-8 bud. (i) Enlargement of the replum region of a stage-8 bud.
(j) Schematic overview of a stage-9 *Eschscholzia californica* gynoecium. The gynoecium is composed of two carpels, one of which has been colour coded: green, ovules; red, placenta; blue, ovary wall with abaxial ridges. Abbreviations: g, gynoecium; ov, ovule; p, petal; pl, placenta; pp, petal primordium; r, replum; se, sepal; sp, stamen primordium; st, stamen. Scale bars: 100 μm.

EcCRC loss-of-function phenotypes result in reduced longitudinal and radial growth of the fruit, and loss of floral meristem termination

To understand the role of *EcCRC* in gynoecium development, we used VIGS to obtain a transient knock-down of *EcCRC* gene expression. We infected 220 poppy plants with a mix of *Agrobacteria* carrying pTRV1 and pTRV2-EcCRC1, and 38 control plants with *Agrobacteria* harboring pTRV1 and pTRV2-E. Of the 220 plants inoculated with pTRV2-EcCRC1, 208 survived the inoculation treatment and 177 flowered. Of the 177 plants that produced flowers, 85 (48%) showed various degrees of defects in fruit development. We phenotypically characterized the first three flowers/fruits of each treated plant, where applicable, totaling 495 analyzed flowers/fruits. Previous studies (Wege *et al.*, 2007) indicated that the phenotypic effect decreases progressively in later formed flowers, and our results indicate a similar trend. When observing only the fruits formed first, we found that 47% show an *EcCRC*-VIGS phenotype, 34% showed wild-type fruits and 19% aborted. Of the fruits formed third, only 16% exhibited an *EcCRC*-VIGS phenotype, 65% did not show a phenotype and 21% aborted at an early developmental stage. Figure S1 shows the detailed distribution of fruit phenotypes of the first three fruits. Inoculation of poppy plants using the alternative construct pTRV2-EcCRC2 resulted in the same phenotypes with very similar ratios (see Figure S1). All plants treated with pTRV1 and pTRV2-E showed a wild-type phenotype.

We observed varying degrees of abnormal phenotypes in the *EcCRC*-silenced plants: in all cases restricted to the gynoecium and fruit development (Figure 3a–d). Mild phenotypes (Figure 3b) show an approximately 50% reduction in fruit length as compared with untreated plants, whereas strong phenotypes (Figure 3d) grew only to ~20% of the wild-type fruit length.

All the *EcCRC*-silenced plants showed a duplication of the fourth floral whorl, resulting in a gynoecium surrounding a second internal gynoecium (Figure 3a,b,e,f). Carpels are initiated at stage 5 of normal *Eschscholzia* flower develop-

ment, before the meristematic activity in the center of the flower ceases. In the case of *EcCRC*-silenced flowers, the carpels initiated correctly at stage 5, but the meristem failed to arrest and continued to produce consecutive carpel whorls (Figure 3f). In several instances a third, and in rare cases even a fourth, gynoecium was observed (data not shown). Later in fruit development, the longitudinal growth and increase in circumference of the inner fruit ruptured the wall of the outer fruit. The additional gynoecia produced viable seeds, albeit less than untreated plants. The normal apical–basal patterning of the fruits was not affected, and carpel fusion was complete. In the more severe phenotypes (Figure 3c, d) the fruit was tightly associated with the seeds, indicating that lateral growth of the fruit was also severely impaired. The number of seeds produced was reduced to a single seed in the most severe cases observed. These most severely affected fruits were also extremely short, growing to a maximum length of 2 cm. A large number of flowers (19%) aborted fruit development (about 10% of the pTRV2-E-treated control plants aborted fruits) even after hand pollination, suggesting that at least some of the most severely affected gynoecia did not develop further into fruits. Transverse sections of the severely affected fruits show that they also contained additional concentric tissue layers adaxial of the inner ovary wall, reminiscent of additional fruits, albeit without any further differentiation (Figure 3e,f). Longitudinal sections show that the center of the developing gynoecium, the floral meristem, continues to produce gynoecia. These inner gynoecia emerge at stage 7 of flower development, and we did not observe additional inner gynoecia in *EcCRC*-silenced plants at earlier developmental stages (Figure 3g,h).

We also tested if the strength of the observed phenotypes correlated with the degree of reduction in the *EcCRC* expression levels, by RT-PCR with *EcCRC*-specific primers on the first floral bud (with a diameter of 1–3 mm), that appeared on a sample of plants, and scored the next fruits to develop. In 175 of the 177 *EcCRC*-VIGS-treated plants examined in the main sample, both the first and second flowers produced the same phenotype (98.9%, e.g. the first and second flowers show a phenotype, or both flowers show no phenotype), thereby allowing us to analyze gene expression in the first flower, and to assess its phenotype based on the phenotype of the following flowers. All four control plants treated with TRV1 and TRV2-E show a strong expression of *EcCRC* in the buds (Figure 3o). Of the 20 plants treated with TRV1 and TRV2-EcCRC, all show either a strongly reduced or no expression of *EcCRC* when compared with TRV2-E-treated plants. All 12 plants that displayed a silencing phenotype observed from the second formed fruit showed no *EcCRC* expression in their first buds, indicating an inter-relationship between the *EcCRC* phenotype and the reduction of *EcCRC* expression. Also, two TRV2-EcCRC-treated plants that still showed expression of *EcCRC* (Figure 3o, nos 2 and 5) did not show a phenotype in their subsequent development. However, in two more plants (Figure 3o, nos 6 and 20) that did not have a phenotype, *EcCRC* expression was also absent, suggesting that an *EcCRC* mRNA concentration below the RT-PCR detection limit is sufficient for proper fruit development. Four more floral buds (Figure 3o, nos 4, 7, 17 and 18) showed no expression, but were the only buds produced, thereby impeding phenotypic assessment.

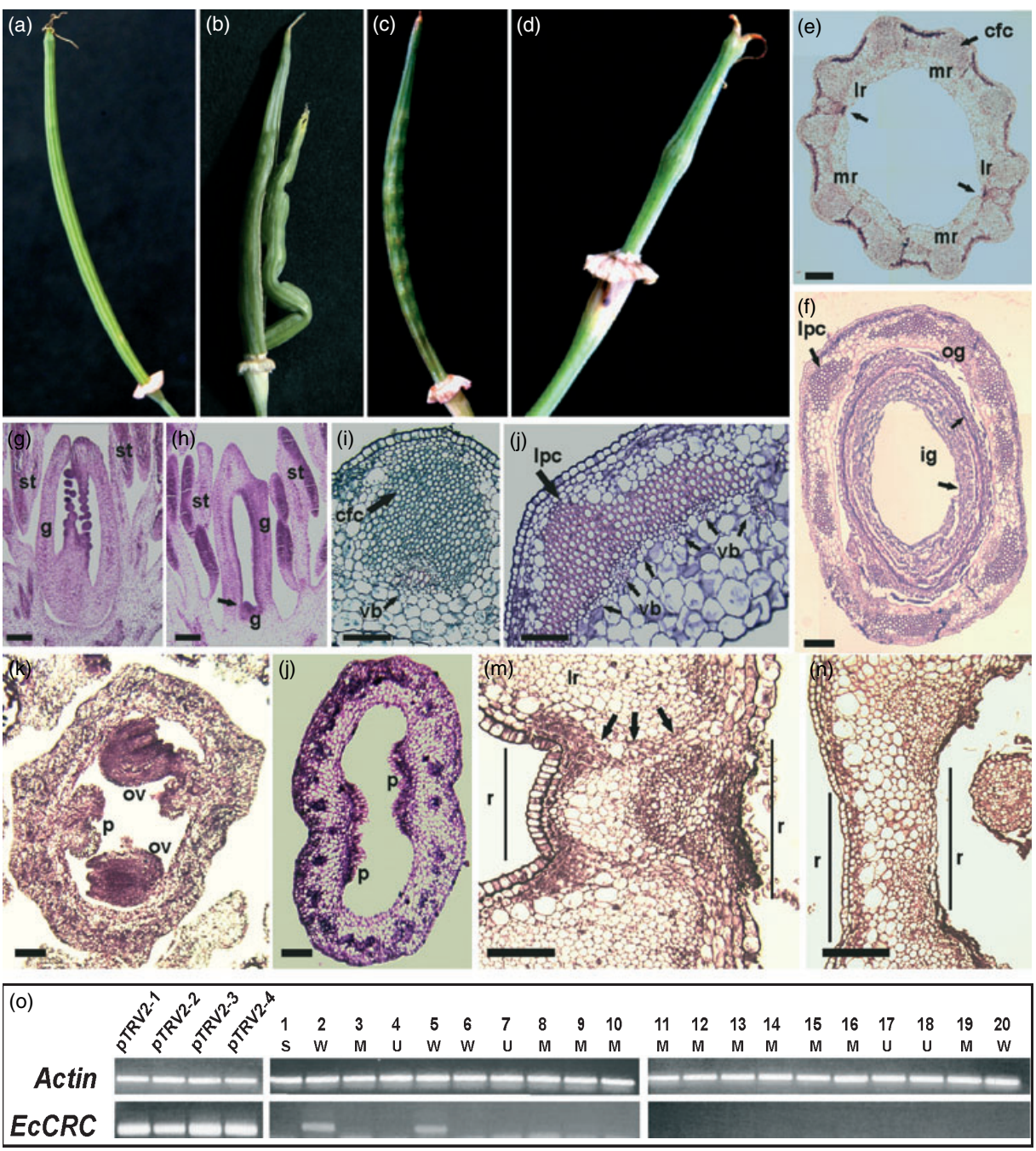
EcCRC expression is required for the elaboration of the abaxial ovary wall

In the late developmental stages of the flower (stage 9), 10 ridges develop at the abaxial surface of the gynoecium, five for each valve, distributed into three medial and two lateral

Figure 3. Phenotype of the *EcCRC*-VIGS plants.
(a) Wild-type fruit of *Eschscholzia californica* (10-cm long, containing 100–120 seeds).
(b) A mild *EcCRC*-VIGS phenotype, showing an apparently normally developed fruit (7.5-cm long) enclosing a second, fully differentiated inner fruit.
(c) A severe phenotype of *EcCRC*-VIGS fruit that is reduced both in length (7.5 cm) and in width, with a highly reduced seed number. Nine seeds are bulging out of the fruit in (c), and only one seed is present in the 1.7-cm long fruit in (d).
(e) Transverse section of wild-type fruit showing medial and lateral ridges protruding from the ovary. Black arrows indicate the replum region from which the seeds have been removed.
(f) Transverse section of an *EcCRC*-VIGS fruit with two additional tissue layers within the outer gynoecium (arrows).
(g) Longitudinal section of a wild-type gynoecium at stage 7, with developing ovules within the gynoecium.
(h) Longitudinal section of an *EcCRC*-VIGS gynoecium at stage 7, showing an active meristem (indicated by an arrow).
(i) Transverse section of a wild-type fruit in the medial ridge region, showing cellulose-fortified cells (green staining), and a single vascular bundle, indicated by an arrow.
(j) Transverse section of an *EcCRC*-VIGS fruit with lignified parenchyma cells (pink staining) and several vascular bundles (arrows).
(k) Transverse section of a wild-type gynoecium showing developing ovules extending from the placenta into the cavity of the gynoecium.
(l) Transverse section of *EcCRC*-VIGS gynoecium lacking the proper development of placental tissue and ovules.
(m) Transverse sections of a wild-type fruit illustrating the lateral ridges embedding the replum region. The arrows mark a file of heavily lignified cells, presumably involved in valve dehiscence.
(n) Transverse section of a mild *EcCRC*-VIGS phenotype fruit that demonstrates a disrupted differentiation of the replum region.
(o) RT-PCR showing the expression of *EcCRC* in young buds, the negative control plants pTRV2-1 to pTRV2-4 have been treated with pTRV1 and pTRV2-E; plants 1–20 were treated with *EcCRC1*-VIGS constructs. Plants showing a severe silencing phenotype are marked with 'S', those with a mild silencing phenotype are marked with 'M', 'U' designates the unknown phenotype, and 'W' refers to the wild-type-like phenotype. Actin was used to normalize the experiment. Abbreviations: cfc, cellulose-fortified cells; g, gynoecium; ig, inner gynoecium; lpc, lignified parenchymatic cells; lr, lateral ridges; mr, middle ridges; og, outer gynoecium; ov, ovules; p, placental tissue; r, replum; st, stamen; vb, vascular bundle. Scale bars: 100 μm.

ridges; the lateral ones are situated next to the replum. The ridges consist of parenchyma cells lined with thick cellulose deposits (collenchymas cells) arranged in an approximately circular manner surrounded by large, irregularly shaped parenchyma cells. The arrangement of collenchyma cells merges adaxially with the vascular bundles, and is abaxially covered with a layer of subepidermal cells (Figure 3i). Strong *EcCRC*-VIGS phenotypes lack the characteristic ridges completely, and show an irregularly spaced array of large patches of lignified cells; however, the subepidermal and epidermal cell layers are not affected (Figure 3f,j). Instead of collenchyma cells in untreated plants, lignified cell walls are found in *EcCRC*-silenced fruits. The localization of the vascular bundles is now oriented towards patches of lignified cells, and several vascular bundles are associated with one patch of lignified cells (Figure 3i,j).

The strong *EcCRC*-VIGS phenotype in Figure 3f shows two additional fruits that emerged as concentric whorls within the outer fruit. These additional whorls appear as two layers of parenchyma cells, without any obvious vascular



bundles or cells with specially fortified cell walls. Adaxial/abaxial tissue differentiation, ridge and replum formation is also absent. This suggests that the ectopic inner fourth whorl organs in the strong *EcCRC*-VIGS phenotypes emerge without adaxial/abaxial and central/lateral polarity.

Taken together, our results indicate that *EcCRC* is necessary for abaxial ridge formation, proper spacing of vascular bundles and the deposition of cellulose in the specialized parenchyma cells of *Eschscholzia* fruits.

EcCRC function is required for ovule initiation

In the developing *Eschscholzia* gynoecia, ovules emerge from two placental tissue strands adaxial to the replum region. The placenta consists of two tissue protrusions, and is covered with a loose array of large club-shaped cells (Figure 3k). The gynoecia of *EcCRC*-VIGS plants showed a strong reduction in seed set, and in some fruits only one seed was produced (Figure 3c, d). We were interested if the reduced seed set was the result of impaired pollination, or the result of a failure of the gynoecium to produce ovules. Transverse histological sections were made of gynoecia of untreated plants in late stage 8, when the ovules have already initiated (Figure 3k), and relatively mild phenotypes of *EcCRC*-VIGS (no double gynoecium) plants of the same stage (Figure 3l). These sections show that the characteristic club-shaped cells are present, but that the placental protrusions are reduced. However, ovules are absent in *EcCRC*-VIGS plants. We were unable to find remnants of aborted ovules in these gynoecia, indicating that ovules, once initiated, will develop fully. Our results indicate that *EcCRC* function is important for ovule initiation.

Replum formation is impaired in EcCRC-VIGS fruits

In ripening fruits at stage 12, two replum regions differentiate between the lateral ridges of the carpels, allowing explosive dehiscence of the two valves to catapult the seeds away. The replum consists of cells markedly smaller in size than neighboring valve cells, some of which are strongly lignified. The replum region is narrower than the valve region of the ovary wall, and the epidermis is shaped like a W facing in the abaxial direction. Heavily lignified cells are located at the base of this W, emanating from a narrow band of lignified cells towards the adaxial side of the replum, which possibly marks the breaking point between the replum and the valve (Figure 3m). Mild *EcCRC*-VIGS phenotypes exhibit a narrow region in the ovary, reminiscent of a replum structure (Figure 3n), and are completely lacking in strong phenotypes (Figure 3f). However, transverse sections of mild phenotypes show a lack of lignified cells and a loss of the characteristic W-shaped indentation of the abaxial ovary wall in the replum region (Figure 3n). Even the fruits of the mild phenotypes have lost their explosive valve dehiscence completely, and need to be opened manually. Our results suggest that *EcCRC* function is necessary throughout gynoecium development, and that *EcCRC* is involved in a wide variety of developmental processes comprising floral meristem termination, longitudinal and radial growth of the gynoecium, ovule initiation, elaboration of the adaxial ovary wall, and replum formation.

Discussion

The molecular mechanisms underlying carpel development have been studied in a number of highly derived species, like the eudicots Arabidopsis and petunia, and in the grass species rice. However, extensive information on organ patterning and tissue differentiation is only available for Arabidopsis (e.g. Sessions *et al.*, 1997; Heisler *et al.*, 2001; Alvarez and Smyth, 2002; Pekker *et al.*, 2005; Sohlberg *et al.*, 2006). The current study aims to dissect the function of one of the key genes involved in carpel development in an evolutionary context. The organization of the *Eschscholzia* gynoecium is to a large extent similar to that of Arabidopsis (Becker *et al.*, 2005). However, the results presented in this work show that the molecular mechanisms governing gynoecium morphogenesis and ovule initiation between superficially rather similar structures, can follow quite different pathways. Moreover, our analysis reveals a complex history of several gains of gene function during the evolution of *CRC*-like genes. Figure 4 schematically summarizes our hypothesis about the history of *CRC*-like gene function acquisition.

Specification of gynoecium abaxial cell identity by *CRC*-like genes

The *Eschscholzia* ovary wall shows clear differentiation along the adaxial/abaxial axis, with prominent cellulose-fortified ridges bulging out of the gynoecium surface (Figure 3e). A loss of *EcCRC* function clearly reduces these abaxial ovary wall elaborations, leading to a complete loss of the ridge structure, and a loss of the regularity in the arrangement of vascular bundles associated with these ridges. Interestingly, the reduction of abaxial cell types did not result in an adaxialization of the gynoecium wall, as a proper epidermis is formed, and the large and highly vacuolized cells usually found on the adaxial side of the ovary wall are not found in the abaxial parts of *EcCRC*-silenced plants (Figure 3f).

All core eudicots for which expression data exist, and the early diverging angiosperm *A. trichopoda*, show *CRC*-like gene expression in the abaxial domain of the gynoecium or carpel (Bowman and Smyth, 1999; Fourquin *et al.*, 2005; Lee *et al.*, 2005b). However, the loss of *CRC* activity in Arabidopsis alone does not lead to obvious adaxial/abaxial polarity defects in the ovary wall, and the cell layers develop

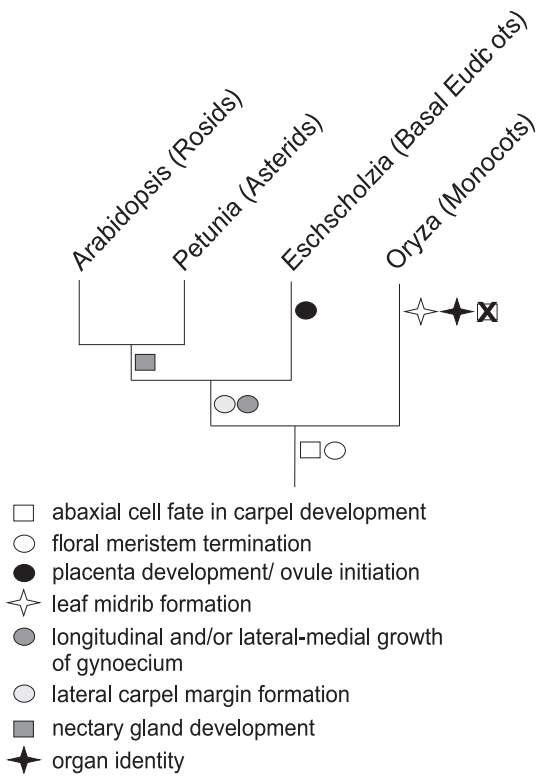


Figure 4. Schematic drawing mapping the history of the gene function acquisitions of *CRC*-like genes.
A simplified phylogeny of the major clades of angiosperms, indicating our theory of the latest time point of the proposed gains/losses of *CRC*-like gene functions during the evolution of flowering plants. The order of the respective symbols on an individual branch does not reflect the order of appearance of the gene function acquisitions/losses. The open circle represents a function in floral meristem termination. An open box indicates the promotion of abaxial cell fate during carpel development. A white star represents a function in the specification of carpel organ identity, and a black star symbolizes a function in leaf midrib formation. A dark-gray circle indicates a function in lateral carpel margin formation; a light-gray circle represents the promotion of longitudinal and/or lateral–medial growth of the gynoecium. Involvement in placenta development and ovule initiation is shown by a black circle. The recruitment of *CRC*-like genes for directing nectary gland development is represented by a light-gray box. The putative loss of function is indicated by the corresponding symbols that are crossed out.

normally, but show earlier vascular differentiation (Eshed *et al.*, 1999; Alvarez and Smyth, 2002). Alvarez and Smyth (2002) argue that earlier vascular differentiation and the observed larger cell sizes might reflect a partial loss of carpel identity, and an acquisition of more sepal- or leaf-like characteristics. This could also be the case in *Eschscholzia*, as *EcCRC* seems to restrict the number and size of vascular bundles, and organizes its association with abaxial ridge structures.

In contrast to the expression pattern detected in dicots, *DL* transcripts are not confined to the abaxial side of the carpel at any developmental stage (Yamaguchi *et al.*, 2004). The lack of abaxial/adaxial differentiation in *DL* expression might represent a gain of adaxial function along the monocot lineage.

The abaxial expression domain of *CRC*-like genes in dicots and *Amborella* is in accordance with the function of other YABBY genes that are all involved in the abaxial cell identity of lateral organs (Eshed *et al.*, 2004). As functional data for *AmbCRC* are not available, we cannot exclude the possibility that no function is assigned to the polar expression pattern of *AmbCRC*. However, as members of the eudicots also share this expression domain, and as a corresponding function was demonstrated for Arabidopsis, and now for *Eschscholzia*, it is very likely that one function of the ancestral *CRC*-like gene is the specification of abaxial cell identity in the gynoecium.

CRC-like genes are involved in floral meristem termination

The *EcCRC*-VIGS plants show the formation of multiple gynoecia in the centre of the flower nested within each other, reminiscent of Russian matrioshka dolls. This striking phenotype indicates that the activity of the floral meristem is prolonged. The strong *crc-1* mutant of Arabidopsis also shows effects in floral meristem termination, albeit only in combination with the *ag+/–* mutant, but another inner whorl of the gynoecia, however, has not been observed in any of the *crc* mutant alleles (Alvarez and Smyth, 1999, 2002).

In Arabidopsis, the floral homeotic class-C gene *AG* is the key regulator responsible for the limitation of stem-cell proliferation in the flower, and *ag* mutants show an indeterminate appearance among other defects in floral morphology. This is because in later stages of flower development *AG*, in addition to an unknown factor, represses the transcription of *WUSCHEL* (*WUS*), a gene specifying stem-cell identity, which leads to the depletion of the stem-cell population in the floral meristem (Mizukami and Ma, 1997; Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). Recently, it has been shown that three genes, *REBELOTE*, *SQUINT* and *ULTRAPETALA* act as modifiers of *CRC* action, and combinations of mutants of these genes show extreme defects in floral meristem termination. These effects are partially the result of a reduction in *AG* expression in the population of cells that is responsible for floral meristem termination (Prunet *et al.*, 2008).

In the monocot rice, the regulation of floral meristem termination involves the class-C gene *OsMADS58*, as well as the *CRC* ortholog *DROOPING LEAF* (*DL*). Plants that are RNA-silenced for *osmads58* show a dramatic loss in floral meristem determinacy, resulting in indeterminate flowers consisting of lodicules, stamens and carpel-like structures. Strong *dl* mutants, however, also show serious defects in floral meristem determinacy, and produce additional ectopic stamens instead of a central carpel, indicating a role for *DL* in

carpel organ identity (Yamaguchi *et al.*, 2006). Presently, the most parsimonious evolutionary path would indicate that the floral meristem termination function of *CRC*-like genes evolved once before the split of the monocot and eudicot lineage, and was maintained in both lineages. However, in the lineage leading to Arabidopsis, additional genes have been recruited to act redundantly to *CRC*, which might indicate a tendency towards a more pronounced homeostasis in the important developmental process of floral meristem termination.

Several functions of *CRC*-like genes are specific to certain angiosperm lineages

Based on functional studies and expression analysis in a phylogenetic context, it becomes apparent that several functions of *CRC*-like genes have been recruited in specific lineages only.

(i) Many representatives of early diverging angiosperm (including *Aquilegia*), monocot and dicot species grow nectaries to ensure maximum pollination success. However, only the core eudicots have recruited *CRC*-like genes for nectary development. Several rosids and asterids have been tested for expression of *CRC*-like genes in nectaries, and it has been demonstrated that flower associated nectaries and extrafloral nectaries express *CRC*-like genes. Nectaries are absent in Arabidopsis, petunia and tobacco if *CRC*-like genes are downregulated. However, nectary development in species outside the core eudicots is not related to *CRC*-like gene expression (Lee *et al.*, 2005b). The California poppy does not develop nectaries (Becker *et al.*, 2005).

(ii) *CRC*-like genes in monocots were recruited for additional functions other than the ones observed in dicots. The *dl* mutant alleles from rice reveal that the *CRC* orthologs in at least part of the lineage leading to grasses have gained specific functions not found in dicots or early diverging angiosperms. *DL* has an important function in the differentiation of the leaf midrib, as *dl* mutants show a strongly reduced mechanical stability of the leaf, resulting in the 'drooping leaf' phenotype.

(iii) The other major function of *DL* in specifying carpel organ identity is not observed to a similar extent in *Eschscholzia*, Arabidopsis or tobacco. However, two mutants of other grass species (*Pennisetum americanum* and *Panicum aestivum*) have also been reported to exhibit the same phenotype combination as *dl* (Yamaguchi *et al.*, 2004).

Non-cell autonomous actions of *CRC*-like genes in carpel margin differentiation

Another feature of the *EcCRC*-VIGS phenotype related to a loss of adaxial/abaxial polarity, is a reduced seed set, most likely caused by the loss of placental tissue differentiation, entailing disrupted ovule initiation. *EcCRC*-VIGS plants lack the characteristic outgrowth of the placentae and only produce ovules in low numbers. An additional characteristic of the *EcCRC*-VIGS phenotype concerning the carpel margins is a severely reduced differentiation of the replum, resulting in fruits that are unable to dehisce. Interestingly, *EcCRC* expression is absent from the placenta and the replum region (Figure 2c, f, g, h). Moreover, not only adaxial but also abaxial tissue differentiation of the carpel margins is affected in *EcCRC*-silenced plants (Figure 3f, k, l). The strong Arabidopsis *crc-1* mutant shows only a mildly affected replum region, and is apparently capable of normal seed dispersal. Also, placenta development and ovule initiation do not seem to be altered, and the reduced seed set is more likely to result from the reduced longitudinal growth of the pollen tubes in *crc-1* gynoecia (Bowman and Smyth, 1999; Alvarez and Smyth, 2002). Thus, the function of *EcCRC* in carpel marginal tissue development is more pronounced than that of *CRC* in Arabidopsis, possibly because of the recruitment of redundantly acting genes. Our data demonstrate that *EcCRC* is necessary for the differentiation of all tissue types originating from the carpel margins, such as placenta, replum and ovules.

Eschscholzia and Arabidopsis are phylogenetically quite distant from each other, and their bicarpellate syncarpous gynoecium architecture is remarkably similar, even though they evolved independently of each other from an apocarpous ancestor (Endress and Igersheim, 1999; Armbruster *et al.*, 2002; Magallon, 2007). However, the functions of *CRC* and *EcCRC* are similar to some extent: the development of tissues derived from the carpel margins is promoted in both species. The most parsimonious explanation for the similarities between the *Eschscholzia* and Arabidopsis *CRC*-like gene function would be that *CRC*-like gene function also promotes carpel margin differentiation in the apocarpous ancestral gynoecium of the eudicot lineage.

How *EcCRC* directs the differentiation of the carpel margins without being expressed there is still to be explained. One possibility is that *EcCRC* promotes carpel lateral domain identity, and inhibits placenta and ovule formation on the abaxial side of the valve margins. This has been shown for the Arabidopsis *CRC* in combination with *GYMNOS* (*GYM*) or *KANADI* (*KAN*). The *crc*, *gym* and *kan* single mutants do not show ectopic ovules on the abaxial side of the ovary wall. However, if *crc* is combined with either *gym* or *kan*, ectopic ovules are observed that develop on the abaxial side of the carpel margins, as a result of the duplication of adaxial tissue types on the abaxial side of the carpel (Eshed *et al.*, 1999). Whether *EcCRC* also acts in combination with orthologs of *GYM* or *KAN* is not known, but could be examined through simultaneous knock-down of *EcCRC* with orthologs of either gene. Another way by which *EcCRC* may influence

replum and placenta differentiation would be a direct or indirect activation of the genes responsible for placenta and replum identity, possibly by providing the valves with the competence to support medial and lateral tissue formation. This hypothesis could account for the significant reduction in placenta and replum development, as well as ovule number, in *EcCRC*-VIGS plants.

YABBY gene duplication and functional diversification at the base of the angiosperm lineage could indicate an important role for YABBY genes in angiosperm evolution. In particular, the establishment of *CRC*-like genes, which are key developmental regulators for the carpel, an autapomorphy of the angiosperms, might have contributed to the evolution of the carpel itself: apparently *CRC*-like genes are involved in promoting the formation of carpel marginal tissue, including the placenta. If one thinks of the carpel as a modified leaf, as Goethe proposed more than 200 years ago (Goethe, 1790), it is the marginal tissue differentiation supporting the placenta, and subsequently the ovules, that accounts for the major difference between the leaf and the carpel.

EXPERIMENTAL PROCEDURES

Cloning of *EcCRC* and phylogenetic analysis

The *EcCRC* gene was isolated using a combination of 3′ and 5′ RACE PCR (Frohmann *et al.*, 1988). Total RNA was isolated from California poppy buds using the RNeasy Plant Kit (Qiagen, http://www.qiagen.com). A 4-μg portion of RNA was reverse transcribed with the Omniscript Kit (Qiagen) using the poly-T anchor primer AB05. A PCR with primers ABCRC06 and the 3′ RACE adapter primer AB07 yielded the 3′ region of the *EcCRC* coding sequence. We then amplified the missing portion of *EcCRC* with 5′ RACE using 2 μg of RNA isolated from buds as a template. The first-strand synthesis was performed with the primer EcCRC5R1 using the Omniscript kit (Qiagen), and a poly-A tail was added to the cDNAs using the NEB terminal transferase (New England BioLabs, http://www.neb.com), following the manufacturer's protocol. Two rounds of nested PCR were performed, using the primers AB05/EcCRC5R2 for the first PCR and the primers AB07/EcCRC5R3 for the second PCR.

The nucleotide sequence has been deposited in the EBI database (acc. no. AM946412). Nucleotide sequences of *CRC* homologs from various other species were kindly provided by John L. Bowman (Lee *et al.*, 2005b). Deduced amino acids were aligned with M-COFFEE (Wallace *et al.*, 2006; Moretti *et al.*, 2007), and were manually adjusted using BIOEDIT (Hall, 1999). Bayesian analysis was performed with MrBAYES 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), according to the general-time-reversal model, with a gamma distribution of site substitution rates and a proportion of invariable sites (GTR + G + I), examined by MrMODELTEST 2.2 (Nylander, 2004).

Expression analysis of *EcCRC* by RT-PCR and *in situ* hybridization

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen), and 1 μg of total RNA was reverse transcribed into cDNA with the SuperScript III Kit (Invitrogen, http://www.invitrogen.com). As an endogenous control for the RT-PCR, the *E. californica* expressed sequence tag (EST) sequence (NCBI accession: CD476630) closest to the Arabidopsis gene *Actin2* was chosen. A total of 35 PCR amplification cycles were used for each RT-PCR, and the expected size of the amplified products was 191 bp for *Actin2* (primer combination: actin2RTQfw/actin2RTQrev) and 192 bp for *EcCRC* (primer combination: eccrcRTQfw/eccrcRTQrev). The primer sequences of this study are listed in Table S1. For the *EcCRC*-VIGS plants, the total RNA of the very first bud (0–3 mm in diameter) was isolated using the RNeasy Micro Kit (Qiagen). As a negative control, we used the first buds of plants inoculated with pTRV1 and the empty pTRV2 (pTRV2-E).

Non-radioactive *in situ* hybridization essentially followed the protocol of Groot *et al.* (2005). The *EcCRC* coding sequence was cloned into the pDrive vector (Qiagen), and digoxigenin-labeled RNA probes were transcribed using T7 RNA polymerase (Roche, http://www.roche.com). A concentration of 5 $\mu g\ \mu l^{-1}$ of Proteinase K was used for treating the tissue before hybridization.

Vector construction and plant inoculation

For the VIGS of *EcCRC*, we amplified a 557-bp fragment containing the major portion of the *EcCRC* open reading frame with a 3′ *Eco*RI and a 5′ *Bam*HI restriction site. The resulting fragment was then cloned into the pTRV2 vector, creating pTRV2-EcCRC1. Additionally, an alternative version of pTRV2-EcCRC1 was produced to exclude the possibility that the observed phenotypes are dependent on the location of the fragment used to silence the *EcCRC* gene. This second fragment of 445 bp, encompassing the 3′ part of the *EcCRC* coding sequence and the 3′ untranslated region (UTR), but excluding the 5′ region of the *EcCRC* coding sequence, was cloned in the same way to produce the vector pTRV2-*EcCRC2*. pTRV2-E is the empty vector and was used as negative control.

The pTRV2-EcCRC1 and pTRV2-EcCRC2 vectors were transformed separately into *Agrobacterium tumefaciens* strain GV3101. The infiltration of *A. tumefaciens* was essentially performed as described previously (Wege *et al.*, 2007), except that 100–150 μl of the combined *A. tumefaciens* strains suspension, containing pTRV1 and pTRV2-EcCRC1 or pTRV2-EcCRC2 plasmids, was injected into the shoot by inserting the 0.45 × 25-mm needle of a 2-ml syringe vertically into the apicalmost region of 3-week-old plants, taking care not to destroy the shoot apical meristem (SAM). The plants were grown under conditions described previously (Wege *et al.*, 2007), and flowers were cross-pollinated by hand to ensure the maximum possible seed set.

Histology and light microscopy

Fresh buds (> 3 mm in diameter) and fruits (> 3 cm in diameter) of untreated and *EcCRC*-VIGS plants were fixed in FAE (3% formaldehyde, 5% acetic acid, 60% ethanol) and embedded in Paraplast Plus (Tyco Healthcare, http://www.tyco.com). Microtome sections of 7 μm thickness were stained with Safranin-O (Carl Roth, http://www.carlroth.com) for 24 h and counterstained with alcoholic Fast-Green (Chroma, http://www.chroma.com) solutions for 3 min.

ACKNOWLEDGEMENTS

We are indebted to David R. Smyth, Günter Theißen and Sinead Drea for their helpful comments and discussions on the manuscript. We also thank Werner Vogel and Angelika Trambacz for their help

with growing the poppy plants. This work was made possible with funding from the German Research Foundation (DFG) grant: BE 2547/6-1 (to AB).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Percentages of *EcCRC*-VIGS phenotypes.

Table S1. List of oligonucleotide sequences.

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REFERENCES

Alvarez, J. and Smyth, D.R. (1999) CRABS CLAW and SPATULA, two Arabidopsis genes that control carpel development in parallel with AGAMOUS. *Development*, **126**, 2377–2386.

Alvarez, J. and Smyth, D.R. (2002) CRABS CLAW and SPATULA genes regulate growth and pattern formation during Gynoecium development in Arabidopsis thaliana. *Int. J. Plant Sci.* **163**, 17–41.

Angiosperm Phylogeny Group (2003) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Bot. J. Linn. Soc.* **141**, 399–436.

Armbruster, W.S., Debevec, E.M. and Willson, M.F. (2002) Evolution of syncarpy in angiosperms: theoretical and phylogenetic analyses of the effects of carpel fusion on offspring quantity and quality. *J. Evol. Biol.* **15**, 657–672.

Becker, A., Gleissberg, S. and Smyth, D.R. (2005) Floral and vegetative morphogenesis in California poppy (*Eschscholzia californica* CHAM.). *Int. J. Plant Sci.* **166**, 537–555.

Bowman, J.L. and Smyth, D.R. (1999) CRABS CLAW, a gene that regulates carpel and nectary development in Arabidopsis, encodes a novel protein with zinc finger and helix-loop-helix domains. *Development*, **126**, 2387–2396.

Crane, P.R., Friis, E.M. and Pedersen, K.R. (1995) The origin and early diversification of angiosperms. *Nature*, **374**, 27–33.

Dilcher, D. (2000) Toward a new synthesis: major evolutionary trends in the angiosperm fossil record. *Proc. Natl Acad. Sci.* **97**, 7030–7036.

Endress, P.K. and Igersheim, A. (1999) Gynoecium diversity and systematics of the basal eudicots. *Bot. J. Linn. Soc.* **130**, 305–393.

Eshed, Y., Baum, S.F. and Bowman, J.L. (1999) Distinct mechanisms promote polarity establishment in Carpels of Arabidopsis. *Cell*, **99**, 199–209.

Eshed, Y., Izhaki, A., Baum, S.F., Floyd, S.K. and Bowman, J.L. (2004) Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. *Development*, **131**, 2997–3006.

Fourquin, C., Vinauger-Douard, M., Fogliani, B., Dumas, C. and Scutt, C.P. (2005) Evidence that CRABS CLAW and TOUSLED have conserved their roles in carpel development since the ancestor of the extant angiosperms. *Proc. Natl Acad. Sci.* **102**, 4649–4654.

Frohmann, M., Dush, M. and Martin, G. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl Acad. Sci.* **85**, 8998–9002.

Goethe, J.W. (1790) *Versuch, die Metamorphose der Pflanzen zu erklären*. Gotha: Ettinger.

Groot, E.P., Sinha, N. and Gleissberg, S. (2005) Expression patterns of STM-like KNOX and Histone H4 genes in shoot development of the dissected-leaved basal eudicot plants Chelidonium majus and Eschscholzia californica (Papaveraceae). *Plant Mol. Biol.* **58**, 317–331.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **41**, 95–98.

Heisler, M.G., Atkinson, A., Bylstra, Y.H., Walsh, R. and Smyth, D.R. (2001) SPATULA, a gene that controls development of carpel margin tissues in Arabidopsis, encodes a bHLH protein. *Development*, **128**, 1089–1098.

Hoot, S.B., Kadereit, J.W., Blattner, F.R., Jork, K.B., Schwarzbach, A.E. and Crane, P.R. (1997) Data Congruence and Phylogeny of the Papaveraceae s.l. Based on four data sets: atpB and rbcL sequences, trnK restriction sites, and morphological characters. *Syst. Bot.* **22**, 575–590.

Huelsenbeck, J.P. and Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754–755.

Itoh, J.-I., Nonomura, K.-I., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H., Kitano, H. and Nagato, Y. (2005) Rice plant development: from Zygote to Spikelet. *Plant Cell Physiol.* **46**, 23–47.

Lee, J.-Y., Baum, S.F., Alvarez, J., Patel, A., Chitwood, D.H. and Bowman, J.L. (2005a) Activation of CRABS CLAW in the Nectaries and Carpels of Arabidopsis. *Plant Cell*, **17**, 25–36.

Lee, J.-Y., Baum, S.F., Oh, S.-H., Jiang, C.-Z., Chen, J.-C. and Bowman, J.L. (2005b) Recruitment of CRABS CLAW to promote nectary development within the eudicot clade. *Development*, **132**, 5021–5032.

Lenhard, M., Bohnert, A., Jurgens, G. and Laux, T. (2001) Termination of stem cell maintenance in Arabidopsis floral meristems by interactions between WUSCHEL and AGAMOUS. *Cell*, **105**, 805–814.

Lohmann, J.U., Hong, R.L., Hobe, M., Busch, M.A., Parcy, F., Simon, R. and Weigel, D. (2001) A molecular link between stem cell regulation and floral patterning in Arabidopsis. *Cell*, **105**, 793–803.

Magallon, S. (2007) From fossils to molecules: phylogeny and the core eudicot floral groundplan in Hamamelidoideae (Hamamelidaceae, Saxifragales). *Syst. Bot.* **32**, 317–347.

Mizukami, Y. and Ma, H. (1997) Determination of Arabidopsis floral meristem identity by AGAMOUS. *Plant Cell*, **9**, 393–408.

Moretti, S., Armougom, F., Wallace, I.M., Higgins, D.G., Jongeneel, C.V. and Notredame, C. (2007) The M-Coffee web server: a meta-method for computing multiple sequence alignments by combining alternative alignment methods. *Nucl. Acids Res.* **35**, W645–648.

Nylander, J.A.A. (2004) *MrModeltest v2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Pekker, I., Alvarez, J.P. and Eshed, Y. (2005) Auxin response factors mediate Arabidopsis organ asymmetry via modulation of KANADI activity. *Plant Cell*, **17**, 2899–2910.

Prunet, N., Morel, P., Thierry, A.-M., Eshed, Y., Bowman, J.L., Negrutiu, I. and Trehin, C. (2008) REBELOTE, SQUINT, and ULTRAPETALA1 function redundantly in the temporal regulation of floral meristem termination in Arabidopsis thaliana. *Plant Cell*, **20**, 901–919.

Ronquist, F. and Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Sessions, A., Nemhauser, J.L., McColl, A., Roe, J.L., Feldmann, K.A. and Zambryski, P.C. (1997) ETTIN patterns the Arabidopsis floral meristem and reproductive organs. *Development*, **124**, 4481–4491.

Sohlberg, J.J., Myrenas, M., Kuusk, S., Lagercrantz, U., Kowalczyk, M., Sandberg, G. and Sundberg, E. (2006) STY1 regulates auxin homeostasis and affects apical-basal patterning of the Arabidopsis gynoecium. *Plant J.* **47**, 112–123.

Soltis, D.E., Soltis, P.S., Chase, M.W. *et al.* (2000) Angiosperm phylogeny inferred from 18S rDNA, rbcL, and atpB sequences. *Bot. J. Linn. Soc.* **133**, 381–461.

Wallace, I.M., O'Sullivan, O., Higgins, D.G. and Notredame, C. (2006) M-Coffee: combining multiple sequence alignment methods with T-Coffee. *Nucl. Acids Res.* **34**, 1692–1699.

Wege, S., Scholz, A., Gleissberg, S. and Becker, A. (2007) Highly efficient virus-induced gene silencing (VIGS) in California Poppy (Eschscholzia californica): an evaluation of VIGS as a strategy to obtain functional data from non-model plants. *Ann Bot*, **100**, 641–649.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y. and Hirano, H.-Y. (2004) The YABBY Gene DROOPING LEAF regulates carpel specification and midrib development in Oryza sativa. *Plant Cell*, **16**, 500–509.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G. and Hirano, H.-Y. (2006) Functional diversification of the two C-Class MADS Box Genes OSMADS3 and OSMADS58 in Oryza sativa. *Plant Cell*, **18**, 15–28.

Accession number for EcCRC: AM946412.

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Floral homeotic C function genes repress specific B function genes in the carpel whorl of the basal eudicot California poppy (*Eschscholzia californica*)

Aravinda L Yellina[1], Svetlana Orashakova[1], Sabrina Lange[1], Robert Erdmann[1], Jim Leebens-Mack[2], Annette Becker[1*]

Abstract

Background: The floral homeotic C function gene *AGAMOUS* (*AG*) confers stamen and carpel identity and is involved in the regulation of floral meristem termination in *Arabidopsis*. *Arabidopsis ag* mutants show complete homeotic conversions of stamens into petals and carpels into sepals as well as indeterminacy of the floral meristem. Gene function analysis in model core eudicots and the monocots rice and maize suggest a conserved function for *AG* homologs in angiosperms. At the same time gene phylogenies reveal a complex history of gene duplications and repeated subfunctionalization of paralogs.

Results: *EScaAG1* and *EScaAG2*, duplicate *AG* homologs in the basal eudicot *Eschscholzia californica* show a high degree of similarity in sequence and expression, although *EScaAG2* expression is lower than *EScaAG1* expression. Functional studies employing virus-induced gene silencing (VIGS) demonstrate that knock down of *EScaAG1* and *2* function leads to homeotic conversion of stamens into petaloid structures and defects in floral meristem termination. However, carpels are transformed into petaloid organs rather than sepaloid structures. We also show that a reduction of *EScaAG1* and *EScaAG2* expression leads to significantly increased expression of a subset of floral homeotic B genes.

Conclusions: This work presents expression and functional analysis of the two basal eudicot *AG* homologs. The reduction of *EScaAG1* and *2* functions results in the change of stamen to petal identity and a transformation of the central whorl organ identity from carpel into petal identity. Petal identity requires the presence of the floral homeotic B function and our results show that the expression of a subset of B function genes extends into the central whorl when the C function is reduced. We propose a model for the evolution of B function regulation by C function suggesting that the mode of B function gene regulation found in *Eschscholzia* is ancestral and the C-independent regulation as found in *Arabidopsis* is evolutionarily derived.

Background

Flowers are complex structures composed of vegetative and reproductive organs that are arranged in concentric whorls in most angiosperms. The vegetative floral organs, the sepals and the petals, develop in the outer whorls while the inner whorls are composed of the pollen-bearing stamens and in the center carpels enclose the ovules. The carpels are the last organs formed in the flower and the floral meristem is consumed in the process of carpel development [1]. As described by the ABCDE model, floral homeotic transcription factors act in a combinatorial fashion to determine the organ identity primordia for the four distinct whorls: A + E class genes specify sepal identity; A + B + E class genes act together to determine petal identity; B + C + E class genes specify stamen identity; C + E class genes together define carpel identity, and C + D + E class genes specify ovule identity [2,3]. Most of these homeotic functions are performed by members of the MADS-box gene transcription factor family. *AGAMOUS (AG), a* C class gene in *Arabidopsis* is necessary for specification and development of stamen and carpals, and floral meristem determinacy [4]. The flowers of the strong *ag-1* mutant shows complete homeotic conversions of stamens into

* Correspondence: annette.becker@uni-bremen.de
[1]University of Bremen, Fachbereich 02 Biology/Chemistry, Evolutionary Developmental Genetics Group Leobener Str., UFT, 28359 Bremen, Germany
Full list of author information is available at the end of the article

petals and carpels into sepals and a recurrence of these perianth organs in a irregular phyllotaxy [5].

Members of the *AG* subfamily of MADS box genes have been identified in all major clades of seed plants but not in more basal, seed-free lineages indicating that the *AG* clade originated around 300 to 400 million years ago in the common ancestor of gymnosperms and angiosperms. In gymnosperm species, *AG* orthologs were found to be expressed in male and female reproductive cones, which is reminiscent of the angiosperm expression in stamens and carpels [6-8]. Gene family phylogenies reveal several duplication events within *AG* clade of MADS box genes (Figure 1 [9,10]). The first duplication event at the base of the angiosperm lineage led to the origins of the *SEEDSTICK* and *AG* clades including ovule specific D class genes and the carpel and stamen specifying C class genes, respectively [10]. A more recent duplication in the C-lineage gave rise to the *PLENA* clade and *euAG* clade, the former containing the Arabidopsis *SHATTERPROOF1* and *2* genes *(SHP1* and *2*), the latter *AG*. This duplication occurred after the ranunculids (basal eudicots in the order Ranunculales) diverged from the lineage leading to the core eudicots [9,11].

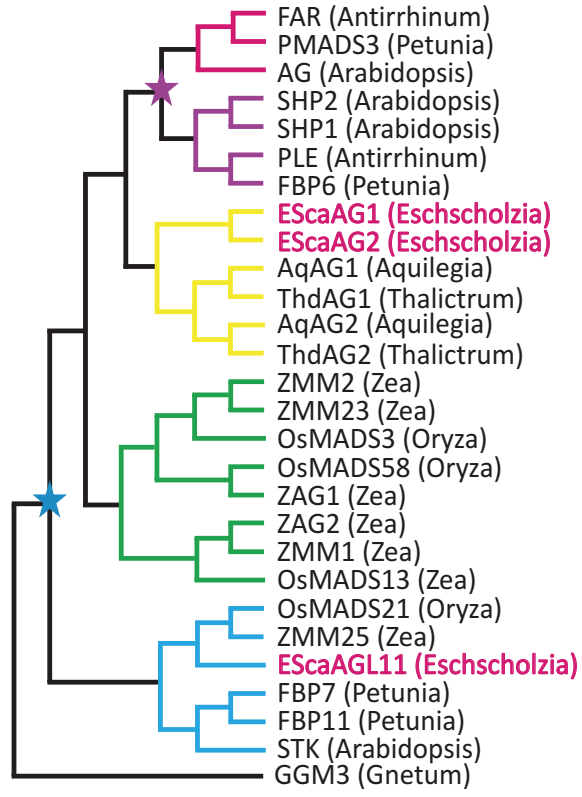


Figure 1 Simplified phylogeny indicating duplication events of the AG lineage in angiosperms based on Zahn *et al.*, 2006 [18]. Red branches denote euAG lineage genes, purple branches the PLE lineage genes, yellow branches symbolize the basal eudicot lineage, green branches denote the monocot C class genes and blue branches denote D class genes. *GGM3* represents the gymnosperm lineage of *AG* homologs. The California poppy genes are marked in red letters. The blue star symbolizes the C/D duplication event and the purple star indicates the EuAG/PLE duplication.

The Arabidopsis members of the *PLENA* clade, *SHP1* and *2* are required for dehiscence zone differentiation in the fruit and consequently for pod shattering [12,13]. Interestingly, *PLENA* itself, a gene in *Antirrhinum majus*, is functionally more similar to *AG* than *SHP1* and *2*, and *FARINELLI (FAR)*, the *Antirrhinum AG* ortholog is required for pollen development. Both *FAR* and *PLENA* are necessary for floral meristem determinacy in *Antirrhinum* [14,15].

Gene duplications and subfunctionalization have also occurred in C-lineage of monocots, but independently of the eudicot duplications (Figure 1). *ZAG1* from maize is required for floral meristem determinacy and *ZMM2* is involved in stamen and carpel identity [16]. The rice homologs *OSMADS3* and *OSMADS58* share common functions, but also show a degree of subfunctionalization. While *OSMADS3* plays a major role in stamen and a minor role in carpel identity, *OSMADS58* has a strong influence on carpel identity and floral meristem determination [17]. Independent duplications of *AG* homologs have been inferred for other flowering plant lineages, but functional analyses of duplicated AG homologs are sorely lacking outside of model core eudicot and grass species.

Here we report functional data of the *AG* homologs of the basal eudicot *Eschscholzia californica* (California poppy, Papaveraceae) that belongs to Ranunculales, a basal eudicot order. Basal eudicots are a sister grade leading to the more diverse core eudicot clade. Investigation of species in this grade can shed light on the divergence of monocots and eudicots and events that may have promoted diversification within the core eudicots.

Two *AG* homologs, *EScaAG1* and *EScaAG2*, and a D lineage homolog, *EScaAGL11* , have been identified in *E. californica*. *EScaAG1* and *EScaAG2* show similar expression patterns, but *EScaAG1* is expressed at a much higher level than *EScaAG*2 [18]. The expression patterns of both genes resembles that of *AGAMOUS* (*AG*) in *Arabidopsis* except that the Eschscholzia poppy *AG* orthologs are expressed earlier in the floral meristem [18,19].

This work presents an experimental investigation of the *EScaAG1* and *EScaAG2* gene function employing VIGS to manipulate transcript concentrations. We map the expression of both genes in more detail than previously published and demonstrate that the down regulation of C function genes in *E. californica* leads to an induction of some floral homeotic B genes in the fourth floral whorl.

Results

EScaAG1 and *EScaAG2* are very similar in sequence and expressed differentially

The two *AG* homologues of *E. californica*, *EScaAG1* and *EScaAG2*, share 66.6% and 61.1% amino acid sequence identity to *AG* of *Arabidopsis*, respectively. These paralogs are very similar throughout the open reading frame and in the 5'untranslated region (UTR) with 75% identity at the nucleotide level and about 81.7% at the amino acid level (Additional file 1). When the two paralogues are compared along their UTR and open reading frame, the *EScaAG*2 nucleotide sequence shows a 45 bp insertion and 14 bp deletion in the 5' UTR and a 10 bp deletion in the 3' part of coding region of *EScaAG1* (data not shown).

Quantitative Reverse Transcriptase (RT)-PCR was carried out on cDNA derived from floral organs at anthesis, young fruits, leaves, and buds of different developmental stages to learn more about the differential expression of *EScaAG1* and *EScaAG2* (Figure 2A). Both genes are expressed in the reproductive organs of the flower, in young fruits and in all tested stages of flower development. *EScaAG1* and *EScaAG2* are expressed in sepals, petals, and leaves at extremely low levels. *EScaAG1* is highly expressed in stamens, carpels, young fruits and later stages of flower development. *EScaAG2* is generally expressed at a lower level than *EScaAG1* with the exception of stamen, where its expression is about 1.5 × higher than that of *EScaAG1*. In young fruits and during bud development, *EScaAG2* transcript abundance is very low in comparison to *EScaAG1*.

The spatial expression patterns of *EScaAG1* and *2* were additionally analyzed through *in situ* hybridizations to obtain a more detailed picture of the expression domains. However, as the open reading frames and UTR's of *EScAG1* and *EScaAG2* are highly similar, we were unable to generate probes that could discriminate between both genes. As a consequence, *in situ* hybridization patterns were nearly identical for these genes. The only difference between the *in situ* hybridization patterns was a much lower level of expression for *EScaAG2* (data not shown). In the following section, we refer to the composite expression of *EScaAG1* and *EScaAG2* as *EScaAG1/*2 expression patterns.

EScaAG1/2 gene expression was first observed in the stage 2 bud before the gynoecium initiates and was visible as lateral domains in a few cells in the floral meristem where later the stamen primordia are initiated (Figure 2B). In a stage 4 bud, the expression expands uniformly in the floral meristem but is excluded from the central primordium where later the gynoecium arises (Figure 2C). By late stage 4, *EScaAG1/2* expression becomes restricted to the boundaries between the stamen anlagen with weak expression at the tip in the floral meristem just before gynoecium initiates (Figure 2D). In stage 6, strong expression is found in the region adjacent to the placenta, the apical part of the medial carpel wall and in the stamens (Figure 2E). Later in late stage 6, *EScaAG1/2* expression is restricted to the adaxial side of the gynoecium and in the stamens (Figure 2F). In transverse sections of the developing flower bud, *EScaAG* expression is confined to the apical part of the ovules but not in the placenta. In later stages of ovule development, the *EScaAG1/2* expression is stronger on the adaxial than on the abaxial side (Figure 2G, H, I). In summary, *EScaAG1/2* genes are expressed during floral meristem initiation at stage 2, during early development of stamen and carpel primordia and later in the developing stamens and ovules.

EScaAG1 and *EScaAG2* confer stamen identity

Virus induced gene silencing (VIGS) was employed to investigate the functions of *EScaAG1* and *EScaAG2* during flower development. This method allows transient down-regulation of gene expression via modified plant viruses, in our case the Tobacco Rattle Virus (TRV). The *E. californica* flower is composed of a single sepal occupying the first floral whorl, two whorls of four petals and a varying number of stamen whorls ranging from four to eight. The inner floral whorl produces a bicarpellate gynoecium (Figure 3A) [20]. Overall, the phenotypic effects of the *EScaAG1* and *EScaAG2* VIGS were restricted to flowers. Treatment plants exhibited a loss of stamen identity, homeotic conversion of stamens into petals, and a loss of carpel characteristics. Additionally, *EScaAG1* and *2* VIGS results in a loss of floral meristem termination. None of the analyzed pTRV2-E (mock treatment, treated with the empty pTRV2 vector) treated or untreated plants showed homeotic conversions or signs of loss of floral meristem termination, and the vegetative habit also did not show any deviations from untreated plants (Table 1 and [21]).

In total, 120 plants were infected with pTRV2-*EScaAG*1, 120 plants with pTRV2-*EScaAG*2, another 120 plants were inoculated with pTRV2-*EScaAG1/2*, and 12 plants were infected with pTRV2-E as a mock control. The first three flowers of each plant were analyzed because the frequency of phenotype decreases in the later formed flowers [21]. The phenotype scores for each treatment are summarized in Table 1: 239 flowers of plants infected with pTRV2-*EScaAG1* were analyzed, of which 122 flowers (51.0%) showed homeotic conversion in the third and fourth whorl floral organs. Of these 122 flowers, 4.5% showed homeotic conversion of all stamens into petal-like organs (Figure 3B). A total of 209 flowers of plants infected with pTRV2-*EScaAG*2 were observed, and of these 118 flowers (56.4%) showed

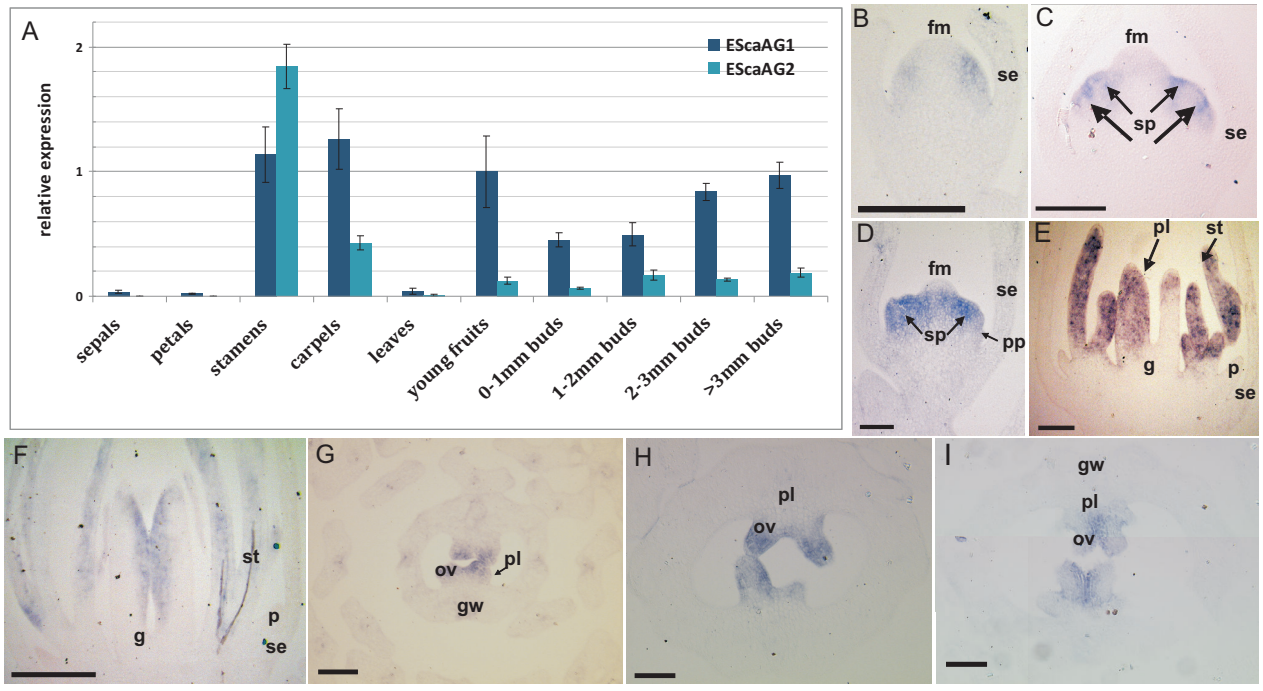


Figure 2 Expression analysis of *EScaAG1* and *EScaAG2* in flowers of untreated plants shown by quantitative RT-PCR and *in situ* hybridization. **(A)** Q-PCR based relative expression analysis of *EScaAG1* and *EScaAG2* in *E. californica*. *Actin* and *GAPDH* were used as reference genes. **(B)** to **(I)** *in situ* hybridization pattern of the *EScaAG* genes using an *EScaAG1* probe. **(B)** Longitudinal section of a bud in stage 2. **(C)** Longitudinal sections of a bud in stage 3. **(D)** Longitudinal sections of a bud in stage 4. **(E)** Longitudinal section of a bud in late stage 6. **(F)** Longitudinal section of a bud in stage 7. **(G)** Transverse section of a bud in stage 7. **(H)** Transverse section of a bud in stage 8. **(I)** Transverse section of a bud in 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.

homeotic transformation of stamens and carpels. Of all flowers developing a silencing related phenotype, 15% exhibited complete homeotic transformation of all stamens into petal-like organs (Figure 3C). Of the 261 flowers of plants infected with pTRV2-*EScaAG*1/AG2, 174 flowers (66.6%) showed homeotic transformation of stamens and carpels and 15% of the latter exhibited complete homeotic transformation of all stamens into petal-like organs (Figure 3D-H, Table 1).

Interestingly, *EScaAG1* and *2* VIGS-treated plants exhibited conversion of stamen to petaloid organs in different stamen whorls (Table 1). Focusing on plants infected with pTRV2-EScaAG1, 64 flowers (95.5% of the flowers with homeotic conversion in the third whorl) showed partial homeotic transformation of only the outer whorls of stamens into petaloid organs (Figure 3I), while the inner stamen whorls maintained a wild type appearance. In contrast, 45 flowers (84.9% of the flowers with homeotic conversions in the third whorl), from plants infected with pTRV2-EScaAG2 showed homeotic conversion of only the inner stamen whorls to petaloid organs (Figure 3J). Plants infected with pTRV2-EScaAG1/2 exhibited composite phenotypes: 96 flowers (84.9% of the flower with homeotic conversion in the third whorl) exhibited partial homeotic conversion of outermost and innermost whorls while retaining wild type stamen morphology in the central stamen whorls (Figure 3K). Homeotic transformations of stamens into petaloid organs occurred in various degrees as we observed phenotypes ranging from complete petal-like organs (Figure 3F) to mosaic staminoid-petaloid structures (Figure 3K).

Histological transverse sections of *EScaAG1* and *2* VIGS-treated plants reveal further details of the homeotic conversions of stamens and gynoecia (Figure 3L, M). In comparison to pTRV2-E treated plants (Figure 3M), the connective of the stamens in the silenced plants is elongated when compared to untreated plants and the theca contain three pollen sacs in a few cases rather than two as seen in untreated plants. The number of vascular bundles in the connective is also increased from one in untreated to five in stamens of VIGS treated plants. Additionally, the gynoecium in the center of the flower of VIGS-treated plants is composed of two fused parts reminiscent of petals. A solid ovary wall is missing in the VIGS-treated plants as well as lateral differentiation of the ovary wall, such as a placenta or ovules (Figure 3L, M).

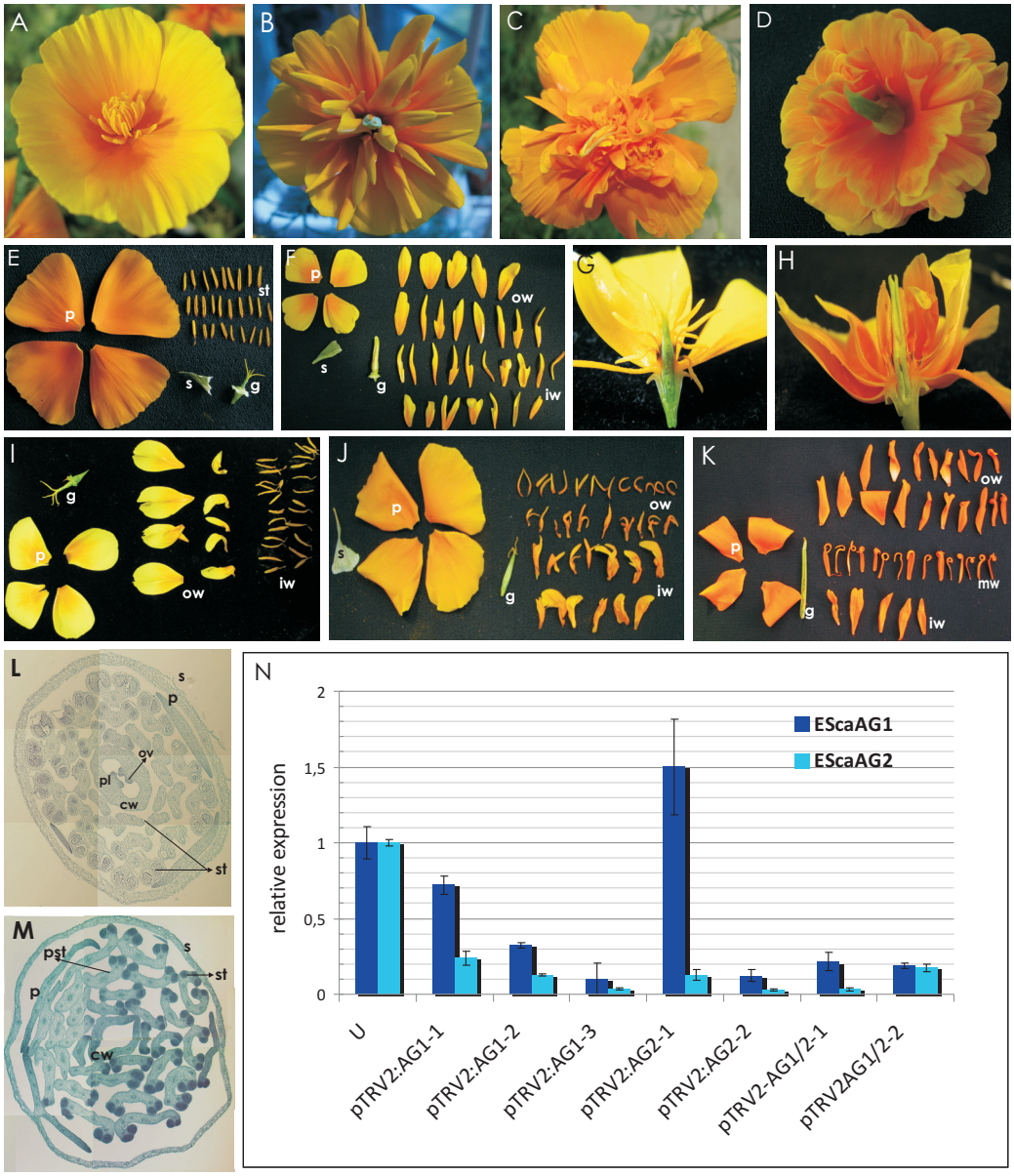


Figure 3 Phenotypes of plants treated with pTRV1 and pTRV2-EScaAG1, pTRV2-EScaAG2, or pTRV2-EScaAG1/2 and expression analysis of the VIGS treated plants. **(A)** Wild type phenotype of an *E. californica* flower treated with pTRV2-E. **(B)** Phenotype of an *EScaAG1* VIGS treated plant showing full homeotic conversions of stamens into petals. **(C)** Phenotype of an *EScaAG2* VIGS treated plant showing full homeotic conversions of stamens and carpels into petal-like structures. **(D)** Phenotype of a flower silenced for *EScaAG1/2* showing homeotic conversions of stamens into petals. **(E)** A mock treated plant with disassembled floral organs. **(F)** Disassembled flower of a plant treated with pTRV2-EScaAG1/2 showing homeotic conversions of stamens into petals. The same phenotype was also achieved with plants silenced for *EScaAG1* or *EScaAG2* individually. **(G)** Transverse hand section of a flower from a mock treated plant. **(H)** Transverse hand section of a flower from a plant silenced for *EScaAG1/2* showing homeotic conversions of stamens into petals. **(I)** Disassembled flower of a plant silenced for *EScaAG1* showing partial homeotic conversions of only the outer whorl stamens. **(J)** Disassembled flower of a plant treated with pTRV2-EScaAG2 showing that the inner whorl of stamens is converted into petal-like structures. **(K)** Disassembled flower of a plant treated with pTRV2-EScaAG1/2 exhibiting homeotic conversions of the innermost and outermost stamens whorls into petals while the middle whorls show mild deviation from wild type stamens while the center whorl stamens remain more stamen-like. **(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, petal-stamen mosaic structures, malformed stamens and a gynoecium lacking tissue differentiation, ovules, and placenta. **(N)** Real-Time PCR analysis of the first bud of individual *E. californica* plants treated with VIGS and untreated (U). Plants were treated with pTRV2-EScaAG1 are abbreviated as VIGS AG1, plants treated with pTRV2-EScaAG2 as VIGS AG2. Numbers below indicated individual plants and the relative expression level of *EScaAG1* and *EScaAG2* in untreated plants was set to 1. Abbreviations: cw, carpel wall; ov, ovule; p, petal; pl, placenta; se, sepal; pst, petaloid stamens; st, stamen.

Table 1 Overview of the observed phenotypes of EScaAG VIGS in California poppy

	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 third and fourth 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%)**

The strength of the observed phenotypes was correlated with the degree of reduction in *EScaAG1* and *2* transcript levels as measured by Q-PCR. The first floral bud (size 1 to 3 mm in diameter) of randomly selected plants treated with the *EScaAG1, EScaAG2, and EScaAG1/2* VIGS vectors was collected and correlated with the phenotype of the next formed flower. In 99% of the cases (n = 414) we observed that when the secondarily formed flower showed a phenotype, the first flower exhibited a phenotype as well. This consistent pattern allowed us to predict the phenotype of the first bud used for quantitative RT-PCR based on the second flower's phenotype (see also [21,22]). The changes in *EScaAG1* and *EScaAG2* expression in the first buds (1 to 3 mm bud diameter) of individual VIGS treated plants are documented in Figure 3N. Targeted silencing of individual *EScaAG* genes was not achieved, suggesting that the overlap in observed phenotypes result from a reduction of expression of both *AG* paralogs. Irrespective of the silencing vector used, *EScaAG1* expression was generally reduced from 70% to 10% of its wild type expression and *EScaAG2* expression was reduced from 25% to less than 5%. The use of the pTRV2-EScaAG1/2 vector resulted in similar reductions in expression levels for both genes. While six plants show silencing of both, *EScaAG1* and *EScaAG2*, one plant (pTRV2:AG2-1) treated with *EScaAG2*-targeted VIGS exhibited reduction of *EScaAG2* expression but increased *EScaAG1* expression relative to untreated plants, demonstrating the variability of VIGS experiments. However, we were able to show a significant reduction of expression in six of seven randomly analyzed buds from individual plants.

VIGS of C-function genes results in homeotic conversions of carpels into petal-like organs

In addition to homeotic conversions of stamens into petal-like structures in plants infected with pTRV2-*EScaAG1* and pTRV2-EScaAG2, we observed changes to the gynoecium morphology. The gynoecia of untreated plants develop as round green cylinders and consist of two fused carpels. This cylinder-like structure was disturbed in *EScaAG1* and *EScaAG2* VIGS-treated plants and the gynoecia of the VIGS treated plants were transformed either into (i) flattened green structures lacking ovules in some cases (Figure 4A Table 1) or (ii) flattened organs showing petal characteristics such as orange pigmentation and petal-like epidermal surface structure (Figure 4B Table 1). The latter was empty (Figure 4A, B) or contained additional floral organs (Figure 4F Table 1).

In order to determine whether the petal-like pigmentation of the gynoecium was associated with a change in cell surface morphology we conducted Scanning Electron Microscopy (SEM) analysis of the carpel whorl. In the wild type, the carpel surface is composed of small compact cells interrupted by stomatal cells (Figure 4C) and the petal surface is composed of long and narrow cells arranged in a parallel manner (Figure 3D) [20]. SEM micrographs of an orange-pigmented gynoecium reveal a mosaic pattern of tubular petal-like cells next to small compact cells typical for a carpel surface scattered with stomata (Figure 3E). This indicates that the gynoecia of *EScaAG1* and *EScaAG2* VIGS treated plants not only show a partially petal-like pigmentation but have also acquired petal-like cell surface characteristics,

supporting the hypothesis that these gynoecia are partially transformed into petal-like organs.

Treating poppy plants with *EScaAG1* and *2* VIGS not only resulted in the loss of stamen and carpel characteristics but also in the addition of petal organ identity to the carpel whorl. We tested the hypothesisis that the expression domains of floral homeotic B genes was extended to the central gynoecium whorl in *EScaAG1* and *2* VIGS treated plants using real-time PCR to assess expression of the three poppy floral homeotic B class genes *EScaDEF1*, *EScaDEF2*, and *EScaGLO* at anthesis and pre-anthesis (Figure 4G, H). B and C gene expression in untreated gynoecia was also characterized (Figure 4D). As expected *EScaAG1* as well as *EScaAG2* were expressed in gynoecia before and at anthesis. Surprisingly, the class B gene ortholog, *EScaDEF1* was expressed in gynoecia at a comparatively high level, although expression levels of two other B-class genes *EScaDEF2* and *EScaGLO* were hardly detectable. Next, the expression of class B and C genes was recorded in the gynoecia of VIGS treated plants (Figure 4G). The relative expression of all analyzed genes was normalized by setting levels to one in gynoecia of untreated plants before anthesis. In the gynoecia of VIGS treated plants (Figure 4H), expression of *EScaAG1* was reduced to 50% and even 20% in the gynoecia of VIGS treated plants and expression of *EScaAG2* was reduced in most gynoecia as well. VIGS treatments had no impact on *EScaDEF1* expression in the gynoecia. However, the expression of *EScaDEF2* was drastically increased between 5.8-fold and 17.7-fold relative to expression in untreated gynoecia. Transcript abundance of *EScaGLO*, also increased significantly upon silencing of C function genes by 2.2 to 5.7 times in the *EScaAG1* and *EScaAG2* VIGS treated plants. These expression analyses indicate that in central whorl organs with reduced expression of C function genes, two B function genes *EScaDEF2* and *EScaGLO* were expressed at significantly higher level in *EScaAG1* and *EScaAG2* VIGS treated than in untreated or mock treated plants.

For the *Arabidopsis* B proteins APETALA3 (AP3) and PISTILLATA (PI) it was shown that their homeotic function requires the formation of AP3-PI heterodimers [23]. EScaDEF2 is an AP3 homolog while EScaGLO is the PI homolog [24] and simultaneous upregulation of the AP3 and PI orthologs in poppy suggests that they might form heterodimers in the central whorl of C function silenced flowers and cause the observed homeotic gynoecium-petal conversions.

EScaAG1 and *EScaAG2* are involved in the regulation of floral meristem termination

The flowers of the plants treated with *EScaAG1* and *EScaAG2* VIGS showed not only homeotic conversions of stamens into petaloid organs, petal-like features in the central whorl, and a reduction in ovule number, but also signs of prolonged floral meristem activity. All treated plants showed increases in floral organ number in the stamen and central whorls. Moreover, flowers exhibiting a strong silencing phenotype showed ectopic structure enclosed inside the gynoecium whorl ranging from carpel like leaves to additional gynoecia and ectopic flowers (Figure 4F).

Interestingly, we observed a significant increase in stamen number in the weaker floral phenotypes characterized by no obvious homeotic organ conversions (Table 2). Untreated plants produced 26.2 stamens per flower on average, *EScaAG1* VIGS-treated plants without any homeotic conversions developed 29 stamens per flower, *EScaAG2* VIGS-treated produced 28.2, and plants treated simultaneously with *EScaAG1/2* produced 28.6 stamens on average. This suggests that whereas a mild reduction in *EScaAG1* and *EScaAG2* expression may not affect floral organ identity any reduction in expression can induce an increase in stamen number.

Discussion

This study is the first functional analysis of floral homeotic C function genes in a basal eudicot. We employed VIGS to transiently down-regulate *EScaAG1* and *EScaAG2* in *E. californica* and observed homeotic conversions of stamens into petals, reduced floral meristem termination, and transformation of the gynoecium into petal-like structures. *EScaAG2* is expressed at lower levels (also observed by [18]) but despite the reduced expression of *EScaAG*2, molecular evolutionary analyses failed to detect evidence of reduced evolutionary constraint (see below).

The two *AG* paralogs of *E. californica*, *EScaAG1* and *EScaAG*2 are quite similar on both protein and nucleotide level including the 5'UTR region indicating that they are duplicates. Generally it is hypothesized, that duplicated genes will not persist over evolutionary time unless sub-, or neofunctionalization results in functional divergence [25-27]. *EScaAG1* and *EScaAG2* share about 81.7% sequence similarity in the open reading frame and are 75.5% identical when the 5'UTR is included. The origin of these paralogs may be associated with an ancient whole genome duplication event that has been inferred on the lineage leading to *Eschscholzia* [28]. Using a penalized likelihood approach [29] we estimated an age of 51 million years for the *EScaAG* duplication. This divergence time was obtained using a maximum likelihood tree for the *AG* subfamily [30] calibrated with taxon ages reported in [31].

No evidence of reduced constraint on *EScaAG2* was inferred from analysis of the ratio of nonsynonymous to synonymous nucleotide substitutions on the branch

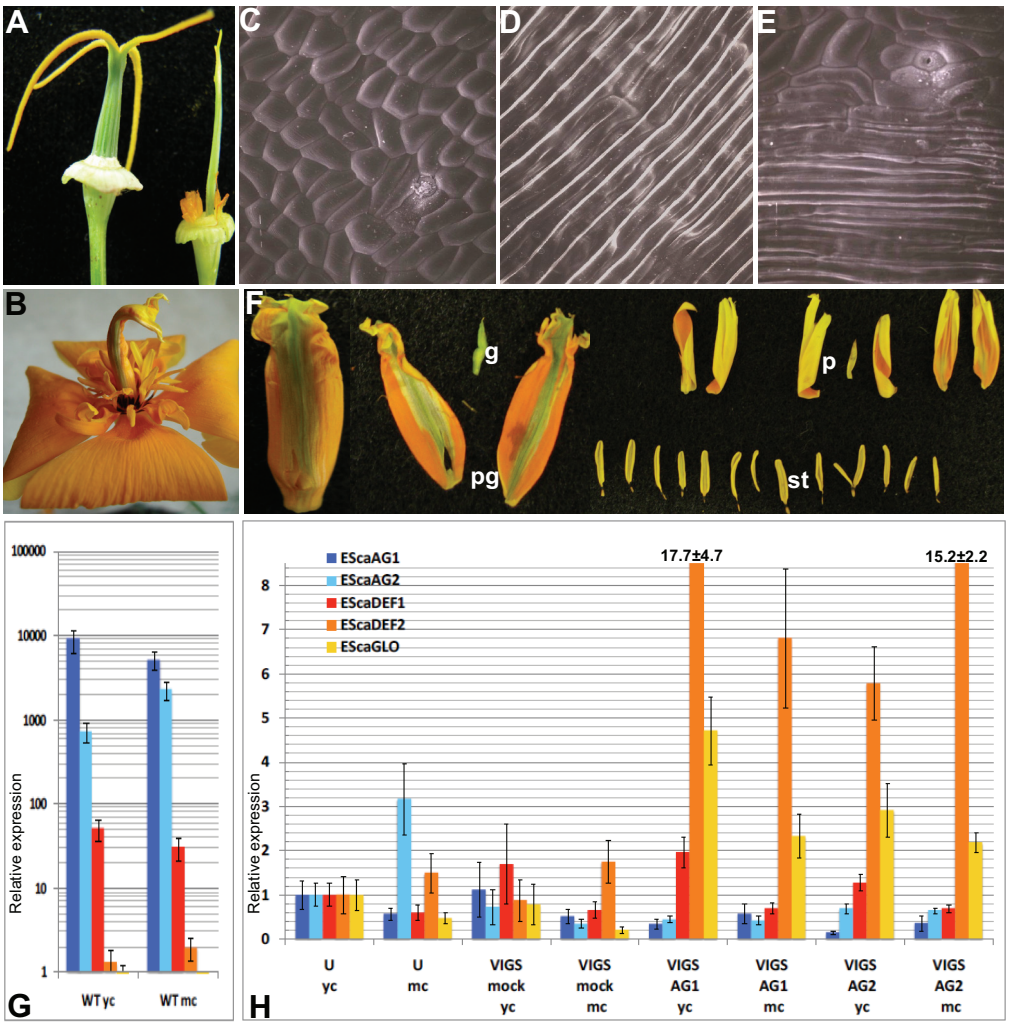


Figure 4 Carpel whorl phenotype of *EScaAG* silenced plants and expression analysis of floral homeotic genes. **(A)** Gynoecium of an untreated plant (left) and of a plant silenced for *EScaAG2* (right) **(B)** Flat orange gynoecium without ovules of a plant treated with pTRV2-*EScaAG1*. **(C)** Scanning electron micrograph (SEM) of the wild type gynoecium surface structure. **(D)** SEM of a wild type petal surface structure. **(E)** SEM of the central floral whorl organ of a plant treated with pTRV2-*EScaAG1* showing a mix of petal and gynoecium surface structures. **(F)** The central whorl floral organ (pg, for petaloid gynoecium) of a plant treated with pTRV2-*EScaAG2* showing petaloid and carpeloid features as well a lack of ovules. This organ encloses an ectopic flower consisting of a remnant gynoecium (g), petals (p) and stamens (st). **(G)** Relative expression of class B and C genes in young carpels before anthesis and mature carpels at anthesis of untreated plants, **(H)** Real-Time RT-PCR expression analysis of *EScaAG1*, *EScaAG2*, *EScaDEF1*, *EScaDEF2*, and *EScaGLO* in the gynoecia of VIGS treated plants. Abbreviations used in (G) and (H): yc, young carpel before anthesis; mc, mature carpel at anthesis; u, untreated plants; pTRV2-E, plants treated with pTRV1 and pTRV2-E; pTRV2-AG1, plants silenced for *EScaAG1*; pTRV2-AG2, plants silenced for *EScaAG2*; pTRV2-AG1/2, plants silenced for *EScaAG1* and *EScaAG2*.

Table 2 Stamen numbers of in EScaAG1, EScaAG2, and EScaAG1/2 VIGS 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)

leading to *EScaAG2* [30]. A recent shift in constraint on *EScaAG2* may not be detectable [32], but the molecular evolutionary analyses indicate that both *EScaAG2* and *EScaAG1* have been evolving under selective constraint for much of the approximately 50 million years since duplication. These results suggest that both *EScaAG1* and *2* have been selectively maintained in the lineage leading to *E. californica*.

Gymnosperm and angiosperm *AG* homologs are highly conserved but gene duplications have spurred functional diversification. The observation that knocking down *EScaAG1* and *2* individually results in overlapping phenotypes can be explained by two alternative scenarios. First, the two poppy *AG* paralogs may be working redundantly in the specification of floral organ identity and floral meristem determinacy. Alternatively, the VIGS method may not be able to individually silence paralogs with highly similar sequences. Our results are not fully consistent with either of these interpretations. Expression analyses of single knock down VIGS plants showed that transcript abundance of both genes was decreased, but *EscaAG2* was silenced more strongly than *EscaAG1*. With respect to the first scenario, the selective maintenance of fully redundant genes over 50 million years is highly unlikely. Full knockouts (vs. knock downs) for each paralog may be required to reveal subtle functional divergence.

Differences in expression between *EScaAG1* and *EScaAG2* (Figure 2A) and deviations in spatial distribution of the homeotic conversions of stamens into petals (Figure 3I-K) hint at some degree of subfunctionalization. However, we were not able to relate the distinct phenotypes of only outer stamen whorl homeotic conversions in the case of *EScaAG1* VIGS and only inner stamen whorl conversion in the *EScaAG2*-silenced flowers to the expression *EScaAG1* and *EScaAG2* expression data. In almost all analyzed floral buds we have simultaneous down-regulation of both genes with always a higher residual *EScaAG1* expression than *EScaAG2* expression, suggesting that subtle spatial expression difference at a very early developmental stage might play a role which we were not able to detect with our expression analysis. A less transient approach such as stable transformation with hairpin RNA constructs that would be able to silence *EScaAG1* and *EScaAG2* expression individually is required to rigorously characterize functional domains for *EScaAG1* and *2*, and test the subfunctionalization hypothesis.

Another characteristic of the *EScaAG1* and *2* VIGS phenotype is the loss of carpel organ identity. The most common phenotype observations were flattened green gynoecia or flat petaloid gynoecia showing an orange pigmentation and cell surface structure typical for petals (Figure 4A-E, Additional file 2.). The latter finding indicates that in *E. californica*, homeotic conversions of gynoecia into petaloid structures can occur when the C function is missing. This homeotic conversion coincides with the expansion of the expression domains of two class B genes, *EScaDEF2* and *EScaGLO*, into the central floral whorl of *EScaAG1* and *EScaAG2* VIGS treated plants. The third B class gene, *EScaDEF1* is also expressed in the gynoecia of untreated plants and expression levels are unaffected by reduction of C class gene expression in VIGS treated plants (Figure 5). These findings suggest that *EScaDEF1* expression is independent of class C gene expression while *EScaDEF2* and *EScaGLO* are negatively regulated by class C genes in the central floral whorl.

Interestingly, *EScaDEF2* and *EScaGLO* are expressed in parallel with the class C genes in the stamen whorl which indicates C independent expression of the two class B genes in stamen whorls in contrast to C dependent expression in the central floral whorl. Thus, a cofactor (X) restricted to the central whorl can be postulated to inhibit expression of *EScaDEF2* and *EScaGLO* expression along with the C class proteins EScaAG1 and EScaAG2 (Figure 5).

This type of C-dependent regulation of B class genes is in contrast to the strong *Arabidopsis ag-3* mutant, where full homeotic conversions of stamens into petals and carpels into sepals are observed. Even in the weaker *ag-4* mutant, the carpel is not converted into a petal-like structure, but rather into a sepal [33]. Single or double mutants *shp1*/*shp2* do not show any floral homeotic functions in *Arabidopsis*. Phenotypic effects are detectable only after fertilization [34]. In contrast, the *Antirrhinum ple-1/far* double mutant shows the type of floral homeotic conversions we observe in poppy: carpels are converted into petal-like structures and additional flower enclosed inside the fourth whorl unlike in the third whorl in Arabidopsis [15]. In the *Arabidopsis ag* mutant, the expression of the B function genes *AP3* and *PI* in the fourth whorl is prevented by the action of *SUPERMAN* (*SUP*) [35] and carpels are converted to sepal-like organs [36]. Therefore, it seems that the regulation of B function genes is independent of C class gene function in *Arabidopsis*. However, at this point we cannot exclude the hypothesis that *AG* together with the closely related *SHP1* and *SHP2* genes work with *SUP* to repress B gene expression in the fourth whorl. In the *Antirrhinum ple-1/far* double mutant, an expansion of the B function expression domain towards the fourth whorl was observed as a result of a C function reduction. It was suggested that the putative *SUP* orthologs in *Antirrhinum, OCTANDRA* (*OCT*) requires *PLE* or *FAR* to exclude B function gene expression from the fourth whorl while *SUP* in *Arabidopsis* acts independently of *AG* [15].

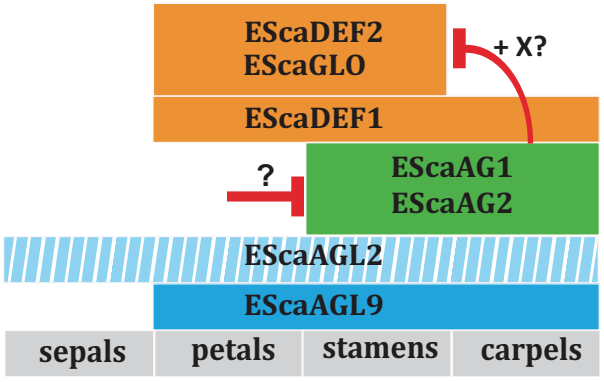


Figure 5 Hypothesis on the regulation of class C dependent B gene expression in *E. californica*. This modified BCE model of *E. californica* floral organ identity specification includes the B class genes (orange boxes) *EScaDEF1*, *EScaDEF2*, and *EScaGLO* that are supposed to be expressed in second and third (stamen) whorl. Expression of *EScaDEF1* is also found in the central whorl. Two class C genes (green boxes) are expressed in the stamen and central whorl, and the two class E genes *EScaAGL2* and *EScaAGL9* (blue boxes) are expressed in all whorls except for the sepal whorl for *EScaAGL9* [43] while no information is available on the expression domain of *EScaAGL2*. Red bars indicate repression of gene expression. It remains unclear by which mechanism *EScaAG1* and *EScaAG2* expression is restricted to the reproductive whorls of the flower and if repression of *EScaDEF2* and *EScaGLO* by the two class C genes is direct or mediated by a co-factor. An A function has not yet been described in California poppy.

Our analysis of the *EScaAG1* and *EScaAG2* VIGS flowers suggests that the regulation of poppy B function genes is more similar to *Antirrhinum* than to *Arabidopsis* because we also find an expansion of petal-like tissues specified by the B function in the fourth whorl. As postulated for *Antirrhinum*, the negative regulation of B function genes in the fourth whorl may involve the activation of an *E. californica SUP* ortholog. A poppy *SUP* ortholog could be positively regulated by *EScaAG1* and *2* or interact with these genes to restrict B function expression to the second and third whorl in wild type plants.

The fact that B gene expression is restricted by C function in *E. californica* as a representative of a basal eudicot lineage and *Antirrhinum*, a member of the asterid clade, in contrast to C independent regulation in *Arabidopsis* indicates that the former regulatory scenario might be ancestral. However, C function dependent regulation of B class genes has also not been reported in monocots such as rice. Down regulation of the rice *AG* homolog, *OsMADS58*, did not result in expansion of the expression domains for B class genes and carpel to lodicule transformation have not been observed in *osmads3* mutants or *OsMADS58* RNAi lines [17].

This suggests 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*. Since class C floral homeotic mutants are not yet available from basal angiosperms or non-grass monocots all three of these scenarios are equally parsimonious.

As reported for *Arabidopsis ag* mutants, a reduction of *EScaAG1* and *2* function in *E. californica* leads to defects in floral meristem termination, albeit in a more complex pattern than observed in *Arabidopsis*. The stamen whorls of *EScaAG1* and *2* VIGS-treated plants are more numerous than in the control plants even if the phenotype is mild, for example, no homeotic conversions of reproductive organs (Figure 3F). These observations support inferences drawn from work on *A. thaliana* and *A. majus* where mild reductions in C-function affect floral meristem determinacy [37,38]. The morphogenesis of *E. californica* flowers differs from most core eudicots, for example, *Arabidopsis*, in that the innermost stamen whorls are still being formed when the central gynoecium is initiated. A ring of cells with meristematic activity around the gynoecium is maintained while the central floral meristem is consumed in the process of gynoecium initiation [20]. This suggests that a mild reduction in *EScaAG1* and *2* expression is sufficient for a prolonged meristem activity in this ring shaped meristem that produces additional stamen whorls in *EScaAG1* and *2* VIGS-treated flowers. Additionally, our results suggest that *EScaAG1* and *2* regulate the termination of meristem activity in *E. californica*. Regulation of meristematic activity was observed in the central floral meristem and the ring meristem that gives rise to stamen whorls independently of the ceasing central floral meristem activity (Table 2). The influence of *EScaAG1* and *2* VIGS on the stamen whorls is especially interesting as stamen numbers in wild type *E. californica* are phenotypically variable, ranging from 18 up to 34 stamens when individuals are grown under identical conditions and constant light [20]. Even slight differences in the timing and dose of *EScaAG1* and *EScaAG2* transcript abundance between plants could account for these stamen number variations in wild type plants. The number of stamens in *E. californica* generally coincides with the plant's stature: as has been reported for *Stellaria media* (chickweed) [39], healthier plants produce

more stamens. Our analyses suggest that the number of stamens produced is dependent on the amount of *EScaAG1* and *EScaAG2* transcript in *E. californica* flowers. This might indicate a stature-dependent regulation of class C floral homeotic genes 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 class C genes 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 subgroup of angiosperms with several stamen whorls and often varying stamen numbers. The duration of class C genes activity in the meristems generating these reiterating stamen whorls might also determine stamen number in these species.

Our study on the function *EScaAG1* and *EScaAG2* in *E. californica* reveal that the VIGS method is suitable to analyze the evolution of gene regulation by enabling gene function analysis in non-model plants for which transgenic approaches are difficult to achieve. This work shows that gene function and the regulation of floral homeotic genes vary among plant lineages. Looking forward, the importance of VIGS for assessing gene function in non-model species will increase as advances in sequencing technologies result in full transcriptome and even genome sequences for an expanding number of species sampled across the plant tree of life. While sequence data will allow characterization of amino acid conservation and gene duplication events, functional studies in non-model species will be required to elucidate the evolution of regulatory networks influencing flowering time and floral form over angiosperm history.

Materials and methods

Expression analysis

Q-PCR

Q-PCR assays were performed on floral organs, leaves, young fruits, and buds of different developmental stages in wild type plants. For expression analysis of the *EScaAG1* and *EScaAG2* VIGS-treated plants, a single bud (1 to 2 mm in diameter) was examined. For the analysis of class C and B genes in VIGS-treated plants, single gynoecia of either buds of 5 to 8 mm diameter (young carpel) or from open flowers (mature carpel) were collected. All samples were analyzed in three technical replicates. One μg of total RNA was reverse transcribed into cDNA using random hexamer primers and the SuperScript III Kit (Invitrogen, Karlsruhe, Germany). A total of 5 μl of 1:50 diluted cDNA was used as a template. *DEF1* and *Actin* primers were designed with the help of the UPL probe program (Roche, Mannheim, Germany); all other primers sets were designed with one intron spanning primer (primer details are in Additional file 3). Paralog specific primer pairs consist of forward primers spanning at least one intron and a reverse primer spanning the deletion part of *EScaAG1* in 3' coding region were used to discriminate between *EScaAG1* and *EScaAG2* and the PCR product was sequenced to confirm primer specificity and the primer melting curves were analyzed. *Eschscholzia Actin2* and *GAPDH* were used as reference genes. The Real-Time PCR reaction mix consisted of: 5 μl of cDNA (1:50 dilution), 10 μl of SYBR Green mix (Roche) and 0.8 to 1.2 pM primers. The UPL Real-Time PCR mix consisted of 5 μl of 1:50 diluted cDNA, 100 nM UPL probe (Roche, #132 for *EScaDEF1* and #136 for *Actin*) and 0.04 pM of each primer. Real-Time PCR was performed using a Light Cycler 480 (Roche) with the following cycle conditions: initial heating of 95°C for 5 minutes, and 45 cycles of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C. Cp values were analysed according to the Genorm manual and accurate normalization was carried out by geometric averaging of multiple internal control genes [40].

In situ hybridisation

Non-radioactive *in situ* hybridization followed essentially the protocol of [41]. The *EscaAG1* and *EScaAG2* coding regions were cloned into the pDrive vector (Qiagen, Hilde, Germany), the digoxigenin-labelled RNA probes were transcribed using SP6 polymerase (Roche) and subsequently hybridized to floral tissue sections.

Virus-induced gene silencing

A 395 bp fragment of *EScaAG1* was amplified from the *EScaAG1* coding region by using the primers VIGSEcAG1A to add a *BamH*I restriction site to the 5' end of the PCR product and EcAG1VIGS to add an *Xho*I restriction site to the 3' end (primer sequences reported in Additional file 3). The amplicon was digested with *BamH*I and *Xho*I and cloned into a similarly cut pTRV2 vector [42]. A 477 bp fragment of *EScaAG2* was amplified from the *EScaAG2* coding region by using the primers VIGSEcAG2A to add a *BamH*I restriction site to the 5' end of the PCR product and EcAG2VIGS to add an *Xho*I restriction site to the 3' end. The amplicon was digested with *BamH*I and *Xho*I and cloned into a similarly cut pTRV2. pTRV2-*EScaAG1/AG2* was constructed by a 190 bp fragment of *EScaAG1* was amplified from the *EScaAG1* coding region by using the primers XbaVIGSEcAG1Bfw to add a *XbaI* restriction site to the 5' end of the PCR product and EcAG1VIGSXhorev to add a *Xho*I restriction site to the 3' end. The amplicon was digested with *XbaI* and *Xho*I. A 214 bp fragment of *EScaAG2* was amplified from the *EScaAG2* coding region by using the primers EcoVIGSEcAG2Afw to add an *EcoRI* restriction site to the 5' end of the PCR product and EcAG2VIGSXbarev to add an *XbaI* restriction site to the 3' end. The amplicon was digested with

*Eco*RI and *Xba*I and was then ligated together with *the EScaAG1* fragment into the *EcoRI* and *XhoI* cut pTRV2 vector producing the pTRV2-*EScaAG1/AG2* plasmid. The vector inserts of the double construct were confirmed by restriction digestion and sequencing. The resulting plasmids were sequenced and transformed into *Agrobacterium tumefaciens* strain GV3101. The agro-inoculation was performed by injecting the *Agrobacterium* suspension into the shoot apical meristem as described by [22].

Scanning electron microscopy and histology

Gynoecia of *EScaAG1* and *EScaAG2* VIGS-treated and untreated plants were analyzed by Scanning Electron Microscopy [14] for changes in the cell surface structure. The gynoecia were incubated in 100% methanol for 10 minutes and subsequently for 10 minutes in 100% ethanol. Then they were kept overnight at room temperature in 100% ethanol and dried with a Critical Point Dryer, gold coated, and examined under the SEM (ISI-100B, International Scientific Instruments, Pleasanton, CA, USA). First formed buds of 1.6 to 2.5 mm in diameter were collected for histological analysis and stained with Safranin and Fast Green as described by [22].

Additional material

Additional file 1: Supplemental Figure 1: Alignment of the EScaAG1 and EScaAG2 protein sequences. Amino acids identical between two paralogs are indicated by dots; dashes indicate deletion of five amino acids located in the C-terminal region of *EScaAG2*. Dissimilar residues are indicated by the respective amino acids.

Additional file 2: Supplemental Figure 2: Phenotypes observed in the gynoecium of *EScaAG* VIGS treated plants. The Y-axis denotes the percentages of different carpel identity phenotypes obtained by VIGS (pTRV2-*EScaAG1*, n = 239; *EScaAG2*, n = 209, *EScaAG1/2*, n = 261 flowers). Differently treated VIGS plants are shown on the X-axis. The green color indicates 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.

Additional file 3: Supplemental Table 1: Sequences of primers used in this study.

Abbreviations

AG: the floral homeotic C function gene *AGAMOUS of A. thaliana*; AP3: the floral homeotic B function gene *APETALA3 of A. thaliana*; EScaAG1: *E. californica* ortholog of *AG*; EScaAG2: *E. californica* ortholog of *AG*; EScaAGL 11: *E. californica* ortholog of the the ovule specific gene *SEEDSTICK* (formerly known as *AGL11*) of *A. thaliana*; EScaDEF1: *E. californica* ortholog of the *A. majus* floral homeotic B function gene *DEFICIENS*; EScaDEF2: *E. californica* ortholog of the *A. majus* floral homeotic B function gene *DEFICIENS* ; EScaGLO: *E. californica* ortholog of the *A. majus* floral homeotic B function gene *GLOBOSA*; FAR: floral homeotic C function gene *FARINELLI* in A. majus; OCT: putative stamen and carpel boundary specifying gene *OCTANDRA* in *A. majus*; OSMADS3: floral homeotic C function gene of *O. sativa*; OSMADS58: floral homeotic C function gene of *O. sativa*; PI: the floral homeotic B function gene *PISTILLATA of A. thaliana*; PLE: the *A. majus* floral homeotic C function gene *PLENA*; Q RT-PCR: Quantitative Reverse Transcriptase polymerase chain reaction; SEM: Scanning electron microscopy; SHP: the *SHATTERPROOF* gene of *A. thaliana*; SUP: the stamen and carpel boundary specifying gene *SUPERMAN of A. thaliana*; TRV: Tobacco rattle virus; UPL: Universal probe library; UTR: Untranslated region; VIGS: Virus induced gene silencing; ZAG1: floral homeotic C function gene of Z. mays; ZMM2: floral homeotic C function gene Z. mays.

Acknowledgements

We thank Zsuzsanna Schwarz-Sommer as well as David Smyth for valuable discussions about the VIGS phenotypes and S.P. Dinesh-Kumar for providing the pTRV vectors. We thank the Becker lab members for lively discussions, Teja Shidore and Gitanjali Darmadhikari for their help with the phenotyping. We also thank Werner Vogel and Angelika Trambacz for poppy plant growth and care. This work was made possible with funding from the German Research Foundation (DFG) grant to A. B. (BE 2547/6-1, 6-2) and funding by the University of Bremen. This paper is dedicated to the memory of the late Zsuzsanna Schwarz-Sommer whose interest in this project was warmly appreciated, and whose contribution to the field was inspirational.

Author details

[1]University of Bremen, Fachbereich 02 Biology/Chemistry, Evolutionary Developmental Genetics Group Leobener Str., UFT, 28359 Bremen, Germany. [2]Department of Plant Biology, University of Georgia, Athens, GA 30602-7271, USA.

Authors' contributions

AYL participated in the expression analysis and phenotype characterization. SL carried out the cloning work and participated in expression analysis and phenotype characterization. SO carried out and interpreted the *in situ* hybridizations. RE analyzed and interpreted the Real-Time PCR data. JLM performed sequence divergence time estimates and molecular evolutionary analyses and edited the manuscript. AB conceived of the study, participated in its design and coordination. AYL and AB wrote this manuscript. All authors read and commented on drafts of the manuscript and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 7 May 2010 Accepted: 1 December 2010
Published: 1 December 2010

References

1. Fletcher JC: **Shoot and floral meristem maintenance in *Arabidopsis*.** *Annual Review of Plant Biology* 2002, **53**:45-66.
2. Coen ES, Meyerowitz EM: **The war of the whorls: genetic interactions controlling flower development.** *Nature* 1991, **353**:31-37.
3. Theissen G, Saedler H: **Floral quartets.** *Nature* 2001, **409**:469-471.
4. Bowman JL, Drews GN, Meyerowitz EM: **Expression of the Arabidopsis floral homeotic gene AGAMOUS is restricted to specific cell types late in flower development.** *Plant Cell* 1991, **3**:749-758.
5. Bowman JL, Smyth DR, Meyerowitz EM: **Genes directing flower development in Arabidopsis.** *The Plant Cell* 1989, **1**:37-52.
6. Rutledge R, Regan R, Nicolas O, Fobert P, Côté C, Bosnich W, Kauffeldt C, Sunohara G, Séguin A, Stewart D: **Characterization of an AGAMOUS homologue from the conifer black spruce that produces floral homeotic conversions when expressed in Arabidopsis.** *The Plant Journal* 1998, **15**:625-634.
7. Winter K-U, Becker A, Munster T, Kim JT, Saedler H, Theissen G: **MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants.** *Proceedings of the National Academy of Sciences* 1999, **96**:7342-7347.
8. Jager M, Hassanin A, Manuel M, Guyader HL, Deutsch J: **MADS-Box Genes in Ginkgo biloba and the Evolution of the AGAMOUS Family.** *Mol Biol Evol* 2003, **20**:842-854.
9. Becker A, Theißen G: **The major clades of MADS-box genes and their role in the development and evolution of flowering plants.** *Molecular Phylogenetics and Evolution* 2003, **29**:464-489.
10. Kramer EM, Jaramillo MA, Di Stilio VS: **Patterns of Gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms.** *Genetics* 2004, **166**:1011-1023.

11. Irish VF: **The evolution of floral homeotic gene function.** *BioEssays* 2003, **25**:637-646.
12. Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL, Yanofsky MF: **SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis.** *Nature* 2000, **404**:766-770.
13. Colombo M, Brambilla V, Marcheselli R, Caporali E, Kater MM, Colombo L: **A new role for the SHATTERPROOF genes during Arabidopsis gynoecium development.** *Developmental Biology* 2010, **337**:294-302.
14. Bradley D, Carpenter R, Sommer H, Hartley N, Coen E: **Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum.** *Cell* 1993, **72**:85-95.
15. Davies B, Motte P, Keck E, Saedler H, Sommer H, Schwarz-Sommer Z: **PLENA and FARINELLI: redundancy and regulatory interactions between two Antirrhinum MADS-box factors controlling flower development.** *EMBO J* 1999, **18**:4023-4034.
16. Mena M, Ambrose BA, Meeley RB, Briggs SP, Yanofsky MF, Schmidt RJ: **Diversification of C-function activity in maize flower development.** *Science* 1996, **274**:1537-1540.
17. Yamaguchi T, Lee DY, Miyao A, Hirochika H, An G, Hirano H-Y: **Functional Diversification of the two c-class MADS box genes OSMADS3 and OSMADS58 in Oryza sativa.** *Plant Cell* 2006, **18**:15-28.
18. Zahn LM, Leebens-Mack JH, Arrington JM, Hu Y, Landherr LL, dePamphilis CW, Becker A, Theissen G, Ma H: **Conservation and divergence in the AGAMOUS subfamily of MADS-box genes: evidence of independent sub- and neofunctionalization events.** *Evolution & Development* 2006, **8**:30-45.
19. Drews NG, Bowman JL, Meyerowitz EM: **Negative regulation of the Arabidopsis homeotic gene AGAMOUS by the APETALA2 product.** *Science* 1991, **65**:991-1002.
20. Becker A, Gleissberg S, Smyth DR: **Floral and vegetative morphogenesis in California poppy (*Eschscholzia californica* CHAM.).** *International Journal of Plant Sciences* 2005, **166**:537-555.
21. Wege S, Scholz A, Gleissberg S, Becker A: **Highly efficient virus-induced gene silencing (VIGS) in California poppy (Eschscholzia californica): An evaluation of VIGS as a strategy to obtain functional data from non-model plants.** *Ann Bot* 2007, **100**:641-649.
22. Orashakova S, Lange M, Lange S, Wege S, Becker A: **The CRABS CLAW ortholog from California poppy (Eschscholzia californica, Papaveraceae), EcCRC, is involved in floral meristem termination, gynoecium differentiation and ovule initiation.** *Plant J* 2009, **58**:682-93.
23. McGonigle B, Bouhidel K, Irish VF: **Nuclear localization of the Arabidopsis APETALA3 and PISTILLATA homeotic gene products depends on their simultaneous expression.** *Genes Dev* 1996, **10**:1812-1821.
24. Zahn LM, Leebens-Mack J, DePamphilis CW, Ma H, Theissen G: **To B or Not to B a flower: the role of DEFICIENS and GLOBOSA orthologs in the evolution of the angiosperms.** *J Hered* 2005, **96**:225-240.
25. Force A, Lynch M, Pickett FB, Amores A, Yan Y-l, Postlethwait J: **Preservation of duplicate genes by complementary, degenerative mutations.** *Genetics* 1999, **151**:1531-1545.
26. Nowak MA, Boerlijst MC, Cooke J, Smith JM: **Evolution of genetic redundancy.** *Nature* 1997, **388**:167-171.
27. Wagner A: **Redundant gene functions and natural selection.** *Journal of Evolutionary Biology* 1999, **12**:1-16.
28. Cui L, Wall PK, Leebens-Mack JH, Lindsay BG, Soltis DE, Doyle JJ, Soltis PS, Carlson JE, Arumuganathan K, Barakat A, Albert VA, Ma H, dePamphilis CW: **Widespread genome duplications throughout the history of flowering plants.** *Genome Res* 2006, **16**:738-749.
29. Sanderson MJ: **r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock.** *Bioinformatics* 2003, **19**:301-302.
30. Shan H, Zahn L, Guindon S, Wall PK, Kong H, Ma H, dePamphilis CW, Leebens-Mack J: **Evolution of plant MADS box transcription factors: evidence for shifts in selection associated with early angiosperm diversification and concerted gene duplications.** *Mol Biol Evol* 2009, **26**:2229-2244.
31. Moore MJ, Bell CD, Soltis PS, Soltis DE: **Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms.** *Proceedings of the National Academy of Sciences* 2007, **104**:19363-19368.
32. Leebens-Mack J, dePamphilis C: **Power analysis of tests for loss of selective constraint in cave crayfish and nonphotosynthetic plant lineages.** *Mol Biol Evol* 2002, **19**:1292-1302.
33. Sieburth LE, Running MP, Meyerowitz EM: **Genetic separation of third and fourth whorl functions of AGAMOUS.** *Plant Cell* 1995, **7**:1249-1258.
34. Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL, Yanofsky MF: **SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis.** *Nature* 2000, **404**:766-770.
35. Sakai H, Medrano LJ, Meyerowitz EM: **Role of SUPERMAN in maintaining Arabidopsis floral whorl boundaries.** *Nature* 1995, **378**:199-203.
36. Jack T, Brockman LL, Meyerowitz EM: **The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens.** *Cell* 1992, **68**:683-697.
37. Mizukami Y, Ma H: **Separation of AG function in floral meristem determinacy from that in reproductive organ identity by expressing antisense AG RNA.** *Plant Molecular Biology* 1995, **28**:767-784.
38. Causier B, Bradley D, Cook H, Davies B: **Conserved intragenic elements were critical for the evolution of the floral C-function.** *The Plant Journal* 2009, **58**:41-52.
39. Haskell G: **Variation in the number of stamens in the common chickweed.** *Journal of Genetics* 1949, **49**:291-301.
40. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: **Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.** *Genome Biol* 2002, **3**:RESEARCH0034.
41. Groot EP, Sinha N, Gleissberg S: **Expression patterns of STM-like KNOX and Histone H4 genes in shoot development of the dissected-leaved basal eudicot plants Chelidonium majus and Eschscholzia californica (Papaveraceae).** *Plant Molecular Biology* 2005, **58**:317-331.
42. Ratcliff F, Martin-Hernandez AM, Baulcombe DC: **Tobacco rattle virus as a vector for analysis of gene function by silencing.** *Plant Journal* 2001, **25**:237-245.
43. Zahn LM, Kong H, Leebens-Mack JH, Kim S, Soltis PS, Landherr LL, Soltis DE, Depamphilis CW, Ma H: **The evolution of the SEPALLATA subfamily of MADS-box genes: a preangiosperm origin with multiple duplications throughout angiosperm history.** *Genetics* 2005, **169**:2209-2223.

doi:10.1186/2041-9139-1-13
Cite this article as: Yellina *et al.*: Floral homeotic C function genes repress specific B function genes in the carpel whorl of the basal eudicot California poppy (*Eschscholzia californica*). *EvoDevo* 2010 **1**:13.

EcSPT, the ortholog of the *Arabidopsis SPATULA* gene in *Eschscholzia californica,* is possibly involved in ovule and seed formation

Svetlana Orashakova and Annette Becker

Evolutionary Developmental Genetics Group, Fachbereich Biology/Chemistry, University of Bremen, Leobener Str., UFT, 28359 Bremen, Germany

Summary

The *Arabidopsis thaliana* gene *SPATULA (SPT)* controls the growth of the carpel margins and the tissues deriving from them. The disruption of the *SPT* function results in strongly reduced growth of the carpel margin tissues, more severe in the apical than in the basal part of the gynoecium, and the transmitting tract completely fails to develop. Here, we report the expression patterns and the transient knock down of *EcSPT*, the *SPT* ortholog in the basal eudicot *Eschscholzia californica* (California poppy). *EcSPT* is widely expressed in floral and non-floral tissues. The highest transcript abundance of *EcSPT* is found in ovules, developing fruits and seeds. Downregulation of *EcSPT* expression results in a reduced seed number suggesting a role for *EcSPT* in *E. californica* in ovule and seed formation.

Keywords: carpel margin development, ovule initiation, seed number, bHLH transcription factor, SPT, *Eschscholzia californica*

Introduction

The complex female reproductive organ, the gynoecium, represents the most characteristic and evolutionary innovative feature of angiosperms. It bears the ovules inside and offers a save environment for fertilization and subsequent for seed formation. After fertilization, the gynoecium develops into the fruit and the ovules into seeds. The fruit protects the developing seeds and after reaching maturity is responsible for their dispersal. The gynoecium in the model core eudicotyledonous plant *Arabidopsis thaliana* consists of two congenitally fused

carpels and arises as a single primordium from the floral meristem at around stage 5 (stages according to (SMYTH *et al.* 1990). Typically for most angiosperms, the mature gynoecium of *A. thaliana* consists along the apical-basal axis of style and stigma (apical tissues), and ovary and gynophore (basal tissues) (FERRANDIZ *et al.* 1999). In transverse perspective, it is obvious that the two carpels are fused laterally at their margins. The apical style and stigma, and the medial replum, placenta with ovules, transmitting tract and septum originate from the carpel margins and are collectively termed marginal tissues. Before fertilization, the false septum differentiates into transmitting tract, which guides the growing pollen tubes from the style to the ovules (DINNENY and YANOFSKY 2005). The carpel marginal tissues and the lateral carpels (valves) of the gynoecium display abaxial-adaxial polarity, as the outer part of the carpel wall is considered as abaxial and the inner as adaxial. Replum is located abaxially, while placenta with ovules, septum and transmitting tract differentiate at the adaxial side.

The *SPATULA (SPT)* gene encodes a basic-helix-loop-helix (bHLH) transcription factor, which controls diverse aspects of plant development in *A. thaliana*. The bHLH gene family is found in animals and plants, where they regulate various developmental processes. In plants, bHLH transcription factors are involved in anthocyanin biosynthesis, phytochrome signaling, fruit and carpel development (BUCK and ATCHLEY 2003). All bHLH gene family members share the highly conserved bHLH domain, consisting of a basic domain at the amino terminus and two α-helixes, separated by a variable loop region, which is thought to be associated with DNA binding and protein dimerization (HEIM *et al.* 2003b; LI *et al.* 2006; PATTANAIK *et al.* 2008). *SPT* is also member of the Phytochrome Interacting Factors/PIF-like (PIF/PIL) family, many members of which regulate different aspects of light signaling (BAILEY *et al.* 2003; HEIM *et al.* 2003a; HEISLER *et al.* 2001; TOLEDO-ORTIZ *et al.* 2003).

In *A. thaliana*, *SPT* regulates the gynoecium size and the development of the carpel margins with the specific tissues deriving from them (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; HEISLER *et al.* 2001). *spt* mutants display severe defects in all marginal tissues of the gynoecium (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; HEISLER *et al.* 2001). The development of style, stigma and septum is severely impaired, particularly in the apical regions of the gynoecium. Furthermore, transmitting tract fails to develop completely and the ovule number is reduced. Also carpel fusion is disturbed in the apical part of the gynoecium. These defects are also apparent later in the fruits, which are much shorter than in wild-type and contain less seeds restricted to the apical side of the silique (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; GROSZMANN *et al.* 2008; HEISLER *et al.* 2001). It has been shown recently that the function of *SPT* in carpel and fruit development is dependent

on *phytochrome B* and that *SPT* action is tightly linked to auxin (FOREMAN *et al.* 2011; NEMHAUSER *et al.* 2000). Phytochromes control the expression and localization of diverse auxin transporters involved in the polar auxin transport (PAT) in the gynoecium (LAXMI *et al.* 2008; WU *et al.* 2010). It has been proposed that an auxin gradient exists in the gynoecium, with declining auxin concentration from the apex to the base, and that this auxin gradient is responsible for establishment of the apical-basal patterning (NEMHAUSER *et al.* 2000). The defects in the apical tissues of *spt* can be complemented by applying of *N*-1-naphthylphthalamic acid (NPA), an inhibitor of the polar auxin transport (NEMHAUSER *et al.* 2000). Moreover, *SPT* seems to be negatively regulated by the auxin response factor *ETTTIN (ETT). SPT* is ectopically expressed in *ett* mutants and possibly contributes, at least partially, to the defects in style, ovary and gynophore in the *ett* mutant gynoecium (HEISLER *et al.* 2001; NEMHAUSER *et al.* 2000; PEKKER *et al.* 2005; SESSIONS *et al.* 1997; SESSIONS and ZAMBRYSKI 1995). Recently, it was shown that *SPT* acts downstream of or parallel with auxin in style growth, but the morphogen function of auxin in elaboration of the apical-basal patterning in the gynoecium is dependent on *SPT* (STALDAL and SUNDBERG 2009). In the control of auxin synthesis and its subsequent distribution along the apical-basal axes of the gynoecium, *SPT* functions possibly in concert with *PHYTOCHROME B* (FOREMAN *et al.* 2011). Beside *SPT* and *ETT, STYLISH1 (STY1)* and three redundantly acting *HECATA1/2/3 (HEC1/2/3)* bHLH genes participate in the control of this auxin gradient in the gynoecium (GREMSKI *et al.* 2007; NEMHAUSER and STEWART 2010; SOHLBERG *et al.* 2006; TRIGUEROS *et al.* 2009).

Additionally to its function in gynoecium and fruit development, SPT also controls seed germination, seedling growth and leaf size in *A. thaliana* (ICHIHASHI *et al.* 2010; PENFIELD *et al.* 2005; SIDAWAY-LEE *et al.* 2010).

In this study, we examined the spatial and temporal expression of the *SPT* ortholog, *EcSPT* and investigated the function of *EcSPT* in the basal eudicot *Eschscholzia californica* (*Papaveraceae*, Ranunculales). Silencing of the *EcSPT* expression by Virus-induced gene silencing (VIGS) led to a reduction of the seed set in the fruits of *EcSPT*-silenced plants, suggesting that that *EcSPT* might be involved in controlling aspects of ovule development and seed formation.

Results

Identification of *EcSPT* in *E. californica*

The sequence of the putative *SPT* ortholog in *E. californica*, *EcSPT,* shares the domain structure characteristic for eudicot *SPT*-like genes (GROSZMANN *et al.* 2008). The coding sequence of *EcSPT* is 1245 bp. The EcSPT protein consists of 445 amino acids and contains the highly conserved bHLH domain (49 amino acids), an amphipathic helix (11 amino acids), an acidic domain (14 amino acids), two nuclear localization sequences (four amino acids each) and one beta strand (nine amino acids) (Figure 1). The amphipathic helix is located close to the N-terminus and upstream of the bHLH domain. The acidic domain, which is predicted to adopt an alpha helical structure, is positioned closely upstream of the bHLH domain (Figure 1). One of the NLS is located immediately upstream of the bHLH domain, whereas the other one is positioned within the bHLH domain. Additionally, nine conserved amino acids, assumed to form a beta strand, are located downstream of the bHLH domain in the EcSPT protein. Within the extended bHLH domain, including both NLS and the beta strand, EcSPT shares 59 (out of 62) conserved amino acids with the *A. thaliana* SPT protein (GROSZMANN *et al.* 2008).

EcSPT is widely expressed in flower and non-flower organs

To analyze the transcript distribution of *EcSPT,* RT-PCR experiments on cDNA from different tissues and different developmental stages were performed (Figure 1A). In flowers at anthesis, *EcSPT* expression is detected in all floral organs, sepals, petals, carpels and stamens with similar intensity. Additionally, transcripts of *EcSPT* appear in leaves, fruits, green and mature seeds. The strongest expression of *EcSPT* is detected in fruits and green seeds. *EcSPT* expression is continuously present in buds from the earliest developmental stages, stage 1-5 (bud size from 0-1 mm in diameter) and is maintained throughout the entire development including the male and female meiosis at stage 8 and 9 (buds' size 3 and more mm in diameter), respectively. The *EcSPT* expression levels rise before and after gynoecium initiation, and during floral organ formation and development (buds' size from 0-1, from one to two, and from two to three mm in diameter), and decline in later stages (buds' size 3 and more mm in diameter) (Figure 1A, stages according to (BECKER *et al.* 2005).

In situ hybridization of *EcSPT* was conducted for more detailed information on its spatial and temporal expression. *EcSPT* expression is detected firstly in buds at stage 2, when the sepal primordia is formed (Figure 1B). The hybridization signal is distributed uniformly in the floral meristem, but is excluded from the just initiated sepal primordia. In early stage 3, *EcSPT* expression in the floral meristem appears stronger, but patchier (Figure 1C). Later in stage 3, the domain of strong *EcSPT* expression becomes restricted to the carpel anlagen, while the expression in the sepal and petal primordial is comparatively weaker (Figure 1D). In early stage 5, when the gynoecium primordium initiates, weak *EcSPT* expression is found in the apical part of the stamen primordia (Figure 1E). Additionally, *EcSPT* transcripts are found in the boundary region between the carpel and stamen primordia (arrows), but are excluded from the arising carpel primordia. Later in stage 5, the expression becomes restricted to the border between stamens and to the center of the gynoecium base, but is excluded from the stamens, petals, sepals, and carpel walls (Figure 1F). In buds at stage 7, the *EcSPT* hybridization signal is distributed throughout the entire ovule primordia and the placenta (Figure 1G). Furthermore, *EcSPT* is expressed in two thin stripes at the carpel margins marking the presumptive replum region. In the same stage, *EcSPT* expression persists also in stamens and in the outermost cell layer of the gynoecium wall (Figure 1G). At stage 8, when male meiosis occurs, the expression of *EcSPT* disappears from the carpel margins and is present only in the funiculus of the ovules (Figure 1H).

Reduced expression of *EcSPT* results in a lower number of seeds

In order to investigate the function of *EcSPT,* VIGS was used to downregulate the *EcSPT* expression. 80 plants of *E. californica* were infected by injecting of *Agrobacterium* suspension containing a mixture of one strain carrying pTRV1 and other containing pTRV2-*EcSPT*. Ten control plants were inoculated with *Agrobacterium* only carrying pTRV1 and the empty pTRV2 (pTRV2-E) vector.

To examine the degree of reduction in the *EcSPT* expression, semi-quantative RT-PCR was performed with *EcSPT*-specific primers on cDNA from the first bud of *EcSPT*-VIGS treated plants. As a control in the RT-PCR experiments, cDNA from the first bud of a plant treated with pTRV1 and pTRV2-E was used. We detected a strong expression of *EcSPT* in the bud of the control plant, whereas the expression of *EcSPT* was reduced in the first buds of ten

EcSPT-VIGS plants compared to the expression of the *Actin* gene used as an endogenous control (Figure 3A).

SPT in *A. thaliana* regulates fruit development and *spt* mutants develop fruits that are shorter than the wild type harbouring fewer seeds (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; HEISLER *et al.* 2001). In contrast to what has been described for *SPT* of *A. thaliana*, the appearance of *EcSPT*-VIGS flowers and fruits was indistinguishable from the wild-type at first sight. In order to quantify the putative contribution of *EcSPT* in fruit and seed development, fruit length and seed number was recorded in the first three fruits of 20 *EcSPT*-VIGS and 20 wild-type plants in a preliminary experiment (Figure 3B, C).

We observed a reduction in the fruit length and seed number in the *EcSPT*-VIGS treated plants when compared to wild-type plants (Fig. 2B). The averages of the fruit length measured in the *EcSPT*-VIGS plants are 5.6 cm (1st fruit), 5.5 cm (2nd fruit) and 5.1 (3rd fruit) compared to 6.6 cm (1st fruit), 6.8 (2nd fruit) and 6.3 cm (3rd fruit) in wild-type plants. The accounted seed number had averages of 15.2 seeds (1st fruit), 12.1 (2nd fruit) and 10.4 (3rd fruit) in the *EcSPT*-silenced fruits compared to 27.1 seeds (1st fruit), 30.45 (2nd fruit) and 29.45 (3rd fruit) in wild-type fruits (Fig.2C). The standard deviations in the fruit length are 1.145 (1st fruit), 1.157 (2nd fruit) and 1.162 (3rd fruit) in the *EcSPT*-VIGS treated plants compared to 1.169 (1st fruit), 1.153 (2nd fruit) and 0.995 (3rd fruit) in wild-type. The standard deviations in the seed number are 13.239 (1st fruit), 9.964 (2nd fruit) and 11.821 (3rd fruit) in the *EcSPT*-VIGS treated plants compared to 16.928 (1st fruit), 14.002 (2nd fruit) and 13.195 (3rd fruit) in wild-type fruits. The high standard deviations in the seed number of *EcSPT*-VIGS and wild-type fruits are due to the strong variation in the seed number formed in both wild type and *EcSPT*-VIGS treated fruits. A one-way ANOVA test did not reveal significant differences between untreated and *EcSPT*-VIGS treated plants. However, the tendency of *EcSPT*-VIGS treated plants to form shorter fruits that contain fewer seeds than the untreated ones can be derived from figures 3B and C.

Discussion

SPT is a key developmental regulator controlling carpel development, seed stratification, light signaling, auxin gradient formation in the gynoecium, and leaf size in *A. thaliana* (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; FOREMAN *et al.* 2011; HEISLER *et al.* 2001;

ICHIHASHI *et al.* 2010; NEMHAUSER *et al.* 2000; PENFIELD *et al.* 2005). However, information on the expression patterns and functions of *SPT*-like genes is limited to *A. thaliana* and *Prunus persica (P. persica)*. Here, we report the isolation, the expression pattern and the phenotype resulting from transient downregulation of *EcSPT,* the ortholog of *SPT* in the basal eudicot *E. californica*.

EcSPT protein shows the same domain organization as SPT (GROSZMANN *et al.* 2008). In *A. thalian*a, additionally to the bHLH domain required for the overall SPT function, also the acidic domain is necessary for the carpel developmental function of SPT, whereas the amphipathic helix only supports it. In order to investigate the importance of the single domains for the carpel developmental function of *EcSPT*, specific domain mutagenesis can be done. Also yeast two hybrid experiments with different deletion variants of the EcSPT protein can be performed.

EcSPT is specifically expressed in the boundaries between gynoecium and stamen primordia

EcSPT is initially expressed in the entire floral meristem at stage 2, when the sepal primordia have initiated. The early *EcSPT* expression resembles that of *SPT* in *A. thaliana*, whose transcripts are also uniformly distributed in the floral meristem at stage 2, when none of the floral organs has been produced yet (HEISLER *et al.* 2001). When carpel primordia start to initiate at stage 5, *EcSPT* is expressed in the boundaries between the carpel and the adjacent stamen primordia, and this expression pattern is maintained also after the gynoecium primordia have developed. The *EcSPT* expression between the two inner organ whorls might suggest a role for *EcSPT* in establishing the boundary between carpel and stamen primordia, possibly in concert with other factors. A reasonable candidate could be a putative ortholog of the *A. thaliana SUPERMAN (SUP)* gene in *E. californica*. *SUP* is similarly expressed in the boundary region between carpel and stamen primordia and is responsible for setting up boundaries between these two whorls early in the flower development of *A. thaliana* (BOWMAN *et al.* 1992; SAKAI *et al.* 1995). It was purposed that *SUP* functions in this by limiting AP3/PI abundance, supporting the proper balance of ternary protein complexes between whorl three and four and consequently, stamen and carpel organ formation, respectively (LIU and MARA 2010).

We detected transcripts of *EcSPT* along the carpel margins of the gynoecium at stage 7, similar to *SPT*, suggesting that both genes might share a function in carpel margin differentiation (HEISLER *et al.* 2001). Also in *E. californica* gynoecia, similar to *A. thaliana*, the region of the gynoecium wall, enclosed by the carpel margins, differentiates into an abaxial (external) replum and a parietal adaxial (internal) placenta bearing the ovules (BECKER *et al.* 2005). In both species, placenta and ovules differentiate from the inner ovary wall. But in contrast to *A. thaliana*, the marginal tissues septum, which originates from the placenta, and transmitting tract are not present in the *E. californica* gynoecium. Septum and transmitting tract are required for proper fertilization in *A. thaliana* as the transmitting tract, which derives from center of the septum, guides the growing pollen tubes from the apical style down to the ovules in the ovary. In *E. californica*, the placenta directs the growing pollen tubes to the ovules (BECKER *et al.* 2005). Furthermore, we detected *EcSPT* expression in the placenta of a gynoecium at stage 7 (Figure 2G). In contrast, *SPT* is not expressed in the placenta, but in septum and transmitting tract since their inception at stage 8 and 11, respectively (HEISLER *et al.* 2001; SMYTH *et al.* 1990). Furthermore, *spt-2* mutants do not exhibit any defects in placenta development, but in stigma, style and septum development and transmitting tract fails to form completely (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002). It is possible that both *EcSPT* and *SPT* function in the differentiation of the carpel marginal tissues responsible for guiding pollen tubes growth and this may suggest a common mechanism underling carpel margin development between *E. californica* and *A. thaliana. EcSPT* might have adopted a function in placenta development, while *SPT* might lost the function in placenta, but gained a new function in the control of the additional marginal tissues, transmitting tract and septum. Alternatively, both *EcSPT* and *SPT* might have independently become subfunctionalized in placenta and transmitting tract/septum development, respectively. In *A. thaliana*, there are many redundantly acting genes in placenta development, which are also absolutely required for style and septum development. Such genes are *AINTEGUMENTA (ANT),* and the transcriptional co-repressors *LEUNIG (LEU)* and *SEUSS (SEU)* (COLOMBO et al. 2010).

The constitutive expression of *EcSPT* in *A. thaliana spt-2* mutants could help to investigate whether the specific function of *SPT* in the development of the marginal tissues specifically responsible for pollen tube growth is conserved between both species. Additionally, promoter studies could be done to investigate the importance of upstream promoter elements for the EcSPT expression. In *A. thaliana*, the *SPT* expression is controlled by two major subregions

located upstream of the SPT transcription start site (GROSZMANN *et al.* 2010). These contain enhancers and silencers driving tissue-specific SPT expression. As we do not have information on the upstream promoter sequence of EcSPT, the SPT promoter region should be fused to the coding sequences of EcSPT and to SPT, and both constructs should be expressed in *spt-2* plants to estimate the degree of complementation of the mutant phenotype. Further, to specifically investigate *EcSPT* expression along the apical-basal axis of the gynoecium, RT-PCR or real-time PCR experiments can be assayed to dissected style, stigma and ovary regions of developing and mature *E. californica* gynoecia.

We observed expression of *EcSPT* in ovules during stages 7 and 8. Ovule expression has been reported also for *SPT* (HEISLER *et al.* 2001). Correspondingly, in *spt-2* mutants, the ovules develop normally, but the ovule number is reduced relative to wild-type, suggesting that *SPT* is involved in ovule initiation in *A. thaliana* (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002). The gynoecium of *spt-2* mutants contains less ovules than those of wild-type plants, as the loss of ovules appears to be restricted to the apical region of the gynoecium. Due to the absence of transmitting tract in *spt-2* plants, which leads to insufficient pollen tube growth, most ovules remain unfertilized and consequently less seeds are formed in the *spt-2* mutant siliques, which are shorter than wild-type ones (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002). Alvarez and Smyth suggested that the reduction in the silique length is possibly a consequence of the reduced development of the septum, which provide a mechanical support in the gynoecium. Also *EcSPT*-silenced plants show a tendency to develop less seeds and the fruits tend to be shorter. The role of *EcSPT* in ovule and seed formation could be revealed by stable *ecspt* mutants or transgenic RNAi lines with stable down regulation of *EcSPT* expression. In *EcSPT*-VIGS silenced plants, the reduced seed number could be a consequence of reduced placenta development, as the placenta is required to guide the growing pollen tubes in *E. californica*. Possible defects in placenta development and/or ovule initiation or development could be revealed by histological sections on floral buds of *EcSPT*-VIGS or *ecspt* mutants in different developmental stages, starting from stage 6, when placenta develops, until stage 11, when anthesis takes place. Also sections on *EcSPT*-VIGS fruits should be done.

Both *EcSPT* and *SPT* are expressed in developing seeds (Figure 1A). In *A. thaliana*, *SPT* controls seed germination in concert with the member of the Phytochrome Interacting Factor-like (PIF/PIL) family PIL5 as a response to light and temperature (OH *et al.* 2004; PENFIELD

et al. 2005). Freshly harvested wild-type seeds of *A. thaliana* are dormant and do not germinate without light and cold stratification. It was suggested that *SPT* represses seed germination in dormant seeds in cold and light stratification (PENFIELD *et al.* 2005). Unlike wild type seeds, seeds of *spt-10* mutants are able to germinate in light conditions even without cold stratification. Previous studies suggested that SPT controls seed germination in response to cold and light via repressing the expression of the gibberellic acid 3-oxidase (*GA3ox*), a key enzyme in the gibberellin biosynthesis probably in concert with PIF5(OH *et al.* 2004; OH *et al.* 2007; OH *et al.* 2006; PENFIELD *et al.* 2005). Additionally, *SPT* also suppresses seedling growth at low daytime temperature but is dispensable for this at high daytime temperatures (SIDAWAY-LEE *et al.* 2010). The role of SPT in the control of seedling growth is very likely dependent on the accumulated SPT protein. The level of SPT protein is elevated at cold daytime temperatures but is reduced at warm daytime temperatures. The strong expression of *EcSPT* in green seeds, revealed via RT-PCR, might hint to some function in the process of seed maturation. The ability of wild-type and *ecspt* seeds to germinate with and without cold and light stratification could be examined. Additionally, the role of EcSPT in seedling growth could be investigated by quantification of the accumulated SPT protein in *E. californica* plants growing at different cold and light conditions.

Our RT-PCR and *in situ* hybridization experiments revealed expression of *EcSPT* in petals and stamens, similar to *SPT* in *A. thaliana* (HEISLER *et al.* 2001). Obvious defects in petal and stamen development were not observed in the *EcSPT*-silenced plants, suggesting that *EcSPT*, despite its expression, is not involved in the development of these floral organs. Also *spt-2* mutants do not display defects in these floral organs (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; HEISLER *et al.* 2001). Due to constant expression of SPT in actively developing petals and stamens, it was suggested that SPT might control cell proliferation in growing stamens and petals (HEISLER *et al.* 2001). It was demonstrated that in leaves, *SPT* restricts cell proliferation and so limits the leaf size but without influencing leaf shape (ICHIHASHI *et al.* 2010; SIDAWAY-LEE *et al.* 2010). Our RT-PCR analyses also revealed *EcSPT* expression in the leaves similar to *SPT* in *A. thaliana*. In plants defective for *SPT*, the leaf size is significantly increased as a consequence of the increased cell number, whereas plants over-expressing *SPT* develop undersized leaves consisting of less and smaller cells (HEISLER *et al.* 2001; ICHIHASHI *et al.* 2010). Although the leaf expression of *EcSPT* suggests a role in leaf development, we did not observed obvious differences in the leaf size between *EcSPT*-silenced plants and the untreated plants. This could be due to the fact that *E.*

californica develops highly dissected leaves with variable morphology and subtle differences in leaf size are difficult to be detected. As *SPT* limits only the leaf size but not the leaf shape it is not excluded that also *EcSPT* functions in leaf development. In order to elucidate a putative role of *EcSPT* in leaf development, leaf size of *ecspt* mutants or hpRNAi lines of *EcSPT* could be measured. Additionally, possible changes in cell size and number can be detected by using SEM or histological sections.

Sepal expression was also revealed for *EcSPT* in RT-PCR, but not *in situ* experiments (Figure 2, 3A). Similarly, *SPT* is also not detected in sepals by *in situ* hybridization (HEISLER *et al.* 2001). In respect to the sepal expression, the discrepancy between our RT-PCR and *in situ* hybridization results is probably due to the different sensitivity of both methods. Probably, *EcSPT* expression in sepals is very weak and can not be detected via *in situ* hybridization, but via the more sensitive RT-PCR. Additional stages of flower development have to be examined and quantitative Real-Time RT-PCR will be helpful to quantify the expression of *EcSPT* in floral and non-floral tissues. The *SPT* ortholog in *P. persica, PPERSPT*, is also widely expressed in all floral (sepals, petals, stamens and carpels) and some non-floral (leaves, seeds and fruits) organs in RT-PCR experiments (TANI *et al.* 2010), suggesting a conservation of expression pattern between higher and early branching eudicots.

In summary, *EcSPT* and *SPT* are expressed differentially within the gynoecium, with the exception of shared expression domains along the carpel margins and in the ovules. This suggests that *EcSPT* also might function in the processes of carpel margin differentiation and ovule initiation. Additionally, *EcSPT* may function in the meristem of *E. californica* due to its expression, but this needs to be investigated. We did not observe any phenotype in the boundary region between third and fourth floral organ primordia, although *EcSPT* expression constantly persists in this region throughout development. Although *EcSPT* is expressed in sepals, petals, and stamens, we did not observed obvious defects in these floral organs. Similarly, also *SPT* is widely expressed outside of the gynoecium, but the mutant phenotype is restricted to the gynoecium and to the fruit (ALVAREZ and SMYTH 1999; ALVAREZ and SMYTH 2002; HEISLER *et al.* 2001). A disadvantage of the VIGS method is that it only transiently down regulates gene expression and residual transcripts of the gene can be still present in the silenced plants. As RT-PCR experiments, conducted on first buds of *EcSPT*-VIGS plants revealed a reduced, but still persisting expression, suggesting that *ecspt* stable mutants could be supportive to reveal all aspects of the *EcSPT* function in *E. californica*.

Another reason for the weak phenotype can be the presence of redundantly acting genes in *E. californica,* i.e. paralogs of *EcSPT,* which can be examined by Southern Blot analysis. Furthermore, additional redundantly acting genes might function in concert with *EcSPT* in carpel margin differentiation and ovule initiation in *E. californica*. Such candidate could be *EcCRC*, the ortholog of the *A. thaliana CRABS CLAW (CRC)* gene in *E. californica,* which is required for proper carpel margin differentiation and ovule initiation/placenta formation (ORASHAKOVA *et al.* 2009). Furthermore, *EcCRC* is also expressed in along the carpel margin, but earlier in the development (ORASHAKOVA *et al.* 2009). In addition, both *EcSPT* and *EcCRC* display continuously overlapping domains of expression in the center of the gynoecium, suggesting that both can directly interact with each other. In *E. californica*, also *EScaAG1/2* control ovule initiation and the investigation of the *EScaAG1/2* expression in *EcSPT*-VIGS plants could reveal, if there is some redundancy of both genes in process of ovule initiation (YELLINA *et al.* 2010).

It could be that pollen tube growth in *E. californica*, which occurs toward the placenta from the style to the ovaries, may represent an ancient mechanism directed by *EcSPT* and possibly other, yet unknown factors. In *A. thaliana*, on the other hand, *SPT* is required for pollen tube growth, although in *Brassicaceae*, septum and transmitting tract have developed as additional specific tissues for proper fertilization.

Materials and Methods

Cloning of *EcSPT* and RT-PCR

The *EcSPT* open reading frame was isolated with a combined 3'- and 5'- RACE approach. For 3' RACE, *E. californica* RNA from floral tissue was reverse transcribed employing the primer AB05 (5'-GACTCGAGTCGACATCTGTTTTTTTTTTTTTTTT-3'). In the 3'-RACE PCR the primers SPTmodi (5'-G(AG)(GT) CTG CTG AAG TTC ATA A(TC)C-3') and AB07 (5'-GACATCGAGTCGACATCTG-3') were used. 5' RACE PCR was carried out as described previously (ORASHAKOVA *et al.* 2009) using RNA from floral tissues with the following oligonucleotides: EcSPT5R1 (5'-GTAACATCCCTGGCAGGTATAATG-3'), EcSPT5R2 (5'-GATGCAAACTCAACCCATTTC-3'), and EcSPT5R3 (5'-CATTGATAACATTTGAACTTGG-3'). All PCR amplified fragments were cloned into pGEM-T (Promega, please check here the location) and subsequently sequenced.

For RT-PCR, total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and subsequently 1 µg of total RNA was reverse transcribed into cDNA with SuperScript III Kit (Invitrogen, Karlsruhe, Germany). A 206 bp fragment was amplified in the coding region of *EcSPT* by using the RTQsptfw (5'-TCCTCCTCTTGACTCGTCTTC-3') and RTQsptrev (5'-CAAATCCTTCCTCGCTTTGGC-3') primers. As an endogenous control for RT-PCR experiments *Actin2* was used as detailed previously (ORASHAKOVA *et al.* 2009). 35 RT-PCR cycles was used for each RT-PCR experiment. For RT-PCR of *EcSPT*-VIGS plants, total RNA was isolated from the very first bud (0-3 mm in diameter) using the RNeasy Micro Kit (Qiagen). As a negative control, cDNAs of the first bud of plants inoculated with pTRV1 and empty pTRV2 (pTRV2-E) were amplified.

***In situ* hybridization**

Non-radioactive in *situ* hybridization following the protocol of Groot et al., 2005 was performed. The probe include the 3'-coding sequence of *EcSPT* and a part of the 3'-UTR untranslated region (UTR), and was positioned downstream of the bHLH domain. The sequence of the probe was amplified using the forward primer EcSPT3f (5'-TTCAGCTTCATCAAGGACAG-3') and the reverse primer EcSPT3rev2 (5'-GCTTGAGTAATAGATGAGAC-3') as the size of the amplified fragment was 334 bp. This was cloned into a pDrive vector and confirmed by sequencing (Qiagen, Hilden, Germany). The digoxigenin-labelled anti-sense RNA probe was synthesized using SP6 RNA polymerase (Roche, Manheim, Germany) and subsequently hydrolysed for the calculated time. The digoxigenin-labelled probe was hybridized to section of floral tissues, digested with Proteinase K (final concentration 5 µg/µl) for 10 min, at 37°C.

Virus-induced gene silencing (VIGS)

For VIGS, a 447 bp fragment of the coding region of *EcSPT,* located upstream of the bHLH domain, was amplified using the forward primer Bam*EcSPT*VIGS (5'-TGTCCTCTGTGTGTTCTTCTGCT-3') containing an incorporated *BamHI* restriction site, and the reverse primer *EcSPT*VIGSXho (5'-TGCTCGAGCGTCAAGATCGTTATCCAC-3') containing an incorporated *XhoI* restriction site. The resulting PCR fragment was digested with *Bam HI* and *XhoI* and cloned into pTRV2 vector also digested with *BamHI* and *XhoI.* The construct was confirmed by sequencing. *Agrobacterium* transformation and plant inoculation were carried out as previously described (ORASHAKOVA *et al.* 2009).

Figure legends:

Figure 1: Domain structure of the EcSPT protein

Schematic representation of the conserved motifs in the EcSPT protein with amphipathic helix (yellow), acidic domain (green), NLS (nuclear localisation signal, blue), bHLH domain (red) and beta strand (purple). Numbers refer to amino acid positions in the EcSPT protein sequence.

Figure 2: Expression analysis of *EcSPT* in wild-type flowers of *E. californica* by RT-PCR and in *situ* hybridization

(A) RT-PCR experiment showing differential expression of *EcSPT* in floral and non-floral organs. *Actin* was used as endogenous control. **(B-H)** DIG-labelled probe hybridizing to *EcSPT* in E. californica floral tissue is documented. **(B)** Longitudinal section of a stage 2 bud. **(C)** Longitudinal section of a bud in early stage 3. **(D)** Longitudinal section of a bud in late stage 3. **(E)** Longitudinal section of a bud in early stage 5. **(F)** Longitudinal sections of a bud at late stage 5. **(G)** Transverse sections of a bud at stage 7. **(H)** Transverse sections of a bud at stage 8. Abbreviations: ca, carpel; cap, carpel primordium; fm, floral meristem; fu, funiculus gynoecium; gw, gynoecium wall; ov, ovules; p, petals; pl, placenta; pp, petal primordium; r, replum; se, sepal; st, stamen; stp, stamen primordium. Scale bars: 100 μm.

Figure 3: Expression of *EcSPT* and phenotype analysis of the *EcSPT*-silenced plants

(A) RT-PCR-based expression of *EcSPT* in young buds of *EcSPT*-VIGS plants. As a negative control, a cDNA from a plant treated only with pTRV1 and pTRV2 was used; 1-10 plants were treated with the *EcSPT* construct. *Actin* was used as an endogenous control. **(B)** Length of the first, second, and third fruits of *EcSPT*-VIGS-treated plants compared to fruits of untreated plants. **(C)** Seed number in the first, second, and third fruits of *EcSPT*-VIGS treated plants compared to untreated plants. Blue colour shows untreated plants and red colour represents EcSPT-treated plants.

References

ALVAREZ, J., and D. R. SMYTH, 1999 CRABS CLAW and SPATULA, two Arabidopsis genes that control carpel development in parallel with AGAMOUS. Development 126: 2377-2386.

ALVAREZ, J., and D. R. SMYTH, 2002 Crabs claw and Spatula genes regulate growth and pattern formation during gynoecium development in Arabidopsis thaliana. International Journal of Plant Sciences 163: 17-41.

BAILEY, P. C., C. MARTIN, G. TOLEDO-ORTIZ, P. H. QUAIL, E. HUQ *et al.*, 2003 Update on the Basic Helix-Loop-Helix Transcription Factor Gene Family in Arabidopsis thaliana. The Plant Cell Online 15: 2497-2502.

BECKER, A., S. GLEISSBERG and D. R. SMYTH, 2005 Floral and vegetative morphogenesis in California poppy (Eschscholzia californica Cham.). International Journal of Plant Sciences 166: 537-555.

BOWMAN, J. L., H. SAKAI, T. JACK, D. WEIGEL, U. MAYER *et al.*, 1992 Superman, a Regulator of Floral Homeotic Genes in Arabidopsis. Development 114: 599-&.

BUCK, M. J., and W. R. ATCHLEY, 2003 Phylogenetic analysis of plant basic helix-loop-helix proteins. J Mol Evol 56: 742-750.

COLOMBO, M., V. BRAMBILLA, R. MARCHESELLI, E. CAPORALI, M. M. KATER *et al.*, 2010 A new role for the SHATTERPROOF genes during Arabidopsis gynoecium development. Developmental Biology 337: 294-302.

DINNENY, J. R., and M. F. YANOFSKY, 2005 Drawing lines and borders: how the dehiscent fruit of Arabidopsis is patterned. Bioessays 27: 42-49.

FERRANDIZ, C., S. PELAZ and M. F. YANOFSKY, 1999 Control of carpel and fruit development in Arabidopsis. Annu Rev Biochem 68: 321-354.

FOREMAN, J., J. WHITE, I. GRAHAM, K. HALLIDAY and E. M. JOSSE, 2011 Shedding light on flower development: phytochrome B regulates gynoecium formation in association with the transcription factor SPATULA. Plant Signal Behav 6.

GREMSKI, K., G. DITTA and M. F. YANOFSKY, 2007 The HECATE genes regulate female reproductive tract development in Arabidopsis thaliana. Development 134: 3593-3601.

GROSZMANN, M., Y. BYLSTRA, E. R. LAMPUGNANI and D. R. SMYTH, 2010 Regulation of tissue-specific expression of SPATULA, a bHLH gene involved in carpel development, seedling germination, and lateral organ growth in Arabidopsis. Journal of Experimental Botany 61: 1495-1508.

GROSZMANN, M., T. PAICU and D. R. SMYTH, 2008 Functional domains of SPATULA, a bHLH transcription factor involved in carpel and fruit development in Arabidopsis. The Plant Journal 55: 40-52.

HEIM, M. A., M. JAKOBY, M. WERBER, C. MARTIN, B. WEISSHAAR *et al.*, 2003a The Basic HelixA?a��a�?LoopA?a��a�?Helix Transcription Factor Family in Plants: A Genome-Wide Study of Protein Structure and Functional Diversity. Molecular Biology and Evolution 20: 735-747.

HEIM, M. A., M. JAKOBY, M. WERBER, C. MARTIN, B. WEISSHAAR *et al.*, 2003b The Basic Helixâ€“Loopâ€“Helix Transcription Factor Family in Plants: A Genome-Wide Study of Protein Structure and Functional Diversity. Molecular Biology and Evolution 20: 735-747.

HEISLER, M. G., A. ATKINSON, Y. H. BYLSTRA, R. WALSH and D. R. SMYTH, 2001 SPATULA, a gene that controls development of carpel margin tissues in Arabidopsis, encodes a bHLH protein. Development 128: 1089-1098.

ICHIHASHI, Y., G. HORIGUCHI, S. GLEISSBERG and H. TSUKAYA, 2010 The bHLH Transcription Factor SPATULA Controls Final Leaf Size in Arabidopsis thaliana. Plant and Cell Physiology 51: 252-261.

LAXMI, A., J. PAN, M. MORSY and R. CHEN, 2008 Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in Arabidopsis thaliana. PLoS One 3: e1510.

LI, X., X. DUAN, H. JIANG, Y. SUN, Y. TANG *et al.*, 2006 Genome-Wide Analysis of Basic/Helix-Loop-Helix Transcription Factor Family in Rice and Arabidopsis. Plant Physiology 141: 1167-1184.

LIU, Z., and C. MARA, 2010 Regulatory mechanisms for floral homeotic gene expression. Seminars in Cell & Developmental Biology 21: 80-86.

NEMHAUSER, J. L., L. J. FELDMAN and P. C. ZAMBRYSKI, 2000 Auxin and ETTIN in Arabidopsis gynoecium morphogenesis. Development 127: 3877-3888.

NEMHAUSER, J. L., and J. L. STEWART, 2010 Do Trees Growon Money? Auxin as the Currency of the Cellular Economy. Cold Spring Harbor Perspectives in Biology 2.

OH, E., J. KIM, E. PARK, J. I. KIM, C. KANG *et al.*, 2004 PIL5, a phytochrome-interacting basic helix-loop-helix protein, is a key negative regulator of seed germination in Arabidopsis thaliana. Plant Cell 16: 3045-3058.

OH, E., S. YAMAGUCHI, J. HU, J. YUSUKE, B. JUNG *et al.*, 2007 PIL5, a Phytochrome-Interacting bHLH Protein, Regulates Gibberellin Responsiveness by Binding Directly to the GAI and RGA Promoters in Arabidopsis Seeds. The Plant Cell Online 19: 1192-1208.

OH, E., S. YAMAGUCHI, Y. KAMIYA, G. BAE, W.-I. CHUNG *et al.*, 2006 Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in Arabidopsis. The Plant Journal 47: 124-139.

ORASHAKOVA, S., M. LANGE, S. LANGE, S. WEGE and A. BECKER, 2009 The CRABS CLAW ortholog from California poppy, (Eschscholzia californica, Papaveraceae), EcCRC, is involved in floral meristem termination, gynoecium differentiation, and ovule initiation. Plant J 58: 682-693.

PATTANAIK, S., C. H. XIE and L. YUAN, 2008 The interaction domains of the plant Myc-like bHLH transcription factors can regulate the transactivation strength. Planta 227: 707-715.

PEKKER, I., J. P. ALVAREZ and Y. ESHED, 2005 Auxin response factors mediate Arabidopsis organ asymmetry via modulation of KANADI activity. Plant Cell 17: 2899-2910.

PENFIELD, S., E.-M. JOSSE, R. KANNANGARA, A. D. GILDAY, K. J. HALLIDAY *et al.*, 2005 Cold and Light Control Seed Germination through the bHLH Transcription Factor SPATULA. Current Biology 15: 1998-2006.

SAKAI, H., L. J. MEDRANO and E. M. MEYEROWITZ, 1995 Role of Superman in Maintaining Arabidopsis Floral Whorl Boundaries. Nature 378: 199-203.

SESSIONS, A., J. L. NEMHAUSER, A. MCCOLL, J. L. ROE, K. A. FELDMANN *et al.*, 1997 ETTIN patterns the Arabidopsis floral meristem and reproductive organs. Development 124: 4481-4491.

SESSIONS, R. A., and P. C. ZAMBRYSKI, 1995 Arabidopsis gynoecium structure in the wild and in ettin mutants. Development 121: 1519-1532.

SIDAWAY-LEE, K., E.-M. JOSSE, A. BROWN, Y. GAN, K. J. HALLIDAY *et al.*, 2010 SPATULA Links Daytime Temperature and Plant Growth Rate. Current biology : CB 20: 1493-1497.

SMYTH, D. R., J. L. BOWMAN and E. M. MEYEROWITZ, 1990 Early Flower Development in Arabidopsis. The Plant Cell Online 2: 755-767.

SOHLBERG, J. J., M. MYRENAS, S. KUUSK, U. LAGERCRANTZ, M. KOWALCZYK *et al.*, 2006 STY1 regulates auxin homeostasis and affects apical-basal patterning of the Arabidopsis gynoecium. Plant J 47: 112-123.

STALDAL, V., and E. SUNDBERG, 2009 The role of auxin in style development and apical-basal patterning of the Arabidopsis thaliana gynoecium. Plant Signal Behav 4: 83-85.

TANI, E., A. TSABALLA, C. STEDEL, C. KALLONIATI, D. PAPAEFTHIMIOU *et al.*, 2010 The study of a SPATULA-like bHLH transcription factor expressed during peach (Prunus persica) fruit development. Plant Physiology and Biochemistry 49: 654-663.

TOLEDO-ORTIZ, G., E. HUQ and P. H. QUAIL, 2003 The Arabidopsis Basic/Helix-Loop-Helix Transcription Factor Family. The Plant Cell Online 15: 1749-1770.

TRIGUEROS, M., M. NAVARRETE-GOMEZ, S. SATO, S. K. CHRISTENSEN, S. PELAZ *et al.*, 2009 The NGATHA genes direct style development in the Arabidopsis gynoecium. Plant Cell 21: 1394-1409.

WU, G., J. N. CAMERON, K. LJUNG and E. P. SPALDING, 2010 A role for ABCB19-mediated polar auxin transport in seedling photomorphogenesis mediated by cryptochrome 1 and phytochrome B. The Plant Journal 62: 179-191.

YELLINA, A., S. ORASHAKOVA, S. LANGE, R. ERDMANN, J. LEEBENS-MACK *et al.*, 2010 Floral homeotic C function genes repress specific B function genes in the carpel whorl of the basal eudicot California poppy (*Eschscholzia californica*). BMC Evo-Devo in press.

Figures:

Figure 1: Domain structure of the EcSPT protein

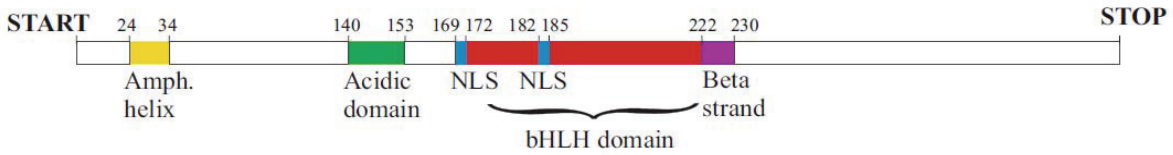


Figure 2: Expression analysis of *EcSPT* in wild-type flowers of *E. californica* by RT-PCR and in *situ* hybridization

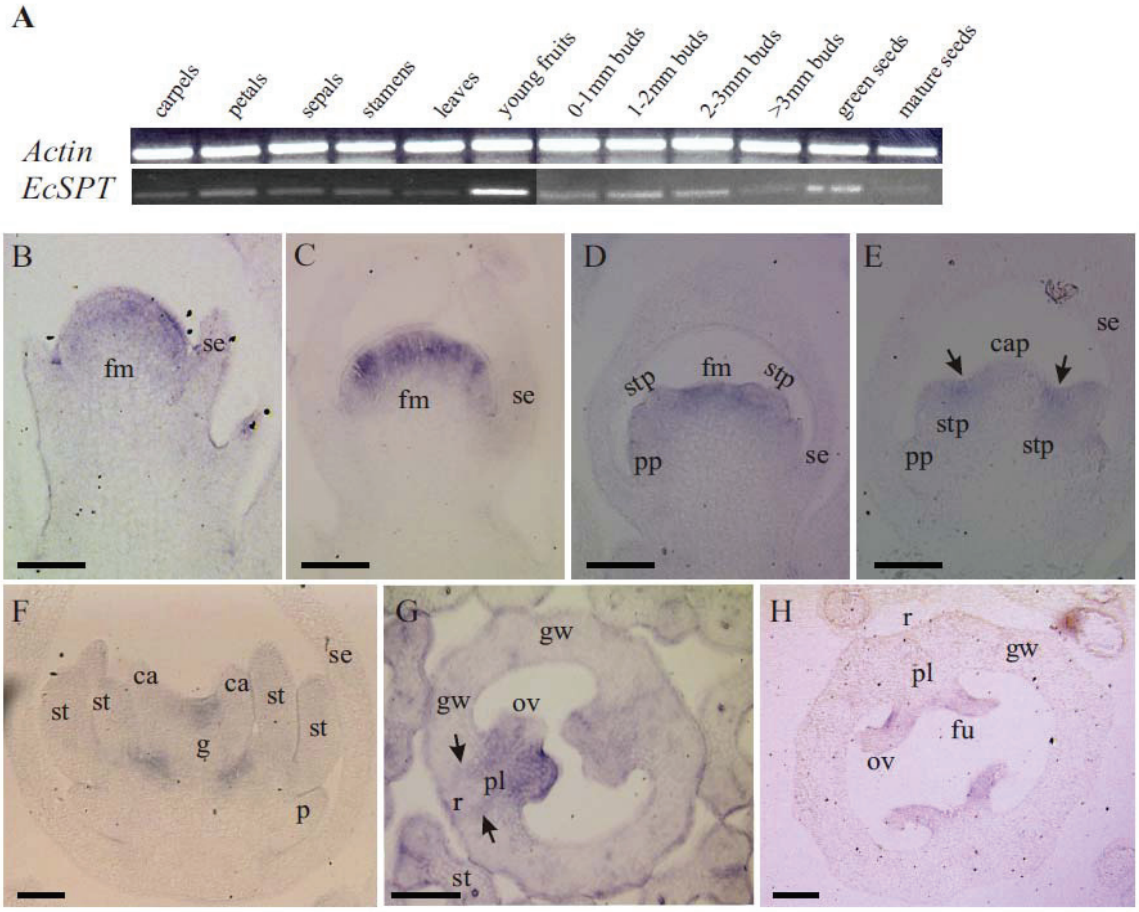
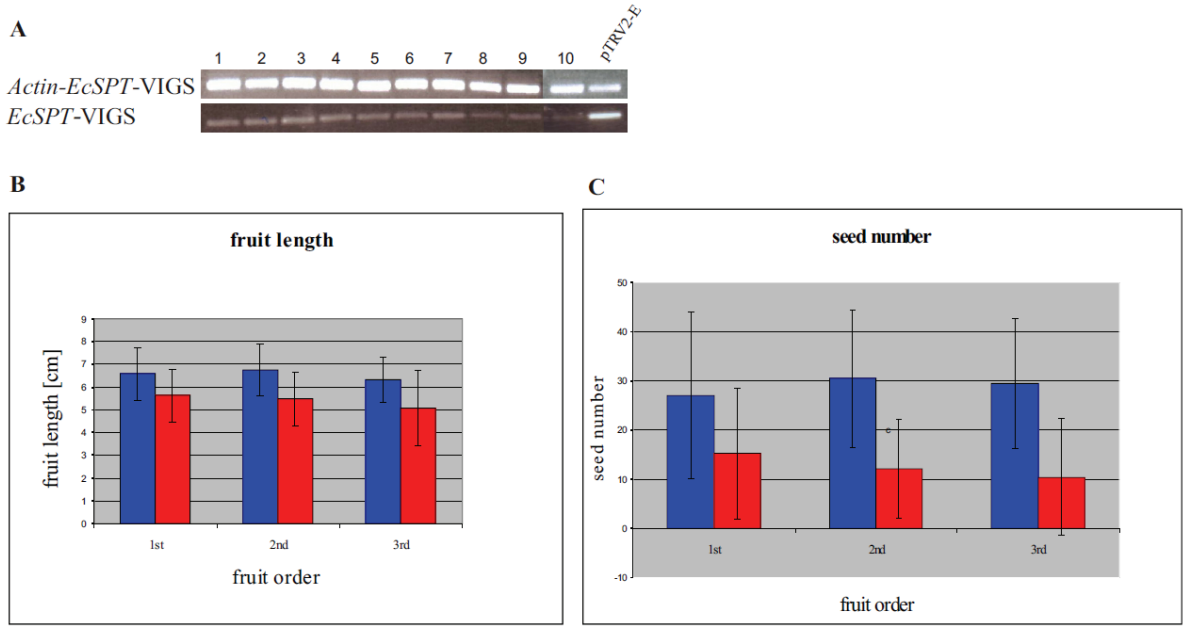


Figure 3: Expression of *EcSPT* and phenotype analysis of the *EcSPT*-silenced plants



Title

The *seirene* B class floral homeotic mutant of California poppy (*Eschscholzia californica*) reveals a function of the enigmatic PI motif in the formation of specific multimeric MADS-domain protein complexes

Authors

Matthias Lange[1], Svetlana Orashakova[1], Sabrina Lange[1], Rainer Melzer[2], Günter Theißen[2] & Annette Becker[1]

Affiliations

[1]University of Bremen, Evolutionary Developmental Genetics Group, Leobener Str., UFT, D-28359 Bremen, Germany

[2] Friedrich Schiller University Jena, Department of Genetics, Philosophenweg 12, D-07743 Jena, Germany

Key words

floral homeotic mutant, *Eschscholzia californica*, SEIRENE, GLOBOSA, evolutionary developmental genetics

Running title

poppy class B mutant

Estimated length of the article

10.9 pages

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Annette Becker (annette.becker@uni-bremen.de)

Abstract

The products of B class floral homeotic genes specify stamen and petal identity, and loss of B function results in homeotic conversions of petals into sepals and stamens into carpels. Here we describe the molecular characterization of *seirene-1* (*sei-1*), a mutant from the basal eudicot California poppy (*Eschscholzia californica*) that shows homeotic changes indicative for floral homeotic B class mutants. *SEI* has been previously described as *EScaGLO*, one of four B class related MADS box genes in *E. californica.* The C-terminus of SEI including the highly conserved PI-motif is truncated in *sei-1* plants when compared to wild type. Similar to the wild type protein, the sei-1 mutant protein is able to bind CArG-boxes specifically and can form homodimers, heterodimers and several ternary complexes with other MADS-domain proteins. However, the mutant protein is not able to mediate ternary complexes consisting of B, C, and E class related proteins indicating that the C-terminal domain of SEI has a function in mediating formation of specific higher-order MADS-domain transcription factor complexes. We present a hypothesis as to why the C-terminal domain of PI in *Arabidopsis* may be functionally different from the one of SEI and propose an evolutionary scenario to explain these functional differences.

Introduction

The regular appearances of the angiosperm flowers require distinct floral homeotic gene functions acting in a combinatorial manner. The ABCE model of flower development explains how four different gene functions can specify organ identity of the four floral organ types. The action of the A function alone specifies the outer whorl sepals and has been found in *Arabidopsis thaliana* and close relatives only. Concerted expression of class A and B governs petal organ identity, B and C function together to specify the stamens and the C gene function alone is required for carpel identities. The E function acts throughout the flower and is required for the determination of all floral organs (Coen and Meyerowitz, 1991; Honma and Goto, 2001; Theissen and Saedler, 2001). The loss of the A, B, C or E function leads to homeotic conversions of floral organs. For example, in *A. thaliana* homeotic B class mutants such as *ap3-3* and *pi-1*, petals are replaced by sepals and stamens are replaced by carpels (Bowman et al., 1989). The genetic factors constituting the ABCE classes have been identified

mainly as floral homeotic MADS domain transcription factors. In *A. thaliana*, the A class genes are *APETALA1* (*AP1*) and *APETALA 2* (*AP2)*, the B class genes *APETALA3* (*AP3)* and *PISTILLATA* (*PI*); *AGAMOUS* (*AG*) carries out the C function and the E function is realized by the four largely redundantly acting genes *SEPALLATA1* to *SEPALLATA4* (*SEP1* to *SEP4*) (Bowman et al., 1989, 1991; Jack et al., 1992; Pelaz et al., 2001). It is proposed that the floral homeotic proteins form multimeric complexes to confer floral organ identity. For example, stamen organ identity is governed by a protein complex consisting of AP3, PI, AG, and SEP proteins according to the floral quartet model (Honma and Goto, 2001; Theissen and Saedler, 2001).

AP3/PI-like genes have been identified from many representatives of diverse angiosperm lineages, but mutant analyses have so far been carried out only in core eudicots and monocots such as *A. thaliana, Antirrhinum majus, Petunia hybrida, Medicago truncatula, Oryza sativa*, and *Zea mays* ; mutants are generally affected in petal and stamen organ identity (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Angenent et al., 1995; Bowman et al., 1999; Ambrose et al., 2000; Nagasawa et al., 2003; Benlloch et al., 2009). In line with this, expression of B class genes was found to be rather conserved in higher eudicots and grassy monocots and is found predominantly in stamens and petals or in the homologous organs of grasses (Sommer et al., 1990; Bowman et al., 1991; Ambrose et al., 2000; Nagasawa et al., 2003; Vandenbussche et al., 2004; Benlloch et al., 2009). Flowers of species outside the core eudicots often have a less well differentiated perianth; correspondingly, the B class genes show a higher degree of expression divergence and B class proteins possess more variation in protein interaction partners (Kramer and Irish, 1999; Kim et al., 2005; Liu et al., 2010).

B class proteins are able to bind to specific DNA sequences named CArG boxes (for CCA/TrichGG; consensus sequence 5'-CC(A/T)$_6$GG-3') only as homo- and heterodimers or in higher-order complexes. While AP3 and PI of *A. thaliana* form obligate heterodimers, many B proteins from gymnosperms, early diverging eudicots, and monocots can also homodimerize (Goto and Meyerowitz, 1994; Winter et al., 2002; Wang et al., 2010). It has been hypothesized that homodimerization is the ancestral state of B lineage MADS-domain protein complex formation since the GGM2

protein of the gymnosperm *Gnetum gnemon* forms homodimers (Winter et al., 2002). The obligate B protein heterodimers in snapdragon and *A. thaliana* are required both for organ identity specification and to maintain B gene expression in an autoregulatory circuit (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Zachgo et al., 1995; Davies et al., 1996; Hill et al., 1998; Tilly et al., 1998)(. In addition to heterodimers of AP3/PI-like proteins, homodimers of AP3 orthologs have been found in basal eudicots like *Aquilegia vulgaris* (columbine) and *Papaver somniferum* (opium poppy) (Drea et al., 2007; Kramer et al., 2007)whereas homodimer formation of PI orthologs has been demonstrated only for monocots like the orchid *Phalaenopsis* (Tsai et al., 2008). However, a function could not be assigned to homodimers formed by either AP3 or PI orthologs.

The B class floral homeotic MADS-domain proteins are of the MIKC type because they possess a characteristic domain structure composed of the MADS, Intervening, Keratin-like and C-terminal domain. The N-terminally located and highly conserved MADS-domain is responsible for DNA binding. It is followed by the only weakly conserved I-domain. The K-domain contains three putative α helices, K1, K2, and K3 which mediate dimerization and the specification of protein-protein interactions (Jack, 2001). The K3- and C-domains of the *Antirrhinum majus* MADS-domain proteins SQUAMOSA, DEFICIENS, and GLOBOSA are required to mediate the assembly of protein multimeric complexes and the C-terminal domain of AP1 of *A. thaliana* encodes a transcriptional activation domain (Cho et al., 1999; Egea-Cortines et al., 1999) .

The C-terminal domain of B class MADS-domain proteins contains lineage-specific sequence motifs. A 16 amino acid long PI motif is found in orthologs of PI. In AP3 orthologs, the eu-AP3 motif occurs in addition to a PI-derived motif (Kramer et al., 1998). The experimental evidence aimed at elucidating the function of these C-terminal motifs is contradictory. Over-expression of truncated versions of AP3 and PI lacking the C-terminal motifs were not able to rescue *ap3* or *pi* mutants of *A. thaliana* (Lamb and Irish, 2003). Another study, however, showed that removing the C-terminal domains of AP3 and PI does not affect their ability to complement the *ap3* or *pi* mutants (Piwarzyk et al., 2007); similar observations have been made with CsAP3, a putative class B protein from the basal angiosperm *Chloranthus spicatus* (Su et al., 2008). These studies led the authors to conclude that the MIK- rather than the C-

terminal domain of AP3-like class B floral homeotic proteins determines functional specificity in the development and evolution of petals (Su et al., 2008). The PI-motif is also dispensable for ternary complex formation including those made up of PI, AP3, and SEP1 proteins when assayed with the yeast three-hybrid system (Piwarzyk et al., 2007). Moreover, *pi* mutants in *A. thaliana* can be rescued with the atypical wild type *Pisum sativum* PsPI protein that lacks the C-terminal domain including the PI motif (Berbel et al., 2005). Also, mutant and RNAi analyses of another legume PI protein, MtPI from *Medicago truncatula* which is similar in structure to PsPI also supports the view that the C-terminal domain is not required for B class protein activity (Tzeng et al., 2004; Benlloch et al., 2009). One wonders, therefore, why the PI-derived and the euAP3 motif within the C-terminal domain of class B proteins have been conserved for probably more than 100 million years of evolution (Su et al., 2008).

Here, we describe the class B floral homeotic mutant *seirene-1* (*sei-1*) in *Eschscholzia californica* (California poppy), a basal eudicot species from the Ranunculales order. The *sei-1* mutant is affected exclusively in floral development, with petals converted to sepals, and stamens to carpels or organs that show a mix of carpel- and sepal-like characteristics. The mutant phenotype results from a fast neutron induced insertion of a DNA fragment into the *EScaGLO* locus (related to *PI* of *A. thaliana* and referred to as *SEI* hereafter) causing the resulting open reading frame to encode a MADS-domain protein that lacks part of the C-terminal domain. The specificity of CArG-box binding of the sei-1 protein and its ability for dimeric protein interactions remain unchanged when compared to the wild type protein. Ternary complexes consisting of different combinations of B and E class proteins are also formed equally well. However, the sei-1 protein is unable to participate in trimeric protein interactions that form putative floral homeotic complexes. Our results suggest that the C-terminal domain mediates formation of ternary complexes when class C proteins are involved. We further hypothesize that the evolutionarily conserved PI motif is required for higher order complex formation in some species but irrelevant in others including *A. thaliana*.

Results

sei shows morphological defects of a B class floral homeotic mutant

Screening the homozygous progeny of a fast neutron irradiated mutant population of *E. californica* revealed the floral homeotic mutant *seirene-1* (*sei*) which is affected exclusively in floral organ formation. The mutant is called after the seirens in greek

mythology, who are females seducing sailors with enchanted singing to shipwreck on inaccessible cliffs of their island.

Wild type flowers of *E. californica* are composed of a sepal in the first whorl that generally dehisces as a cap during bud opening and four orange petals arranged in two whorls, six to eight whorls of stamens and two fused carpels constituting the gynoecium in the center of the flower (Fig. 1A and B). *sei-1* flowers (Fig. 1C, D, E) show a sepal with wild type morphology in the first whorl. The next two inner whorls comprise four sepal-like organs instead of petals, indicating a petal to sepal floral homeotic conversion. In the more central whorls chimeric organs are produced that show a mix of sepal and carpel characters (Fig. 1E). While the base of these organs is sepal-like they exhibit yellow stigmatic papillae on their apices (Fig. 1 C – E). Towards the center of the flower, the chimeric organs accumulate more carpel characteristics but are all unfused. These carpel-like organs also show a carpel-specific surface structure (Suppl. Fig. 1). The central whorl consists of a gynoecium of almost wild type appearance but with incomplete carpel fusion (Fig. 1D, E). The unfused or partially fused carpels grow into tube-like structures that are open at the top and thus fail to develop seeds as the ovules dry prematurely. In spite of the homeotic organ conversions, the organ number in *sei-1* mutants does not deviate significantly from the wild type (Fig. 1E).

Siblings of the *sei-1* mutant line in the heterozygous F_1-generation (heterozygous for *sei-1*) were inter-crossed to analyze the mode of inheritance. 92 F_2 flowering plants were observed and 22 of them exhibited the *sei* phenotype (χ^2= 0.056, p= 0.8129, df=1) indicating that the *sei* phenotype is caused by a mutation at a single locus. Heterozygous *sei-1*/*SEI* plants show a phenotype not deviating from the wild type, except for less than 5 % of the heterozygote plants in which one, or at most two, stamens develop into slightly petal-like organs, indicating that the *sei-1* mutation is recessive, albeit slightly incomplete.

SEI encodes the EScaGLO protein

At least four *AP3*- and *PI*-like and thus putative B class proteins are encoded in the *E. californica* genome: *EScaDEF1*, *EScaDEF2*, *EScaDEF3*, and *EScaGLO*. Except for *EScaDEF3*, these putative B class genes have been reported previously (Zahn et al., 2005). Phylogeny reconstructions (Suppl Fig. 2) based on a large dataset comprising many Ranunculales putative B class genes shows that *EScaDEF2* and *EScaDEF3* are

very closely related paralogs and closely related to *AP3-1* of *Papaver somniferum*. This suggests that they originated from a recent duplication event that occurred after the lineage leading to *E. californica* separated from the lineage leading to *P. somniferum*. *EScaDEF1* is the most likely ortholog of *AP3-2* of *P. somniferum*, and both genes fall into an orphan group of Ranunculales genes that does not form a well supported clade and includes genes from Papaveraceae as well as Ranunculaceae species. None of the three *E. californica* genes or their *P. somniferum* homologs cluster within the three groups of *AP3*-like genes observed from Ranunculaceae by (Kramer et al., 2003). The PI-like gene *SEI* forms a well supported clade with the Papaveraceae species *Sanguinaria canadensis ScPI*, but is only distantly related to the two *GLO*-like genes from *P. somniferum* (Suppl. Fig. 2).

Investigations of B class gene coding sequences in the *sei-1* mutant revealed changes in the transcript and protein sequence of the *EScaGLO* gene, termed *SEIRENE* (*SEI*) from now on (Fig. 1F and G). Sequencing 3' RACE PCR products revealed that the mutant plant's *SEI* transcripts include a premature stop codon at nucleotide position 590 of the coding sequence and an altered nucleotide sequence starting at position 539. As a consequence, the sei-1 mutant protein contains 17 changed amino acids and is 22 amino acids shorter than the wt SEI (Fig. 1F). Moreover, the highly conserved PI-motif found in the vast majority of angiosperm PI orthologs is absent in *sei-1* (Fig. 1F). Analyses of the genomic locus of *sei-1* revealed no sequence deviation compared to the wild type *SEI* locus in the first four exons, four introns, and 612 bp of sequence upstream of the start codon (Fig. 1G). However, the Fast Neutron irradiated plants have a DNA fragment originating from an unknown locus inserted into exon five after nucleotide 47. This inserted DNA sequence introduces a premature stop codon as well as a polyadenylation signal leading included in the *sei-1* transcripts (Fig. 1G, Suppl. Fig. 3).

The Fast Neutron irradiated mutant population of *E. californica* yielded only one mutant allele of *sei*. To obtain confirmatory evidence that the phenotype is associated with this gene, Virus-Induced Gene Silencing (VIGS) was employed to transiently down regulate *SEI* gene expression in *E. californica* (Suppl. Fig. 4A). The three first formed flowers of 62 plants treated with SEI-VIGS were observed morphologically (Suppl. Fig. 4A, B). Of the 186 flowers scored, 67 flowers did not show deviations from

the wt morphology, 81 flowers exhibited a mild phenotype that is characterized by petals that are slightly reduced in size and in shape intermediate between sepals and petals with orange wt color. In addition, in the position where in wt stamens develop, the SEI-VIGS plants show chimeric organs that develop stamen characters in combination with petaloid and carpeloid features. 38 flowers showed a strong phenotype upon SEI-VIGS treatment. This is characterized by partial homeotic conversions of petals into sepals, such that the petals are sepaloid in size and shape but retain the wt orange coloration except for a broad green stripe in the center. Organs that develop in the position of outer stamen whorls show the morphology of petaloid organs albeit with reduced size. In place of inner whorl stamens, single, unfused carpel-like organs develop (Suppl. Fig. 4B). RT-PCR was carried out on floral tissue at anthesis showing a reduced expression of SEI in the VIGS treated plants. This demonstrates that even a reduction in *SEI* expression is sufficient to induce homeotic changes as observed in the *sei-1* plants, albeit to a lesser extent (Suppl. Fig. 4C).

Expression of *AP3*-like and *PI*-like genes is decreased in the *sei-1* mutant

The *sei-1* mutant morphology is reminiscent of the class B floral homeotic mutants of *A. thaliana* and *A. majus* and thus expression analysis of the four putative class B floral homeotic genes of *E. californica EScaDEF1, EScaDEF2, EScaDEF3* (all *AP3-like),* and *SEI* (*PI*-like) was carried out by quantitative RT-PCR in wild type and mutant flowers one day before anthesis (Fig. 1H). In wild type plants, none of the class B genes is expressed at a significant level in sepals. In petals, *EScaDEF2, 3*, and *SEI* are expressed strongly, *EScaDEF1* only weakly. In inner (central) and outer (lateral) stamens the expression of B genes is generally lower than in petals with *EScaDEF1* being the most weakly expressed gene. No expression of B class genes is detected in the wild type gynoecium. In *sei-1* flowers, expression of all four B class genes is strongly reduced in the second whorl, the sepal-carpel intermediate organs and in carpels. While the expression of *EScaDEF1* is weak also in the wild type organs, expression of *EScaDEF2 and EScaDEF3* is significantly reduced in most sei-1 whorls and *SEI* expression is hardly detectable in the *sei-1* mutant.

In situ hybridization was carried out with an *SEI*-specific probe to obtain information on its expression pattern with a high temporal and spatial resolution (Fig. 1I-J). In stage 2 flowers (developmental stages of *E. californica* flower development according to

Becker et al., 2005) expression of *SEI* is restricted to the periphery of the floral meristem (Fig. 1I). The sepal primordium and the center of the floral meristem are devoid of *SEI* expression throughout all observed developmental stages (Fig. 1I-L). In buds of stage 3 when the petal primordia are initiated, *SEI* is expressed in the petal and stamen anlagen (Fig. 1). In stage 4, when the stamen primordia appear, *SEI* is restricted to petal and stamen primordia (Fig. 1K, L). In early and late stage 5 (Fig. 1L and M, respectively) when the gynoecium primordium emerges and the carpel walls elongate, *SEI* is expressed evenly throughout the developing stamens and the petal primordia.

The wild type SEI and the mutant sei-1 protein are capable of similar dimeric interactions and are both able to bind sequence-specifically to CArG boxes

The mutant sei-1 protein shows differences in sequence and length of the C-terminal domain when compared to the wild type sequence. Consequently, three experimental approaches were chosen to analyze how these changes may affect protein interaction behavior with all known putative floral homeotic proteins of *E. californica* (Table 1 and Suppl Fig. 5): First yeast two-hybrid (Y2H) analyses were carried out with the *E. californica* MADS domain proteins SEI, sei-1, EScaDEF1, EScaDEF2, EScaDEF3, the AGAMOUS-like proteins EScaAG1ΔC and EScaAG2ΔM (EScaAG1ΔC lacks the C-terminal domain which shows transcriptional autoactivation, and EScaAG2ΔM lacks the MADS domain), and the SEPALLATA3-like protein EScaAGL9. Further interaction assays were carried out, secondly by Bifluorescence Complementation (BiFC) and thirdly, by Electophoretic Mobility Shift Assays (EMSA). Interactions shown in the BiFC and EMSA assays were not quantified.

Taken together our data show that most protein interactions are not consistently observed with all three methods employed. But more importantly, differences in the protein dimerization behavior could not be detected between SEI and the mutated protein sei-1 in any of the assays. A summary of all data is presented in Table 1, the original data are found in Suppl. Figs 5, 6, and 7.

More detailed analyses show that all three DEF-like proteins are able to form homodimers in the Y2H system, but SEI, sei-1, EScaAGL9, EScaAG1ΔC, and

EScaAG2ΔM are incapable of homodimerization in the Y2H assays. However, SEI and sei-1 do show homodimerization in EMSA and BiFC assays. AGAMOUS-like and SEPALLATA-like proteins were not able to consistently form homodimers in all experiments such that EScaAG2 only homodimerized in the Y2H system, while EScaAG1 homodimerized only in BiFC and EMSA, and EScaAGL9 only when bound to DNA in EMSA assays.

Heterodimers consisting of SEI and any of the DEF-like proteins as well as of sei-1 and any of the DEF-like proteins were observed in Y2H and BiFC experiments, and for EScaDEF1 and 2 EMSA experiments were carried out that suggest similar interactions for SEI and sei-1. BiFC analyses show that EScaAGL9 can interact with EScaDEF1 and ESaDEF2 but not with EScaDEF3, SEI or sei-1.
All B class proteins, except SEI and sei-1 are also able to form heterodimers with EScaAG2ΔM but not with EScaAG1ΔC and EScaAGL9 in the Y2H assay.

The sei-1 protein is unable to form specific ternary protein complexes
As floral homeotic function very likely requires the formation of higher order protein complexes (Honma and Goto, 2001; Theissen and Saedler, 2001), trimeric interactions of the B, C, and E class proteins of *E. californica* were analyzed using the Yeast Three-Hybrid (Y3H) and a modified BiFC approach (Fig. 2 A-E). Ternary complex formation was observed in the Y3H system upon the addition of EScaAGL9. Four ternary complexes were observed: (i) EScaDEF1-EScaAG1-EScaAGL9 (B-C-E protein complex), (ii) EScaDEF2-EScaAG1-EScaAGL9 (B-C-E protein complex), (iii) SEI-EScaAG1-EScaAGL9 (B-C-E protein complex), and EScaDEF2-EScaAG2-EScaAGL9 (B-C-E protein complex). Remarkably, the complex comprising the mutant sei-1 protein (sei-1-EScaAG1-EScaAGL9) was not formed.

A modified BiFC approach, termed TriFC was carried out with all protein combinations that did not form dimers in the BiFC assay. A third protein was then delivered to the *in planta* assay system by simultaneous transformation of three plasmid constructs from which the proteins assayed are expressed. In some cases, the third protein was able to mediate interaction between the two proteins fused to

either the N-terminal or the C-terminal half of YFP inducing the reconstitution of the fluorescent YFP protein.
Table 2 shows a summary of all interactions analyzed by TriFC. With the TriFC assay, 13 ternary complexes were consistently observed to form *in planta* containing putative B, C, and E class proteins. Most importantly, we were able to detect similar differences in ternary complex formation between the SEI and sei-1 protein with TriFC and Y3H assays. A ternary B-C-E complex was formed when EScaAG1, EScaAGL9 and SEI were participating which failed to form when sei-1 was added instead of the wild type protein (Fig. 2 D,E). This suggests that the formation of specific ternary MADS complexes requires the C-terminal domain of SEI. Our combined Y3H and TriFC results show that the B-C-E complex consisting of EScaAG1-EScaAGL9-SEI which is most likely required for stamen identity is ablated when the C-terminal domain is deviant in sequence.

Two other complexes were also unable to form in the TriFC assay when the mutated protein was added instead of SEI: the C-B-B complex EScaAG1-EScaDEF2-SEI (Fig. 2 B, C) and the E-B-B complex EScaAGL9-SEI-EScaDEF1 are formed only with the wild type but not with the mutant protein (table 2), suggesting that also these complexes, when formed in *E. californica*, require an intact C-terminal domain. Interestingly, two out of three complexes sensitive to sequence deviation in the C-terminal domain contain the class C protein EScaAG1 indicating that the C-terminal domain of SEI is required to mediate the formation of floral homeotic complexes that incorporate class C floral homeotic proteins.

In other combinations the changed C-terminal domain of sei-1 appears to be not relevant for trimeric interaction (Table 2). Not surprisingly, we found that the ability for ternary complex formation is dependent on the orientation of the proteins in the complex. Our results show that several complexes such as EScaAGL9-EScaDEF3-SEI are only formed when SEI is not fused to a YFP fragment suggesting that this complex formation depends on the proper positioning of the proteins and that this positioning is disturbed by the YFP fusions. Another alternative explanation for impaired protein complex formation in the TriFC assay could be that while MADS domain proteins form complexes they pull the YFP fragments too far apart for reconstitution. However, a general pattern such that a specific protein cannot interact

when fused to YFP is not deducible from our data and it apparently depends on the individual complex to be formed.

Variations in the PI motif sequence result in changes of protein interaction abilities

As the C-terminal domain of PI orthologous proteins contains the PI motif, we investigated sequence and function of this highly conserved stretch of amino acids. Firstly, an alignment of 36 PI orthologous proteins from all major groups of angiosperms was produced and secondly, a sequence logo representing the conserved amino acids within the PI motif was produced. The most highly conserved amino acids are Pro at position 2 of the PI motif, the three amino acids Val, Glu, and Pro, spanning positions 7-9 in the central region of the motif and the two residues Asn and Leu near the C-terminal end of the PI motif at positions 13 and 14. Between these positions, stretches of less conserved residues were identified (Fig. 2F).
The alignment shows that different species of angiosperms exhibit lineage specific changes within the PI motif, apparently and most dramatically in the Brassicaceae family, where the PI protein of *A. thaliana* significantly differs at the N-terminal end of the PI motif, with GQFGY in the first five positions instead of the consensus sequence MPFAF (Fig. 2G). This difference in amino acid sequence is shared among the Brassicaceae family, but is absent from the PI motif of the GLO-like protein of *Cleome spinosa*, which is a member of the Cleomaceae, the most closest relative of the Brassicaceae (Suppl. Fig. 9). Solanaceae PI proteins, GLO from *A. majus* as well as PI proteins of many rosids, such as poplar, apple, and papaya share the PI motif's consensus sequence. Monocots, particularly grasses and orchids have a slightly diverged PI motif at positions three and four, and additionally at amino acid positions 11 and 12 of the PI motif (Suppl. Fig.9). The SEI protein includes the highly conserved N-terminal part but differs in the C-terminal part of the PI motif, which is otherwise highly conserved among PI proteins (Fig. 2F).

To investigate the role of the PI motif in the formation of multimeric complexes of B, C, and E class proteins, the PI motif of SEI was replaced by the PI motif of the *A. thaliana* PI protein by site directed mutagenesis. Specifically, the first five amino acids of the *A. thaliana* PI motif were introduced into the SEI protein and the multimer formation was observed with our modified TriFC. These amino acid replacements resulted in the

failure of the modified SEI to form multimers with EScaAGL9 and EScaAG1, very similar to what has been observed for sei-1 (Fig. 2H-J). This indicates that the first five amino acids of the PI motif are required for multimerization of the poppy B, C, and E proteins into a putatively homeotic complex required for stamen identity (Fig. 2H).

Discussion

The *sei-1* mutant is a B class floral homeotic mutant

We have described the mutant phenotype of *sei-1*, the structure of the *SEI* wild type and *sei-1* mutant locus and carried out expression analysis of the four putative B class genes in *E. californica* to help characterize the *sei-1* mutant in detail and to understand the molecular mode of action of the *SEIRENE* gene. The *sei-1* phenotype resembles that of a typical B class mutant, as in *sei*, petals are replaced by sepals and stamens are transformed into carpels. In position between these two ectopic organ types, mosaic organs are regularly formed, which are morphologically intermediate between sepals and carpels. Our data show that the mutation causing the *sei-1* phenotype is due to an insertion of genomic DNA in the *PI*-like gene *SEI.* This insertion changes the open reading frame to encode a MADS domain protein with a shortened and modified C-terminal domain.

Intriguingly, this mutant is a eudicot class B floral homeotic mutant in which the petal and the stamen whorls both are converted and that produces the same number of organs as the wild type. In *Arabidopsis*, strong *ap3* and *pi* mutants exhibit a similar homeotic conversion of petals and stamens as in *sei,* but the number of floral organs is reduced in both mutants (Bowman et al., 1989, 1991; Jack et al., 1992; Tröbner et al., 1992). In the *def* and *glo* mutants of *A*. *majus*, the number of converted floral organs is variable and the fourth whorl wild type gynoecium is missing (Sommer et al., 1990; Tröbner et al., 1992; Riechmann et al., 1996a; Riechmann et al., 1996b). In the maize *AP3*-like gene mutant *silky1* (*si1*), the number of floral organs formed does not seem to be affected, but organ identity is impaired in that the stamens do not abort in the ear spikelets as in wild type but develop into pistil-like organs (Ambrose et al., 2000). Also the *superwoman1* mutant of rice shows an unaltered number of floral organ primordia in whorl three and four (Nagasawa et al., 2003).

It appears likely that mutations in the higher eudicot B function, but not in the monocots and early diverging eudicot genes also impair the ability of the floral meristem to produce the proper wild type floral organ number, resulting in a lower or

varying number of floral organs in class B mutants. This suggests that a new level of gene regulation was established which links B gene expression to floral meristem activity after the basal eudicot lineages diverged from the lineage leading to the eudicot crown group.

B-B and B-B-E protein complexes are not sufficient for transcriptional autoregulation of B gene expression

The *sei-1* mutant transcript shows an altered nucleotide sequence in the 3' part of its CDS encoding a shorter protein that lacks the PI motif (Fig. 1F). *SEI* is likely the only *PI*-like gene while three *AP3*-like genes, *EScaDEF1*, *EScaDEF2*, and *EScaDEF3* are present in the *E. californica* genome. In the *sei-1* mutant, the expression of *SEI* as well as of the three *DEF*-like genes is severely reduced possibly because organ identity of the petals and stamens, organs of strong B class gene expression in the wild type cannot be established, and so these organs are replaced by carpels and sepals in the *sei-1* mutant.

Heterodimerization between SEI and EScaDEF proteins is supported by all three assays carried out and these heterodimers are also able to bind to CArG boxes. Thus, the mutant sei-1 protein's heterodimerization and CArG box binding abilities do not severely deviate from the wild type protein at a level detectable by the analyses carried out.

Previous work has shown that several B-class proteins such as AP3 actively maintain their expression by binding of AP3/PI-like heterodimers to CArG-box motifs in their own promoter (Tröbner et al., 1992; Hill et al., 1998; Honma and Goto, 2000). However, in *A. thaliana*, the overexpression of AP3 and PI alone is not able to transform leaves into petals, and is unable to activate the transcriptional autoregulatory loop outside of the flower, suggesting that the AP3-PI dimer alone is unable to regulate target genes. The concerted overexpression of AP3, PI, and SEP3 is able to convert leaves into petals and for transcriptional autoregulation of B genes indicating that SEPALLATA proteins are important to form floral homeotic complexes and for transcriptional autoregulation in *A. thaliana* (Goto et al., 2001; Honma and Goto, 2001; Pelaz et al., 2001). Our data show that all *E. californica* B genes are significantly down-regulated in the mature floral organs of the *sei-1* mutant suggesting

that the transcriptional auto activation is interrupted. However, the sei-1 mutant protein is able to participate in B-B-E complexes similar to the wild type SEI protein. If the regulation of B gene expression is similar to what has been observed in *A. thaliana* we would expect a wild type expression of all B genes in the *E. californica sei-1* mutant. As this is not the case we can hypothesize that a sei-1-EScaDEF-EScaAGL9 protein complex is not sufficient to activate B gene expression in *E. californica* and propose a B-C-E complex for transcriptional auto activation for the B genes. Alternatively, complexes including sei-1 instead of SEI might not be stable *in planta* or only insufficiently activating transcription.

Floral homeotic protein complexes incorporating C class proteins require the C-terminal domain of SEI

While the function of the PI motif of the *A. thaliana* PI seems difficult to elucidate, our combined Y3H and TriFC data allow an assessment of the PI motif in the *E. californica* SEI protein (Fig. 2A-E and table 2). Based on TriFC experiments we are able to show that the sei-1 mutant protein interacts in B-B-E complexes of all tested orientations similar to the wild type protein (Table 2). This indicates that, also in *E. californica*, the C-terminal domain of SEI is not required for B-B-E complex formation. The only exception is the E-B-B complex composed of EScaAGL9-YFP^n, SEI-YFP^c, and EScaDEF1 which is only formed with the wild type SEI and not with the mutant protein (Table 2). However, as EScaDEF1 is expressed at a very low level in all floral organs (Fig. 1F) this interaction is possibly of no relevance and the role of EScaDEF1 remains obscure generally.

Our combined Y3H and TriFC data show that participation in ternary complex formation of B and C or B, C, and E proteins differs between the wild type and mutant sei-1 proteins. More specifically, complexes composed of EScaAG1, SEI, and EScaAGL9 or EScaAG1, EScaDEF2, and SEI can be formed in the Y3H and TriFC systems only with the wild type protein but not with the mutant sei-1 protein (Fig. 2B-E). These findings indicate that the C-terminal domain of SEI is specifically required for mediating higher order complexes incorporating C function proteins but is of less importance when a ternary complex is formed that comprises B and E proteins only. Only when a C-function protein is added to form a floral homeotic complex the importance of the PI-motif hidden in the C-terminal domain becomes obvious. Thus,

our observations provide insight into the selective formation of floral homeotic complexes specifying stamen organ identity *in planta*.

The PI motif represents a rapidly evolving Short Linear Motif (SLIM)

Our data indicate that the sei-1 protein fails to participate in higher order protein complexes with EScaAG1 and EScaAGL9 unlike the wild type SEI protein which strongly suggests that that the C-terminal domain mediates these interactions. Surprisingly, previous studies largely concluded that the C-terminus of PI is of no importance for the protein's function in rosids but is required for higher order complex formation in *A. majus*. The sei-1 mutation provided a tool to study the function of the C-terminal domain and to possibly understand the reason for the conflicting results on the function of the PI motif in an evolutionary way.

The PI motif is highly conserved in sequence and position in almost all angiosperm PI orthologous proteins (Fig. 2F and Suppl. Fig. 9), with only two exceptions: (i) basal angiosperm PI-like proteins from *Amborella* and *Nuphar* deviate mostly in the N-terminal conserved part of the PI motif, (ii) Fabaceae PI proteins have a deleted C-terminus including the PI-motif, and (iii) PI-like proteins from the Brassicaceae family including *A. thaliana*, which also show an altered amino acid composition at the N-terminal part of the PI motif resulting in the loss of otherwise highly conserved residues (Suppl Fig.9).

This suggests that the difference in the *A. thaliana* PI motif may translate directly into differences in protein interaction abilities. To test this hypothesis we transformed the N-terminal part of the PI motif of the SEI amino acid sequence into the corresponding sequence of PI from *A. thaliana* by four amino acid exchanges (Fig. 2G). Similarly to the sei-1 mutation, this amino acid exchange results in a protein that is unable to mediate interactions with C and E class proteins in a ternary complex (Fig.2 H-J). The *A. thaliana* PI motif has apparently lost the ability to mediate formation of these specific homeotic protein complexes suggesting that the C-terminal domain of the *A. thaliana* PI protein is indeed not required for mediating specific interactions within floral homeotic complexes. This hypothesis is supported by previously published data on the ability of the *PI* ortholog *PsPI* from *P. sativum* that lacks a C-terminus to fully complement the *pi-1* mutant (Berbel et al., 2005; Piwarzyk et al., 2007). Moreover, the

genomes of other Fabaceae such as *M. truncatula* and *Lotus japonicus* also encode PI proteins which lack a large portion of the C-terminal domain including the PI motif and are still able to carry out floral homeotic B function suggesting that in a larger fraction of the rosids the PI motif is not required for the B function in these species (Dong et al., 2005; Benlloch et al., 2009).

Detailed sequence analysis of the PI motif reveals lineage-specific differences in the extremely conserved amino acid residues in the N-terminal part of the motif (Suppl Fig. 9). While rosids like *Populus trichocarpa* or *Carica papaya* show the consensus residues, the Brassicaceae accumulated lineage-specific changes leading to the loss of a hydrophobic residue and a proline and the legumes lost the PI motif altogether. These two amino acids lost in the Brassicaceae are conserved in PI orthologs of all other randomly selected representatives of the magnoliids, monocots, basal eudicots, and asterids (Suppl Fig. 9).

We hypothesize that the N-terminal part of the PI motif represents a Short Linear Motif (SLiM). SLiMs are short stretches of three to ten amino acids that are often part of an otherwise unstructured region of the protein and they play crucial roles in protein interaction networks. In many examples they were shown to mediate specific protein interactions. SLiMs show different degrees of sequence conservation, some positions are more tolerant to exchanges allowing for a high degree of evolutionary plasticity, while others are extremely conserved (Neduva and Russell, 2005; Diella et al., 2008; Wagner and Lynch, 2008)). A very well described example of protein modification via SLiM evolution is the Hox/HOM protein FUSHI TARAZU (FTZ). In the phylogenetically basal grasshopper *Schistocerca*, FTZ interacts only with the protein EXTRADENTICLE (EXD) via the short YPWM motif, and this interaction confers homeotic function. In the beetle *Tribolium*, FTZ additionally acquired the short motif LXXLL that mediates interaction with the protein FTZ-F1, a protein-protein interaction required for proper segmentation. However, the *Drosophila* FTZ protein has lost the ancient motif YPWM and hence, the ancestral interaction with EXD and consequently also its homeotic function while it has retained the motif LXXLL and now functions exclusively in segmentation in these flies (Löhr et al., 2001; Löhr and Pick, 2005).

Our sequence analysis suggests that the SLiM hidden in the N-terminal part of the PI motif required for B-C-E complex formation evolved in the angiosperm lineage after the Amborellaceae and Nymphaeaceae diverged from the lineage that led to all other angiosperms, but before the magnoliids evolved (Fig. 3, Suppl. Fig. 9). It was then maintained in at least one PI-like protein in the different angiosperm lineages, except that the lineage leading to the Brassicaceae after their split from the Cleomaceae lost the important residues for specifically mediating floral homeotic complexes. Independent from the loss of only a few conserved residues in the Brassicaceae, at least part of the Leguminosae lost the C-terminal domain completely (Fig. 3, Suppl. Fig. 9).

The loss of the protein interaction SLiM which is crucial for establishing floral homeotic complexes required for stamen organ identity will ultimately lead to sterility of the affected plant. However, compensatory mutation in the other participating proteins, such as SEP-like or AG-like proteins may be able to overcome this failure in protein complex formation and can be hypothesized to have arisen in Fabaceae and Brassicaceae.

The PI motif has been conserved during evolution for dozens of millions of years, but its functional importance remained controversial. In conclusion, our results demonstrate what a big difference a subtle small change, concerning an enigmatic sequence element hidden in the C-terminal domain, can make for floral organ identity.

Materials and Methods

Establishment and characterization of an *E. californica* mutant library

Seeds exposed to Fast Neutron irradiation were recorded for their germination and survival rates carried out in growth rooms at 20 °C with 16 hours light at 70 µmol s^{-1} m^{-2} over a period of 21 days. Seeds irradiated with 40 Gy were used for all further experiments and cultivated as explained in detail earlier (Wege et al., 2007). Two F_0 plants were crossed with each other to produce the F_1 generation (*E. californica* is self-sterile). F_1 sibling plants were then interbred to produce homozygous recessive genotypes in the F_2 generation.

EScaDEF3 identification and expression analysis of *AP3/PI*-like genes

RNA was extracted from tissue samples of all floral whorls of *E. californica* wild-type and *sei* with the OLS Plant RNA isolation kit (OLS Life Sciences, Bremen, Germany). 500 ng of total RNA with an Oligo (dT) primer was used to synthesize first strand cDNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). EScaDEF3 coding sequence was amplified with 3`RACE using the RACE-DEF3fw primer which was derived from NG sequencing data (Wall et al., 2009), and AB07 rev, cloned into pGEM and sequenced.

cDNA pools were diluted 1:50 for subsequent RTq-PCR analysis, using *Actin2* and *GAPDH* gene expression as reference genes. RTq-PCR assay design and analysis has been published previously and follows the MIQE guidelines (Bustin et al., 2009; Yellina et al., 2010). Primer sequences for *EScaDEF1, EScaDEF2, EScaDEF3*, and *SEI* as well as UPL probe sequences (Roche, Germany) are provided in Suppl. table 1. Expression was measured with three technical replicates for each of the two biological replicates. *In situ* hybridization of *SEI* transcripts was carried out on sections of floral buds of consecutive developmental stages with DIG-labeled probe encompassing nucleotides 509 of the *SEI* coding sequence to nucleotide 53 into its 3'UTR (198 nt total length) as described earlier (Orashakova et al., 2009).

Phylogeny reconstruction and PI-motif analysis

Nucleotide sequences of *AP3* and *PI* orthologs from Ranunculales, *GLO* and *DEF* of *A. majus,* and *PI* and *AP3* of *A. thaliana* were gathered from the NCBI database (http://www.ncbi.nlm.nih.gov) and translated *in silico* with BioEdit (Hall, 1999). The amino acid sequences were aligned using CLUSTALW2 using default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2). Neighbor-Joining analysis using observed distances and 1000 bootstrap replicates were employed on the protein alignment spanning amino acid position 25 of the conserved MADS domain to the end of the K domain with the program SEAVIEW 4 (Gouy et al., 2010). The *GGM2* sequence of the gymnosperm *Gnetum gnemon* was used as an outgroup representative.

A collection of 37 PI amino acid sequences from all major angiosperm lineages was aligned using CLUSTALW2 (http://www.ebi.ac.uk/-Tools/msa/clustalw2) and used to build a sequence logo representation of the conservation of individual positions in the alignment using default parameters employing Weblogo 2.8.2 (http://weblogo.berkeley.edu) (Crooks et al., 2004).

Molecular characterization of the *sei-1* mutant locus

3'RACE: Coding sequences of *EScaDEF3* and *SEI* from wild-type and *sei-1* mutants were PCR amplified from cDNA pools with gene-specific forward and the AB07 reverse primer. Amplified fragments were cloned using the pDRIVE cloning kit (Qiagen, Hilden, Germany) and sequenced. The EScaDEF3 sequence has been deposited in GenBank (acc. no. HE573239)

RAGE: Genomic DNA from wild-type and *sei* plants was isolated with the Peqlab Mini Gold kit (Peqlab, Erlangen, Germany). DNA was treated with the restriction enzymes BamHI, EcoRI, HindIII, XbaI, XhoI, and blunt-ended with T4 DNA polymerase and the blunt-ended DNA was ligated to the RAGE-adaptor (Siebert et al., 1995). Cloning of the *SEI* locus was performed in a nested PCR approach with the PHUSION polymerase (Finnzymes, Espoo, Finland). The cycling profile: 94°C for 25 sec, 67°C for 3 min for 7 cycles and 94°C for 25 sec, 65°C for 3 min for 35 cycles was used. The secondary PCR was done with the following PCR conditions: 94°C for 25 sec, 67°C for 3 min for 5 cycles and 94°C for 25 sec, 65°C for 3 min for 20 cycles. The obtained genomic DNA fragments were sequenced.

Virus-induced gene silencing (VIGS): A fragment of the *SEI* cDNA (nucleotide positions 215 of the coding sequence to position 43 of the 3'UTR (489 nucleotides) were amplified with primers containing restriction sites. *SEI* was digested with *Bam*HI and *Xho*I and cloned into the equally digested pTRV2 vector to create pTRV2-*SEI*. *Agrobacterium tumefaciens* GV3101 was used to inoculate 3 week old *E. californica* seedlings as described previously (Orashakova et al., 2009) and plants were grown under the conditions described in (Wege et al., 2007).

Protein interaction analysis

Yeast Two-Hybrid (Y2H): *EScaDEF1*, *EScaDEF2*, *SEI* and *sei-1* open reading frames *(*ORFs*)*, all lacking their MADS box*,* were amplified from cDNA and cloned in-frame into the yeast expression vectors pGADT7 and pGBKT7 (Clontech, Mountain View, USA). Y2H analyses were carried out as described previously (Erdmann et al., 2010). EScaAG1 ΔC lacking the the C-terminal domain (ΔC) and EScaAG2ΔM lacking only the MADS domain were assayed. The full-length EScaAGL9 was cloned into both yeast expression vectors.

The strength of interaction as observed in the Y2H experiments was classified in three categories: strong, when yeast growth on SD media (-Leu/-Trp/-His) with 3 mM 3-AT at 30°C was observed in all dilutions, all yeast colonies were stained blue after the ß-gal assay, and the interaction was observed regardless of the vector the protein was expressed from; and no interactions. Weak interactions show yeast growth in undiluted and 1:10 dilutions only, all colonies were stained blue after the ß-gal assay and protein interactions were observed in at least one vector combination. The Y2H experiments were carried out in at least three biological replicas.

Yeast Three-Hybrid (Y3H): *EScaAG1ΔM* and *EscaAG2ΔM* ORFs were cloned into the pGADT7 and the full-length *EScaAGL9* ORF was cloned into the ternary vector pTFT1 (Egea-Cortines et al., 1999) and the *AP3/PI*-like ORFs were used in pGBKT7 without their MADS box. All tested combinations were co-transformed into AH109 yeast cells and selected on SD media lacking Leu, Trp and adenine (Ade). To quantify the interaction of putative B class proteins with EScaAG1, EscaAG2 and EScaAGL9, yeast-three-hybrid β-Galactosidase liquid assays using ONPG as substrate were employed (Miller, 1972). Three to six independent clones for every combination and three technical replicates for each clone were used to determine the β-Galactosidase activity.

Electrophoretic mobility shift assays (EMSA) were conducted as described previously (Melzer and Theissen, 2009) except that approximately 400 ng of polydI/dC instead of salmon sperm DNA was used as nonspecific competitor for every binding reaction. Full length coding sequences of *EScaDEF1*, *EScaDEF2*, *SEI*, *sei1, EScaAG1,* and *EScaAGL9* were amplified and cloned into the *in vitro* translation vector pSPUTK. 2 µl of *in vitro* translated protein and about 0,1 ng of labelled DNA probes were used per reaction. Co-translation was performed when two proteins were assayed for heterodimer formation. The CArG-box encoded on the DNA probe was derived from the regulatory intron of *AGAMOUS* from *A. thaliana*. Sequence of the complete probe was 5'- AATTC GAAAT TTAAT TATAT TCCAA ATAAG GAAAG TATGG AACGT TGAAT T-3' (CArG-box is underlined). As specificity control, a probe with the same nucleotide composition but in randomized order was used. Sequence of this probe was 5'-AATTC ATAAA ACGGC AAGGA GAATT ATATT TTTAT GATGA ACATA TGAAT T-3'.

Bifluorescence Complementation (BIFC) was carried out according to (see (Hu et al., 2002). Full length sequences of *EScaDEF1, EScaDEF2, EScaDEF3, SEI,*

sei1, EScaAG1, and EScaAGL9 were cloned, with their native stop codon deleted, into the BiFC vectors pNBV-YC and pNBV-YN (Walter et al., 2004). All pNBV-YC and pNBV-YN vector constructs were verified by sequencing and subsequently cloned into the plant expression vector pMLBART by *Not*I digestion. All pMLBART constructs were transformed into the *A. tumefaciens* strain GV3101. As a positive control the vector constructs pSPYCE-35S/bzip63yc and pSPYNE-35S/bzip63yn were employed (Walter et al., 2004). The silencing suppressor protein p19 under the control of the ubiquitous 35S promoter (pBIN61-P19) was kindly provided by David Smyth, Monash University, Australia. Leaves of four week old *Nicotiana benthamiana* plants were inoculated with mixtures of *A. tumefaciens* strains carrying pMLBART-YN and pMLBART-YC constructs in different protein combinations and additionally an *A. tumefaciens* strain harbouring the p19 plasmid to suppress RNA silencing response in transformed plant cells. To detect trimeric interactions, a third coding sequence without YFP fragment was expressed under the control of the 35S promoter from the pMLBART vector that was co-transformed into *N. benthamiana*. The YFP fluorescence signal demonstrating protein-protein interactions in living plant cells was observed 3-4 days after inoculation. The BiFC experiments were carried out in at least three biological replicas.

Site directed mutagenesis was done according to (Wang and Malcolm, 1999) , two sets of primers (Suppl.Tab1) were use to introduce multiple nucleotide substitutions simultaneously into the SEI open reading frame to change the N-terminal sequence of the PI motif of SEI into the N-terminal part of the PI motif of the *A. thaliana* PI protein. The resulting ORFs is SEImPIn in which the first five aa of the *A. thaliana* PI motif replace the *E. californica* PI motif. A two-stage PCR was employed, and the resulting PCR products were digested with DpnI to remove non-mutated vector of the original PCR template. Mutated variants were sequenced and cloned into pMLBART as described above.

Supplemental online material

Supplemental Figure 1: SEM of wildtype organs and *sei-1* carpel-like organs

Supplemental Figure 2: Extended phylogeny of Ranunculales AP3/PI-like proteins.

Supplemental Figure 3: Amplification of the sei-1 mutant transcript

Supplemental Figure 4: SEI-VIGS phenotypes and RT-PCR expression analysis of the SEI-VIGS treated plants.

Supplemental Figure 5: Yeast-two hybrid growth assay of floral homeotic proteins of *E. californica*

Supplemental figure 6: Protein dimerization analysis by BiFC.

Supplemental figure 7: DNA binding of *E. californica* MADS domain proteins.

Supplemental figure 8: Ternary protein complex formation was analyzed by TriFC.

Supplemental figure 9: Alignment of PI protein sequences and their PI motif.

Supplemental table 1: A list of oligonucleotides used in this study

Figure Legends

Figure 1: The *sei-1* phenotype and expression analysis of class B genes in California poppy. (A) Wild type flower, **(B)** wild type California poppy floral organs. **(C)** *sei-1* flower showing homeotic conversions of petals into sepals and stamens into carpels, **(D)** *sei-1* flower with ectopic sepals peeled away, **(E)** Overview of the *sei-1* floral organs. The arrows indicate the central gynoecium. **(F)** Amino acid alignment of the wild type SEI and the mutant sei-1 proteins. Regions of sequence identity are highlighted in grey, the MADS-, I-, and C-domains, and the proposed amphipathic helices of the K domain are indicated by boxes, and the conserved c-terminal *PI* motif is underlined. The start position of the protein sequence change caused by the genomic DNA insert is marked by an asterisk **(G)** Organization of the *SEI* genomic locus in the wild type and *sei-1* mutant plants. Protein-coding portions of exons are shown as black boxes, 3'UTR as white boxes, insertion of random genomic DNA of *E. californica* in the *sei-1* locus is marked with crosses. The numbers above the exons indicate exon length. The start codon is symbolized by a horizontal arrow, the stop codon and poly-adenylation (pA) site by vertical arrows. **(H)** Expression analysis by qRT-PCR indicating the relative expression levels of *EScaDEF1*, *EScaDEF2, EScaDEF3,* and *SEI* in wild type (left) and *sei-1* (right) floral organs. Abbreviations: ca, organs with only carpel-like characteristics; gyn, central gynoecium; se, organs with only sepal-like characteristics; se/ca, organs with a mix of sepal and carpel characteristics. Stars above the bars indicate significant decrease of expression in *sei-1* when compared to wild type expression. *sei*-se was compared to wt petals and *sei*-ca was compared to wt inner and outer stamens. **(I)** - **(M**) *in situ* hybridization pattern of *SEI* in longitudinal sections of Californica poppy buds of stage 3 (I), early stage 4 (J), late stage 4 (K), early stage 5 (L), and late stage 5 (M). Stages according to (Becker et al., 2005).

Abbreviations: fm, floral meristem; gyn, central gynoecium; pe, petals; pp, petal primordia; se, sepals; st, stamens; stp, stamen primordia. Scale bar is 100 µm.

Figure 2: Analysis of the ternary complexes of B, C, and E class proteins formed in planta and yeast (A) – (E) and analysis of the PI motif of SEI and PI (F) – (G). **(A)** Ternary complex formation of MADS domain proteins was analyzed with the Y3H system and quantified with the β-galactosidase assay. The light grey columns show interaction strength of the proteins expressed from the bait vector pGBKT7 (BD) with proteins expressed from the prey vector pGADT7 (AD) together with the empty ternary vector pTFT1. The black columns show interaction strength when pTFT1 contains the EScaAGL9 CDS. Stars above the columns indicate significant differences in reporter gene activation between empty pTFT1 and pTFT1-EScaAGL9 interactions, indicating formation of ternary complexes. **(B**) **–(E)** Multimeric BiFC experiments showing trimeric complex formation of B, C, and E class proteins. The partial YFP fusion constructs are as follows: **B)** EScaAG1:YFP^N- EScaDEF2: YFP^C-SEI ; **C)** EScaAG1:YFP^N- EScaDEF2: YFP^C-sei-1; **D)** EScaAG1:YFP^N- EScaAGL9: YFP^C-SEI , and **E)** EScaAG1:YFP^N- EScaAGL9: YFP^C-sei-1. **(F)** Sequence logo representation of the PI motif from selected PI-like proteins across angiosperms listed in Suppl. Fig. 9. Numbers refer to the positions of the amino acid within the PI motif alignment. **(G)** The sequence of the PI motifs of PI and SEI are shown and the differing amino acids are marked in red. **(H) – (J)** TriFC interactions of *E. californica* with the modified SEI proteins. **(H)** EScaAG1:YFP^N- EScaAGL9: YFP^C-SEI, **(I)** EScaAG1:YFP^N- EScaAGL9: YFP^C-sei-1, **(J)** EScaAG1:YFP^N- EScaAGL9: YFP^C- SEImPIc.

Figure 3: Hypothesis of gain and loss of the PI motif SLIM. Schematic and highly simplified representation of the phylogeny of angiosperms (based on (Soltis et al., 2011) indicating the postulated appearance of the PI-motif SLIM and independent losses of PI-motif parts within the Rosids based on the sequences listed in Suppl. Fig. 9. Above the branches species names are given and the clades they represent are shown in brackets. The SLIM required of B-C-E ternary complex formation appeared before the divergence of the Magnoliidae from the rest of the angiosperm lineages and is indicated by an arrow. While it is conserved in sequence and position in representatives of the Magnoliidae, Monocotyledonae, Ranunculales, and Asteridae, it was lost in several Rosidae species. A white star indicates loss of a large portion of

the C-terminal domain of the PI-like genes including the entire PI motif. The grey star symbolized the loss of the C-terminal part of the PI motif and the black star represents loss of the three amino acid residues within the postulated SLIM found in all analyzed Brassicaceae PI orthologs.

Acknowledgements: We are very grateful for the help of Robert Erdmann with the initial yeast two-hybrid analysis, and Tezz Quon and Martin O'Brian for the thorough introduction to Bifluorescence Complementation. Anna Fees cloned and sequenced the *EScaDEF3* gene sequence. We thank Christian Gafert and Kenny Peuker for help with the EMSAs. Ralf Dringen allowed the very much appreciated use of his epiluorescence microscope for BiFC and TriFC. David Smyth has provided the pNBV vectors and numerous very helpful suggestions and encouragement. Angelika Trambacz and Werner Vogel help with taking care of the poppy plants is warmly appreciated. This work was made possible by the DFG grant BE 2547/6-1 and 6-2 and support of the University of Bremen to A.B.

Author contributions: ML performed all experiments except for the *in situ* hybridizations, some BiFC and TriFC, and EMSA assays; SO performed the *in situ* hybridization; SL performed parts of the BiFC and TriFC; RM performed the EMSAs; GT and AB analyzed data; AB wrote the paper with the help of all coauthors.

Table 1: Summary of dimeric protein interaction between the putative B, C, and E class proteins of *E. californica*. The proteins used in the respective assays are listed in the left column and in the top row. Protein dimer formation was assayed with Y2H (left box) and BiFC (middle box). Additionally, the abilities of the protein dimers to interact with CArG boxes were analyzed with EMSA (right box). The original data on which this table is based can be viewed in the supplemental figures five to seven. A black box displays strong interactions, a grey box medium interactions and white boxes no detectable interactions in the Y2H assays. Interaction strength was not quantified in EMSA and BiFC experiments. Proteins listed on the left side were fused to the activation domain in the Y2H system and to YFPn in the BiFC system. Proteins listed on top were fused to the binding domain of the Y2H system and to YFPc in the BiFC assay. Full-length proteins were used for the BiFC and EMSA assays, all proteins for the Y2H experiments were lacking the MADS domain.

	ESca DEF1	ESca DEF2	ESca DEF3	SEI	sei-1	ESca AG1	ESca AG2	ESca AGL9
ESca DEF1	■□□							
ESca DEF2	▒■□	■■□						
ESca DEF3	▒□	■□	■□					
SEI	■■■	■■■	▒■	□■■				
sei-1	■■■	■■■	▒■		□■■			
ESca AG1	□■	□■	□□	□■	□■	□■■		
ESca AG2	▒	■	▒	▒	▒	□	■	
ESca AGL9	□■	□■	□□	□□	□□	□■	□	□□■

Table 2: Summary of TriFC assay results analyzing the ability of trimeric complexes formation of MADS domain proteins. Fusion proteins used for the TriFC: YFP^N-protein 1; YFP^C-protein 2; protein 3, abbreviations of the protein names: AGL9, EScaAGL9; C, EScaAG1 and EScaAG2; AG1, EScaAG1; DEF1, EScaDEF1; DEF2, EScaDEF2; DEF3, EScaDEF3; SEI/sei-1, SEIRENE and sei-1. Protein complexes that show differences between the wild type SEI and the mutant sei-1 protein are marked with grey boxes.

Multimer composition	**Multimer formation**	**No multimer formation**	**Test not possible: dimeric interactions of nYFP – cYFP fusion proteins**
B – B - C			DEF-like – SEI – C DEF-like – sei-1 - C SEI – DEF-like – C sei-1 – DEF-like - C
C – B – B	**AG1 - DEF2 – SEI**	**AG1 – DEF2– sei-1**	AG1 – SEI/sei-1 - DEF2
		AG1 - DEF1 - SEI	AG1 – SEI/sei-1 - DEF1
		AG1 - DEF1 - sei-1	
	AG1 - DEF3 - SEI		AG1 - SEI/sei-1 - DEF3
	AG1 - DEF3 – sei-1		
	AG1 - DEF3 - SEImPI		
B – C - B		DEF3 - AG1 - SEI	All other combinations
		DEF3 - AG1 - sei-1	
B – E – B	DEF2 – AGL9 – SEI	DEF3 - AGL9 - SEI	DEF1 - AGL9 - SEI/sei-1
	DEF2 – AGL9 – sei-1	DEF3 - AGL9 - sei-1	
C – E – B	AG1 - AGL9 - DEF2	AG1 - AGL9 - DEF1	All other combinations- DEF3 not tested
	AG1 - AGL9 - SEI	**AG1 - AGL9 - sei-1**	
		AG1 - AGL9 - SEImPI	
E – B - B	**AGL9 –SEI – DEF1**	**AGL9 - sei-1- DEF1**	
	AGL9 – SEI - DEF2		AGL9 - DEF2 - SEI/sei-1
	AGL9 - sei-1 - DEF2		
	AGL9 – DEF3 – SEI	AGL9 - DEF1 - SEI	
	AGL9 – DEF3 – sei-1	AGL9 - DEF1 -sei-1	
E – C - B			AGL9 - AG1 – SEI/sei-1

References

Ambrose, B.A., Lerner, D.R., Ciceri, P., Padilla, C.M., Yanofsky, M.F., and Schmidt, R.J. (2000). Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. Molecular Cell **5,** 569-579.

Angenent, G.C., Busscher, M., Franken, J., Dons, H.J.M., and Vantunen, A.J. (1995). Functional Interaction between the Homeotic Genes Fbp1 and Pmads1 during Petunia Floral Organogenesis. Plant Cell **7,** 507-516.

Becker, A., Gleissberg, S., and Smyth, D.R. (2005). Floral and vegetative morphogenesis in California poppy (Eschscholzia californica Cham.). International Journal of Plant Sciences **166,** 537-555.

Benlloch, R., Roque, E., Ferrandiz, C., Cosson, V., Caballero, T., Penmetsa, R.V., Beltran, J.P., Canas, L.A., Ratet, P., and Madueno, F. (2009). Analysis of B function in legumes: PISTILLATA proteins do not require the PI motif for floral organ development in Medicago truncatula. Plant Journal **60,** 102-111.

Berbel, A., Navarro, C., Ferrandiz, C., Canas, L.A., Beltran, J.P., and Madueno, F. (2005). Functional conservation of PISTILLATA activity in a pea homolog lacking the PI motif. Plant Physiology **139,** 174-185.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1989). Genes Directing Flower Development in Arabidopsis. Plant Cell **1,** 37-52.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1991). Genetic Interactions among Floral Homeotic Genes of Arabidopsis. Development **112,** 1-20.

Bowman, J.L., Baum, S.F., Eshed, Y., Putterill, J., and Alvarez, J. (1999). Molecular genetics of gynoecium development in Arabidopsis. Current Topics in Developmental Biology, Vol 45 **45,** 155-205.

Bustin, S.A., Benes, V., Garson, J.A., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., and Hellemans, J. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry **55,** 611-622.

Cho, S., Jang, S., Chae, S., Chung, K., Moon, Y.-H., An, G., and Jang, S. (1999). Analysis of the C-terminal region of Arabidopsis thaliana APETALA1 as a transcription activation domain. Plant Molecular Biology **40,** 419-429.

Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: genetic interactions controlling flower development. Nature **353,** 31-37.

Crooks, G.E., Hon, G., Chandonia, J.-M., and Brenner, S.E. (2004). WebLogo: A Sequence Logo Generator. Genome Research **14,** 1188-1190.

Davies, B., Egea-Cortines, M., de Andrade Silva, E., Saedler, H., and Sommer, H. (1996). Multiple interactions amongst floral homeotic MADS box proteins. EMBO J **15,** 4330-4343.

Diella, F., Haslam, N., Chica, C., Budd, A., Michael, S., Brown, N.P., Trave, G., and Gibson, T.J. (2008). Understanding eukaryotic linear motifs and their role in cell signaling and regulation. Frontiers in Bioscience **13,** 6580-6603.

Dong, Z.-c., Zhao, Z., Liu, C.-w., Luo, J.-h., Yang, J., Huang, W.-h., Hu, X.-h., Wang, T.L., and Luo, D. (2005). Floral Patterning in Lotus japonicus. Plant Physiology **137,** 1272-1282.

Drea, S., Hileman, L.C., de Martino, G., and Irish, V.F. (2007). Functional analyses of genetic pathways controlling petal specification in poppy. Development **134,** 4157-4166.

Egea-Cortines, M., Saedler, H., and Sommer, H. (1999). Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is

involved in the control of floral architecture in Antirrhinum majus. The EMBO Journal **18,** 5370–5379.

Erdmann, R., Gramzow, L., Melzer, R., Theissen, G., and Becker, A. (2010). GORDITA (AGL63) is a young paralog of the Arabidopsis thaliana B(sister) MADS box gene ABS (TT16) that has undergone neofunctionalization. Plant Journal **63,** 914-924.

Goto, K., and Meyerowitz, E.M. (1994). Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. Genes & Development **8,** 1548-1560.

Goto, K., Kyozuka, J., and Bowman, J.L. (2001). Turning floral organs into leaves, leaves into floral organs. Curr Opin Genet Dev **11,** 449-456.

Gouy, M., Guindon, S.p., and Gascuel, O. (2010). SeaView Version 4: A Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. Molecular Biology and Evolution **27,** 221-224.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp.Ser. **41,** 95-98.

Hill, T.A., Day, C.D., Zondlo, S.C., Thackeray, A.G., and Irish, V.F. (1998). Discrete spatial and temporal cis-acting elements regulate transcription of the Arabidopsis floral homeotic gene APETALA3. Development **125,** 1711-1721.

Honma, T., and Goto, K. (2000). The Arabidopsis floral homeotic gene PISTILLATA is regulated by discrete cis-elements responsive to induction and maintenance signals. Development **127,** 2021-2030.

Honma, T., and Goto, K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409,** 525-529.

Hu, C.D., Chinenov, Y., and Kerppola, T.K. (2002). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol Cell **9,** 789-798.

Jack, T. (2001). Plant development going MADS. Plant Molecular Biology **46,** 515-520.

Jack, T., Brockman, L.L., and Meyerowitz, E.M. (1992). The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. Cell **68,** 683-697.

Kim, S., Koh, J., Yoo, M.-J., Kong, H., Hu, Y., Ma, H., Soltis, P.S., and Soltis, D.E. (2005). Expression of floral MADS-box genes in basal angiosperms: implications for the evolution of floral regulators. The Plant Journal **43,** 724-744.

Kramer, E.M., and Irish, V.F. (1999). Evolution of genetic mechanisms controlling petal development. Nature **399,** 144-148.

Kramer, E.M., Dorit, R.L., and Irish, V.F. (1998). Molecular evolution of genes controlling petal and stamen development: Duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages Genetics **149,** 765-783.

Kramer, E.M., Di Stilio, V.S., and Schluter, P.M. (2003). Complex Patterns of Gene Duplication in the APETALA3 and PISTILLATA Lineages of the Ranunculaceae. International Journal of Plant Sciences **164,** 1-11.

Kramer, E.M., Holappa, L., Gould, B., Jaramillo, M.A., Setnikov, D., and Santiago, P. (2007). Elaboration of B gene function to include the identity of novel floral organs in the lower eudicot Aquilegia (Ranunculaceae). Plant Cell **Epub ahead of print**.

Lamb, R.S., and Irish, V.F. (2003). Functional divergence within the APETALA3/PISTILLATA floral homeotic gene lineages. Proceedings of the National Academy of Sciences of the United States of America **100,** 6558-6563.

Liu, C.J., Zhang, J.A., Zhang, N., Shan, H.Y., Su, K.M., Zhang, J.S., Meng, Z., Kong, H.Z., and Chen, Z.D. (2010). Interactions among Proteins of Floral MADS-Box Genes in Basal Eudicots: Implications for Evolution of the Regulatory Network for Flower Development. Molecular Biology and Evolution **27,** 1598-1611.

Löhr, U., and Pick, L. (2005). Cofactor-Interaction Motifs and the Cooption of a Homeotic Hox Protein into the Segmentation Pathway of Drosophila melanogaster. Current Biology **15,** 643-649.

Löhr, U., Yussa, M., and Pick, L. (2001). Drosophila fushi tarazu: a gene on the border of homeotic function. Current Biology **11,** 1403-1412.

Melzer, R., and Theissen, G. (2009). Reconstitution of 'floral quartets' in vitro involving class B and class E floral homeotic proteins. Nucleic Acids Res **37,** 2723-2736.

Miller, J.H. (1972). Experiments in molecular genetics. (Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory).

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H., and Nagato, Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. Development **130,** 705-718.

Neduva, V., and Russell, R.B. (2005). Linear motifs: evolutionary interaction switches. FEBS Lett **579,** 3342-3345.

Orashakova, S., Lange, M., Lange, S., Wege, S., and Becker, A. (2009). The CRABS CLAW ortholog from California poppy, (Eschscholzia californica, Papaveraceae), EcCRC, is involved in floral meristem termination, gynoecium differentiation, and ovule initiation. Plant J **58,** 682-693.

Pelaz, S., Tapia-Lopez, R., Alvarez-Buylla, E.R., and Yanofsky, M.F. (2001). Conversion of leaves into petals in Arabidopsis. Current Biology **11,** 182-184.

Piwarzyk, E., Yang, Y., and Jack, T. (2007). Conserved C-terminal motifs of the Arabidopsis proteins APETALA3 and PISTILLATA are dispensable for floral organ identity function. Plant Physiol **145,** 1495-1505.

Riechmann, J.L., Krizek, B.A., and Meyerowitz, E.M. (1996a). Dimerization specificity of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA, and AGAMOUS. Proc Natl Acad Sci U S A **93,** 4793-4798.

Riechmann, J.L., Wang, M., and Meyerowitz, E.M. (1996b). DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. Nucleic Acids Res **24,** 3134-3141.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W.E., Saedler, H., and Sommer, H. (1992). Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J **11,** 251-263.

Siebert, P.D., Chenchik, A., Kellogg, D.E., Lukyanov, K.A., and Lukyanov, S.A. (1995). An improved PCR method for walking in uncloned genomic DNA. Nucl. Acids Res. **23,** 1087-1088.

Soltis, D.E., Smith, S.A., Cellinese, N., Wurdack, K.J., Tank, D.C., Brockington, S.F., Refulio-Rodriguez, N.F., Walker, J.B., Moore, M.J., Carlsward, B.S., Bell, C.D., Latvis, M., Crawley, S., Black, C., Diouf, D., Xi, Z.X., Rushworth, C.A., Gitzendanner, M.A., Sytsma, K.J., Qiu, Y.L., Hilu, K.W., Davis, C.C., Sanderson, M.J., Beaman, R.S., Olmstead, R.G., Judd, W.S., Donoghue, M.J., and Soltis, P.S. (2011). Angiosperm Phylogeny: 17 Genes, 640 Taxa. American Journal of Botany **98,** 704-730.

Sommer, H., Beltran, J.P., Huijser, P., Pape, H., Lonnig, W.E., Saedler, H., and Schwarzsommer, Z. (1990). Deficiens, a Homeotic Gene Involved in the Control of Flower Morphogenesis in Antirrhinum-Majus - the Protein Shows Homology to Transcription Factors. Embo Journal **9,** 605-613.

Su, K.M., Zhao, S.H., Shan, H.Y., Kong, H.Z., Lu, W.L., Theissen, G., Chen, Z.D., and Meng, Z. (2008). The MIK region rather than the C-terminal domain of AP3-like

class B floral homeotic proteins determines functional specificity in the development and evolution of petals. New Phytologist **178,** 544-558.

Theissen, G., and Saedler, H. (2001). Plant biology. Floral quartets. Nature **409,** 469-471.

Tilly, J.J., Allen, D.W., and Jack, T. (1998). The CArG boxes in the promoter of the Arabidopsis floral organ identity gene APETALA3 mediate diverse regulatory effects. Development **125,** 1647-1657.

Tröbner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lonnig, W.E., Saedler, H., Sommer, H., and Schwarz-Sommer, Z. (1992). GLOBOSA: a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. EMBO J **11,** 4693-4704.

Tsai, W.-C., Pan, Z.-J., Hsiao, Y.-Y., Jeng, M.-F., Wu, T.-F., Chen, W.-H., and Chen, H.-H. (2008). Interactions of B-class complex proteins involved in tepal development in Phalaenopsis orchid. Plant Cell Physiol. **49,** 814-824.

Tzeng, T.Y., Liu, H.C., and Yang, C.H. (2004). The C-terminal sequence of LMADS1 is essential for the formation of homodimers for B function proteins. J Biol Chem **279,** 10747-10755.

Vandenbussche, M., Zethof, J., Royaert, S., Weterings, K., and Gerats, T. (2004). The Duplicated B-Class Heterodimer Model: Whorl-Specific Effects and Complex Genetic Interactions in Petunia hybrida Flower Development. Plant Cell **16,** 741-754.

Wagner, G.P., and Lynch, V.J. (2008). The gene regulatory logic of transcription factor evolution. Trends Ecol Evol **23,** 377-385.

Walter, M., Chaban, C., Schutze, K., Batistic, O., Weckermann, K., Nake, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K., and Kudla, J. (2004). Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J **40,** 428-438.

Wang, W., and Malcolm, B.A. (1999). Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange site-directed mutagenesis. BioTechniques **26,** 680-682.

Wang, Y.Q., Melzer, R., and Theißen, G. (2010). Molecular interactions of orthologues of floral homeotic proteins from the gymnosperm Gnetum gnemon provide a clue to the evolutionary origin of 'floral quartets'. The Plant Journal **in press**.

Wege, S., Scholz, A., Gleissberg, S., and Becker, A. (2007). Highly efficient virus-induced gene silencing (VIGS) in California poppy (Eschscholzia californica): an evaluation of VIGS as a strategy to obtain functional data from non-model plants. Ann Bot (Lond) **100,** 641-649.

Winter, K.U., Saedler, H., and Theissen, G. (2002). On the origin of class B floral homeotic genes: functional substitution and dominant inhibition in Arabidopsis by expression of an orthologue from the gymnosperm Gnetum. Plant Journal **31,** 457-475.

Yellina, A., Orashakova, S., Lange, S., Erdmann, R., Leebens-Mack, J., and Becker, A. (2010). Floral homeotic C function genes repress specific B function genes in the carpel whorl of the basal eudicot California poppy (*Eschscholzia californica*). BMC Evo-Devo **in press**.

Zachgo, S., Silva, E.D., Motte, P., Trobner, W., Saedler, H., and Schwarzsommer, Z. (1995). Functional-Analysis of the Antirrhinum Floral Homeotic Deficiens Gene in-Vivo and in-Vitro by Using a Temperature-Sensitive Mutant. Development **121,** 2861-2875.

Zahn, L.M., Leebens-Mack, J., dePamphilis, C.W., Ma, H., and Theissen, G. (2005). To B or Not to B a Flower: The Role of DEFICIENS and GLOBOSA Orthologs in the Evolution of the Angiosperms. J Hered **96,** 225-240.

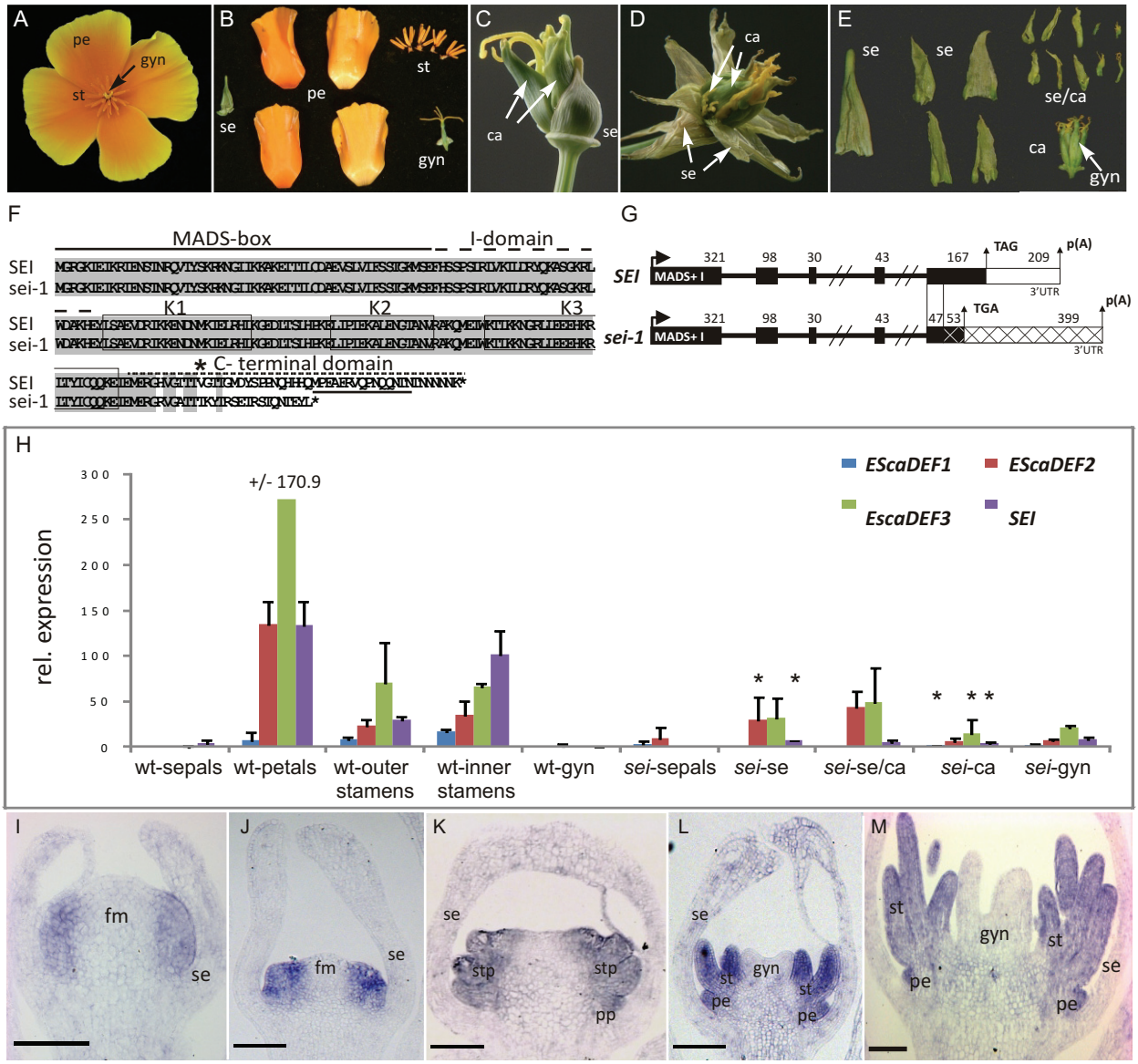

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Fig. 1

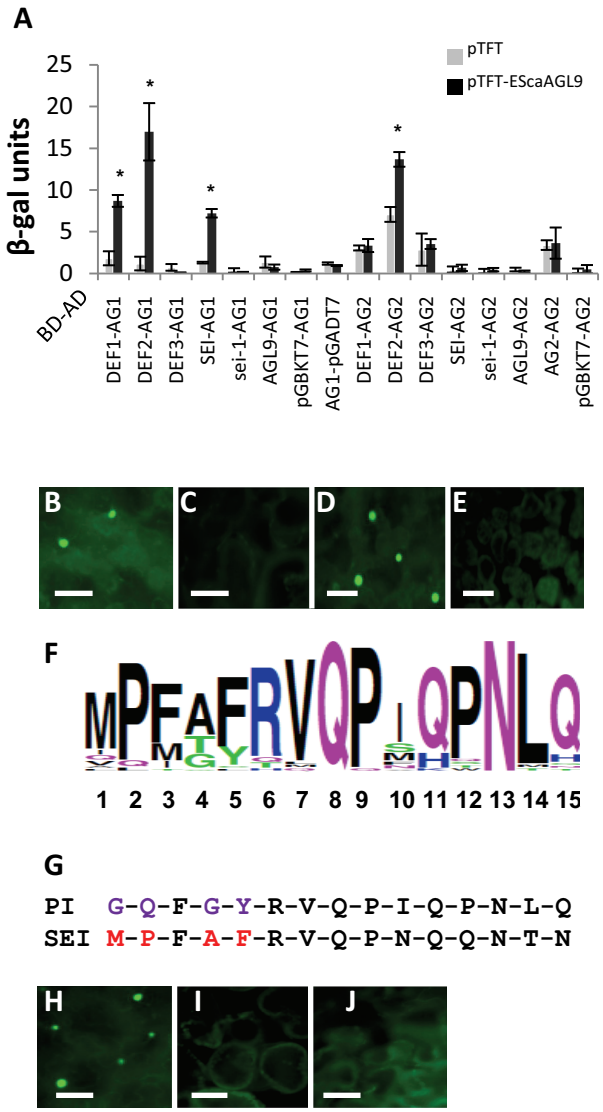
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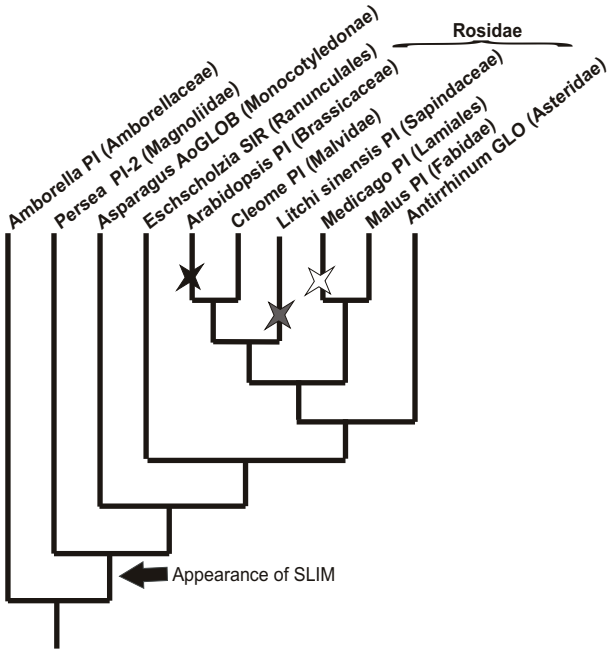
Fig. 2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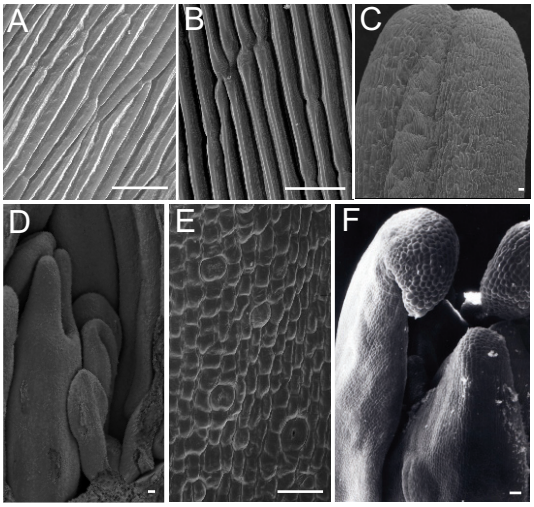
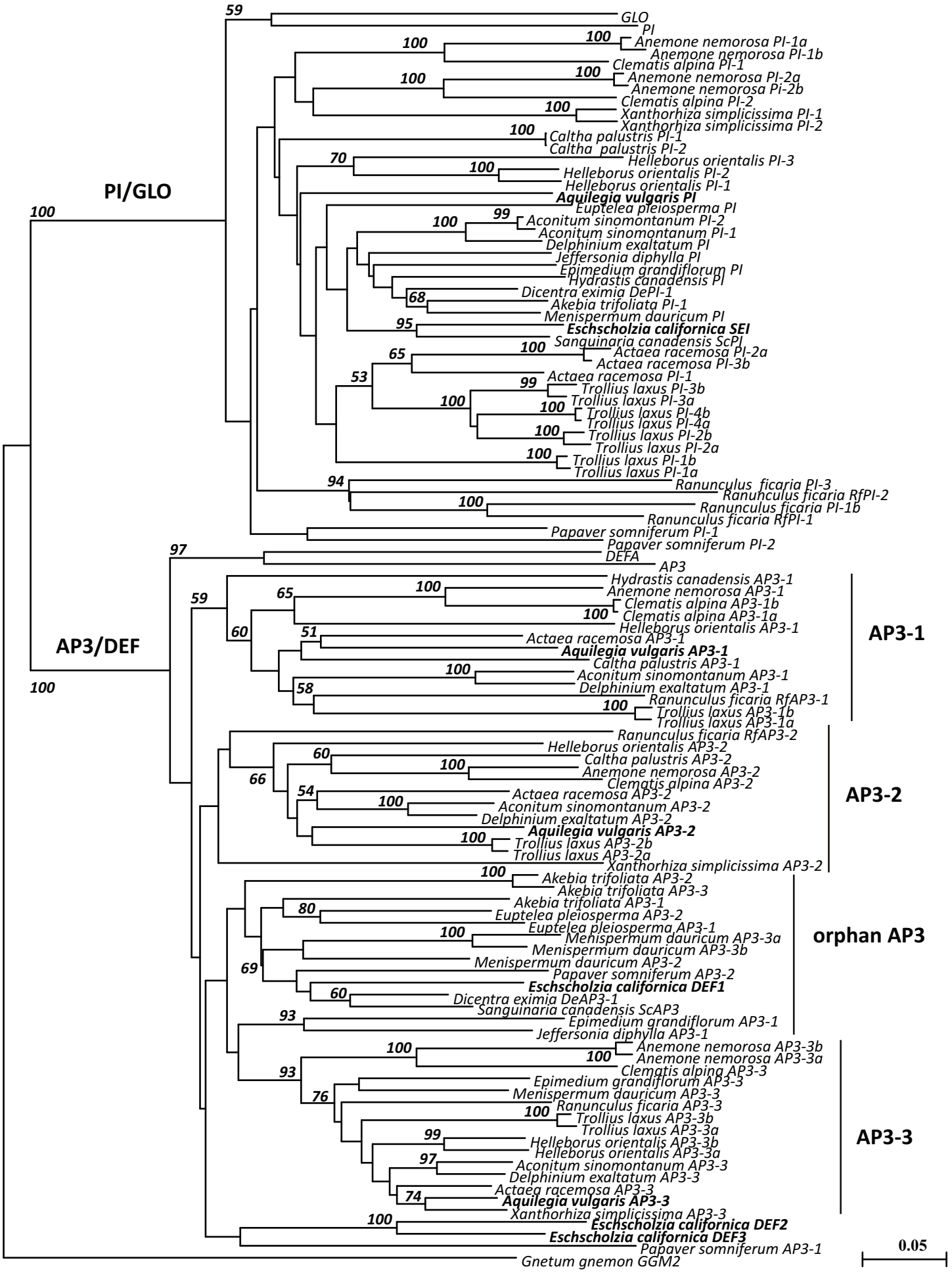
Asparagus AoGLOB (Monocotyledonae)
Eschscholzia SIR (Ranunculales)
Arabidopsis PI (Brassicaceae)
Cleome PI (Malvidae)
Litchi sinensis PI (Sapindaceae)
Medicago PI (Lamiales)
Malus PI (Fabidae)
Antirrhinum GLO (Asteridae)
Appearance of SLIM

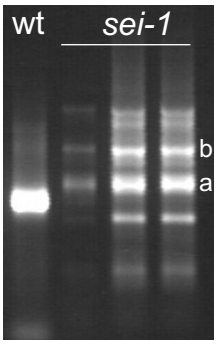
Fig. 3



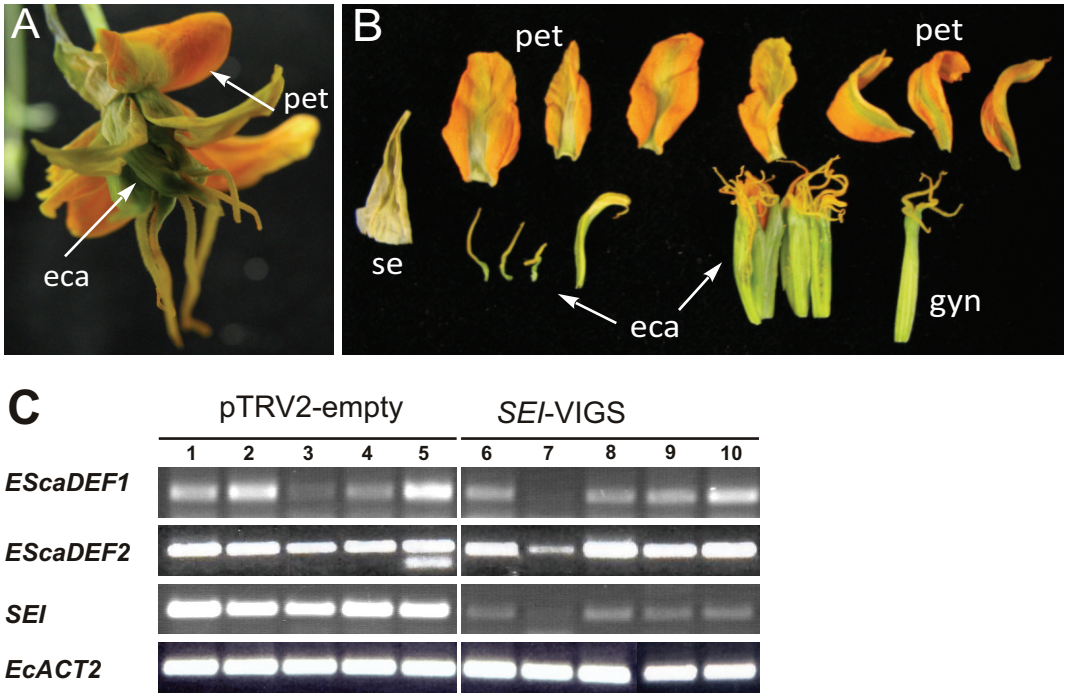
Supplemental Figure 1: SEM of wildtype organs and sei-1 carpel-like organs
(**A-E**) wild-type and (**F**) *sei* mutant. **A**) Outer surface of sepals, **B**) inner surface of petals, **C**) view on the anther filament structure, **D**) gynoecium at stage 6 showing the central gynoecium and surrounding stamens, **E**) close view of the outer surface of a gynoecium, **F**) bulk of unfused carpels surrounding the centrally located carpels in *sei* flowers at stage 6. Scale bar is 10 µm



Supplemental Figure 2: Extended phylogeny of Ranunculales AP3/PI-like proteins. Neighbor joining tree with 1000 bootstrap replicates of Ranunculales, as well as selected higher eudicot AP3/PI-like protein sequences. The major clades of AP3- and PI-like proteins are denoted on the tree. Ranunculales *AP3* subclades according to Kramer et. al (2003) are noted on the right side of the phylogeny. Bootstrap support values above 50% are indicated on the respective branches. *E. californica* and *A. vulgaris* sequences are highlighted in bold along the tree.

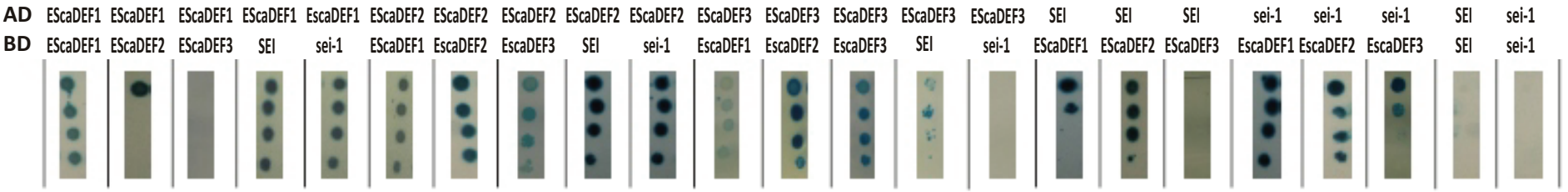


Supplemental Figure 3 3' RACE was carried out with SEI-specific primers on wild type (left) and three individual *sei-1* plants. While only one fragment was amplified in the wild type, several amplification products are documented for the *sei-1* plants. All fragments were cloned and sequenced but only the ones labeled a) and b) were *sei-1* specific and correspond to two transcripts which are similar in coding sequence but deviate in their 3' UTR.

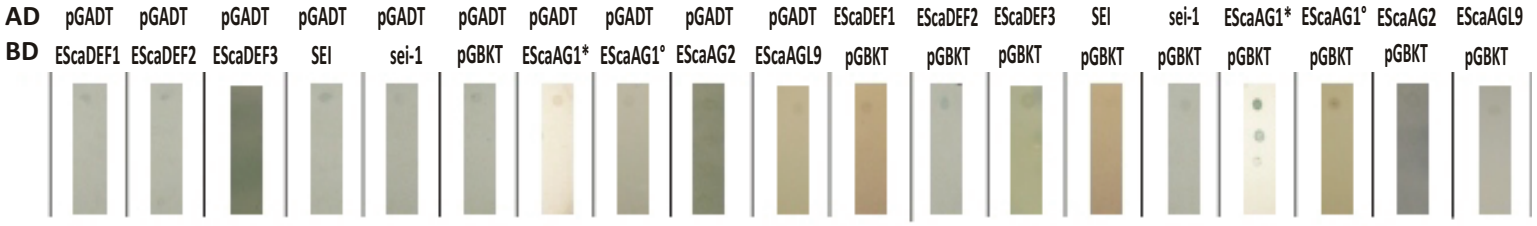


Supplemental Figure 4: SEI-VIGS phenotypes and RT-PCR expression analysis of the SEI-VIGS treated plants. (A) Flower of a pTRV2-*SEI* treated *E. californica* plant. (B) Floral organs of a pTRV2-*SEI* treated plant showing petaloid organs with a sepal-like green stripe in the center and stamens replaced by organs with carpeloid characters. (C) Expression of *EScaDEF1*, *EScaDEF2, SEI*, and *EcACT2* in five plants treated with the empty pTRV2 vector and in five plants treated with pTRV2-*SEI* VIGS showing a specific reduction of *SEI* transcripts in SEI VIGS treated plants. Abbreviations: eca, ectopic carpels; gyn, gynoecium; pet, sepaloid petals; se, sepals

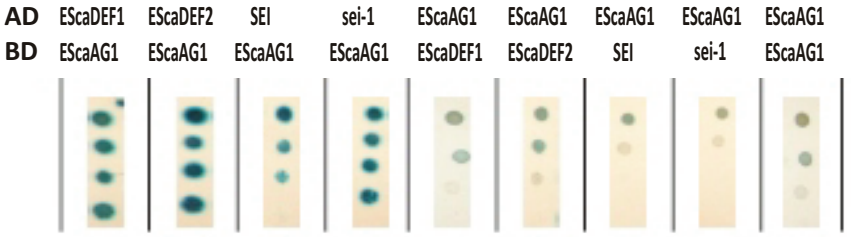
A) EScaDEF1, EScaDEF2, EScaDEF3, SIR and sir-1 (ΔMADS)



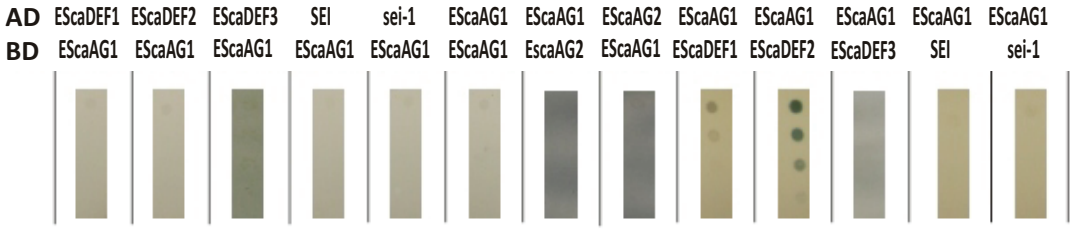
B) empty vector controls of B class proteins (ΔMADS), EScaAG1 (*ΔMADS, °ΔC), EScaAG2 (ΔMADS), and EScaAGL9



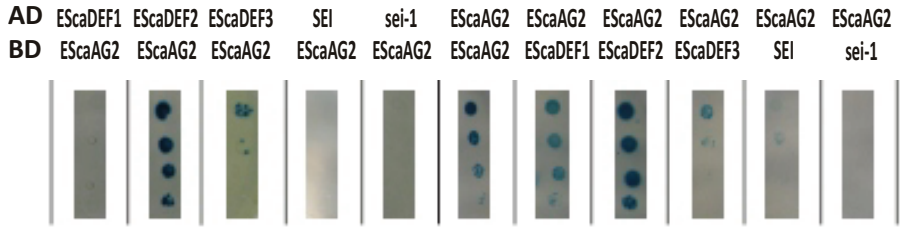
C) B class proteins (ΔMADS) and EScaAG1/2 (ΔMADS)



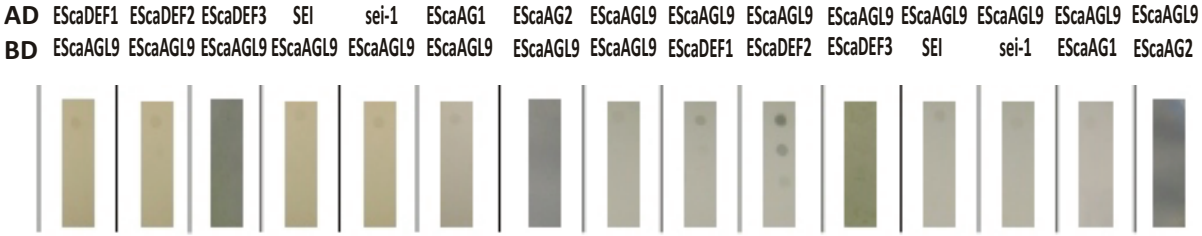
D) B class proteins (ΔMADS), EScaAG1 (ΔC), and EScaAG2 (ΔMADS)



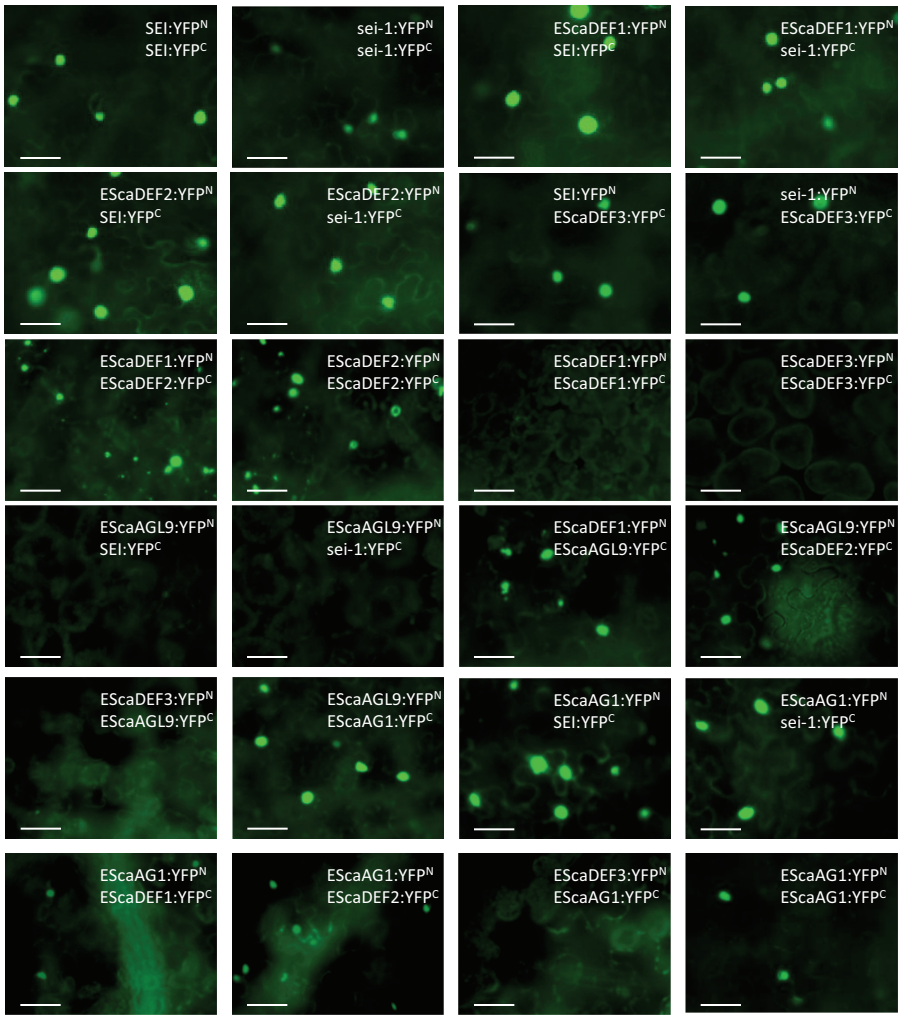
E) B class proteins (ΔMADS) and EScaAG2 (ΔMADS)



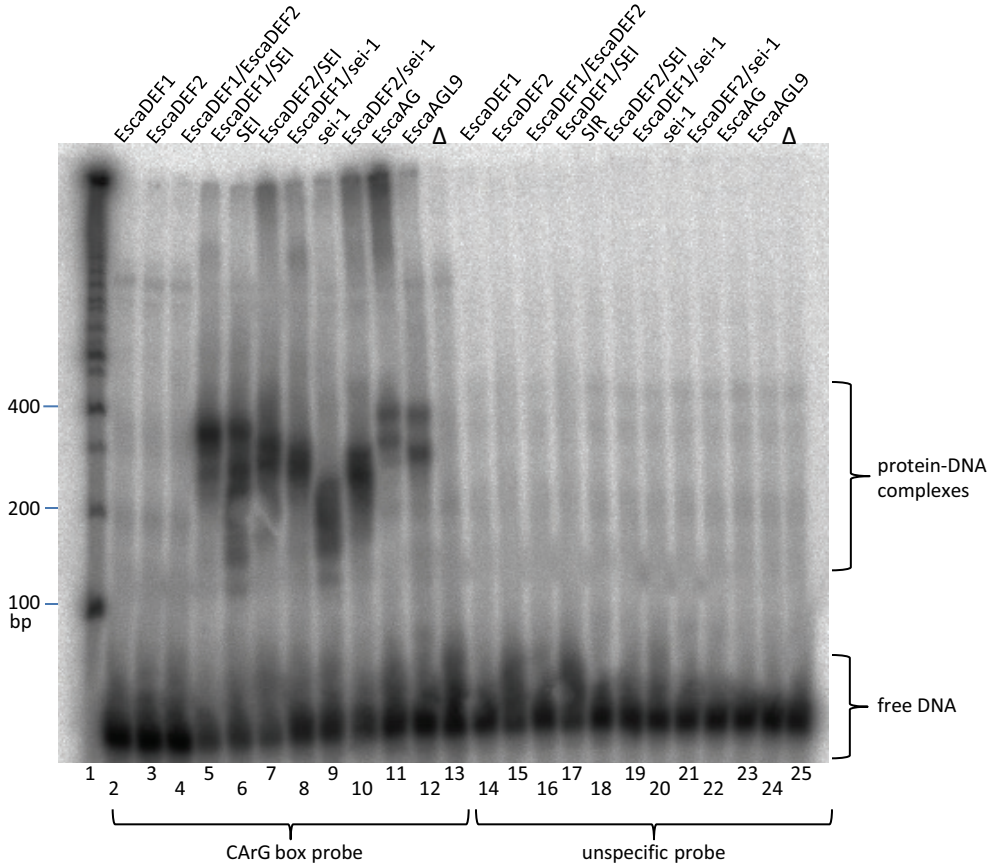
F) B class proteins (ΔMADS), EScaAG1 (ΔC), EScaAG2 (ΔMADS), and EScaAGL9



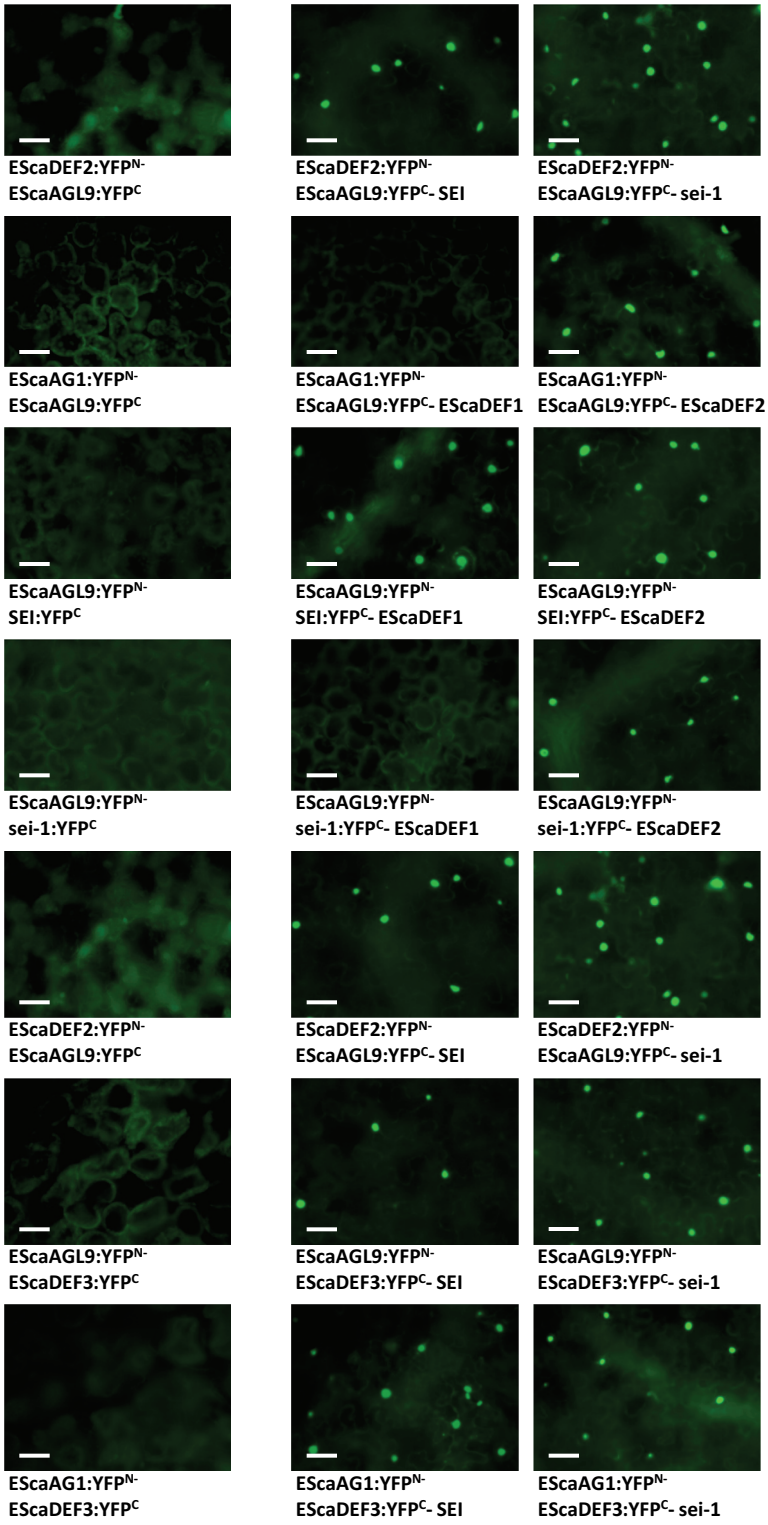
Supplemental Figure 5: Yeast-two hybrid growth assay of floral homeotic proteins of California poppy. Protein-protein interaction was measured as growth of transformed yeast colonies on media lacking leucine, tryptophane, and histidine, supplemented with 3 mM of 3-amino-1,2,4-triazole (3-AT) at 30°C for 3-5 days. Yeast cells were spotted in tenfolrd serial dilutions from to do down and the growing colonies were also tested for LacZ activity, where weak protein-protein interaction show light blue and strong interactions dark blue colors. The protein combinations tested are indicated above the pictures. AD, activation domain vector pGADT7; BD, binding domain vector pGBKT7; ΔMADS, MADS domain has been deleted from proteins; ΔC, C-terminus has been deleted from proteins.



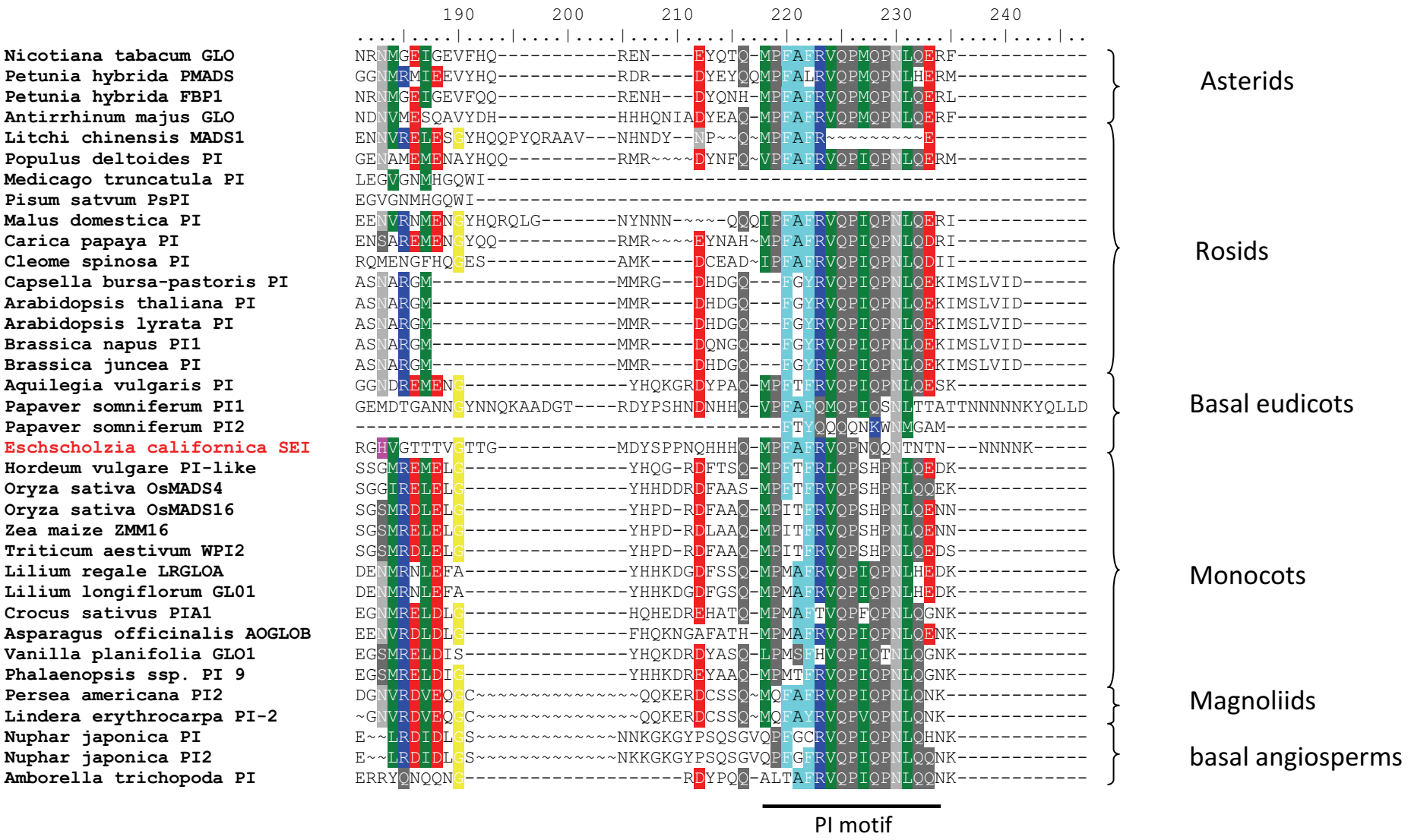
Supplemental figure 6: Protein dimerization analysis by BiFC.
Only selected protein combinations tested are shown here. A comprehensive list of BiFC results is given in Table 1. Names of the fusion constructs is given in the upper right corner of each subfigure. YFP^N denotes fusion with the N-terminal part of YFP, YFP^C denotes fusion with the C-terminal of YFP. Bright green, nuclear localized YFP fluorescence is scored as interaction. Chloroplast auto-fluorescence is visible in several subfigures.



Supplemental figure 7: DNA binding of *E. californica* MADS domain proteins. Proteins applied are denoted above the lanes. In lanes 2 to 13, a probe on which a CArG box is encoded was used, in lanes 14 to 25, a DNA probe having the same nucleotide composition as the CArG box probe but with nucleotides reordered randomly was used as a specificity control. Δ denotes a negative control in which the *in vitro* translation extract was programmed with an 'empty' pTNT *in vitro* translation vector. 'M' denotes a radioactively labelled molecular size marker (100 bp ladder, NEB).
When two cotranslated proteins constituted a protein-DNA complex with an electrophoretic mobility different from that of the individual proteins bound to DNA, formation of DNA-binding heterodimers was concluded (compare electrophoretic mobility of the SEI-DNA complex (lane 7) with that of the EscaDEF2/SEI-DNA complex (lane 8), for example). A potential EscaDEF1/SEI-DNA complex had an electrophoretic mobility very similar to that of the SEI-DNA complex (compare lanes 6 and 7). However, the complex formed when EscaDEF1 was applied together with SEI appeared to migrate slightly faster through the gel compared to SEI alone, what tempted us to assume that EscaDEF1/SEI-DNA complexes were formed.
Some of the proteins form more than one protein-DNA complex (EscaAG1, for example). The reasons for that are unknown. However, similar observations have been made also for other MADS-domain proteins (Wang et al., 2010).



Supplemental figure 8: Ternary protein complex formation was analyzed by TriFC. Only selected protein combinations tested are shown here. A comprehensive list of TriFC results is given in Table 2. Names of the fusion constructs is given in the upper right corner of each subfigure. YFP^N denotes fusion with the N-terminal part of YFP, YFP^C denotes fusion with the C-terminal of YFP Bright green, nuclear localized YFP fluorescence is scored as interaction. Chloroplast auto-fluorescence is visible in several subfigures.



Supplemental figure 9: Alignment of PI protein sequences and their PI motif.

The C- terminal part of 37 angiosperm PI-like proteins is shown with the conserved PI motif underlined. Conserved amino acid residues are highlighted in the same colour. The affiliation of each species listed to phylogenetic clades is given in brackets on the right side of the alignment.

Supplemental table 1: Oligonucleotides used in this study

Primer name	Method	Sequence 5`-3`
AB05	3'RACE	GACTCGAGTCGACATCTGTTTTTTTTTTTTTTTTT
AB07	3'RACE	GACTCGAGTCGACATCTG
RACE-SIR fw	3'RACE	TCAGAAGGCTTCAGGGAAGA
RACE-SIR fw2	3'RACE	TACCATGGGGAGGGGTAAGATAGAG
RACE-DEF3fw	3'RACE	GGGTCGTGGAAAGATTGAGA
RTq-EScaDEF1 fw	real-time qPCR	GGATGGGAGAGGATTTGGAT
RTq-EScaDEF1 rev	real-time qPCR	TTCCAGATTTTGCTCAAGACTTC
RTq-EScaDEF2fw	real-time qPCR	ATTTGGTGGAGGAGATGATGAG
RTq-EScaDEF2rev	real-time qPCR	TTTTGAAGATTGGGATGGCTA
RTq-EScaDEF3 fw	real-time qPCR	TCCTCGGCACTCAAAGTGA
RTq-EScaDEF3 rev	real-time qPCR	TCCACCACCAAAAGCATGTA
RTq-SIR-sir1 fw	real-time qPCR	TCTAGCACTGGCAAGATGTC
RTq-SIR-sir1 rev	real-time qPCR	TTGATTCTATCCACTTCAGCAC
RTq-Actin fw	real-time qPCR	AAGAGCTCGAAACTGCCAAG
RTq-Actin rev	real-time qPCR	CATCGGGAAGCTCGTAATTT
RTq-GAPDH fw	real-time qPCR	GCTTCCTTCAACATCATTCC
RTq-GAPDH rev	real-time qPCR	AGTTGCCTTCTTCTCAAGTC
UPL probe ACT #136	real-time qPCR	GCTCATCA
UPL probe EScaDEF1 #132	real-time qPCR	GAGCAGGA
UPL probe EScaDEF3 #69	real-time qPCR	GGAGGAAG
Insitu SIR fw	in situ hybridization	TAGAAATGGAACGCGGTCATG
Insitu SIR rev	in situ hybridization	TGCTCGAGGCACCATCACTTAGTCCCTTG
AP1	adaptor primer, RAGE	GTAATACGACTCACTATAGGGC
AP2	adaptor primer, RAGE	ACTATAGGGCACGCGTGGT
SIRdown-GSP1	RAGE downstream	TGAGTGCTGAAGTGGATAGAATCAAGAA
SIRdown-GSP2	RAGE downstream	ATCAAGAAAGAGAATGACAACATGAAGATT
SIRupGSP1	RAGE upstream	CAGAATCTCTTGAATCCAGTGGTACAAAAC
SIRupGSP2	RAGE upstream	AGTCCCTTGATAGAACTACTAATACTAGCAGC
sir1upGSP1	RAGE upstream	GAGATACAAGAGTCCCACGAGGAAGTAACG
sir1upGSP2	RAGE upstream	TAACCAGCACCACCATCAGATGCCGTTC
DEF1ΔMFw	Yeast 2 hybrid	CTCCATGGAGTTCTCTGAATATATCAGTCCTTCT
DEF1ΔMrev	Yeast 2 hybrid, EMSA	AGGATCCTCATGCAAGGCGTAGATCGTG
DEF2ΔMFw	Yeast 2 hybrid	CTCCATGGAGTTTGCTGAATATATTAGCCCT
DEF2ΔMrev	Yeast 2 hybrid, EMSA	TGGATCCTCATTCAAAGTGTAGATTGTATG
SIR-sir1ΔMFw	Yeast 2 hybrid	TACCATGGAGTCTGAGTTTCATTCTTC
SIRΔMrev	Yeast 2 hybrid, EMSA	AGGATCCTATTTGTTGTTGTTGTTGTTGG
sir1ΔMrev	Yeast 2 hybrid, EMSA	AGGATCCTCTAATAAATGCAAGCTCCCTTA
AG1ΔMFw	Yeast 2 hybrid	TAGGATCCGTGCCAATAACAGTGTGAAATCC
AG1ΔMRev	Yeast 2 hybrid	AGGGATCCCTAACCAAGTTGGAGAGTTGTCTG
AG1ΔC_fw3	Yeast 2 hybrid	CTACATATGACGGATTTCCAAAGTCAAGTA
AG1ΔC_rev3	Yeast 2 hybrid	AGGGATCCCTACTCTTCTGCATGTACTCG
EcAGL9fw_BamHI	Yeast 2 hybrid	AGGATCCATGGGAAGAGGAAGAGTTG
AGL9y2hR	Yeast 2 hybrid, EMSA	AGGATCCCTACCATCCTGGTCCTG
DEF3ΔM_FW	Yeast 2 hybrid	CTCCATGGTCACTGAATATATCAGTC
DEF3ΔM_rev	Yeast 2 hybrid	AGGGATCCTCATTCAATTTGGAGATTGTA
AG2 ΔM FW	Yeast 2 hybrid	CACCATGGCTTATGCTAACAACAGTGTAAG
AG2 ΔM rev	Yeast 2 hybrid	AGGGATCCCTAACCAAGTTGGAGGGATG
VIGS-SIR-fw-Bam	VIGS	TGGATCCTCAGAAGGCTTCAGGGAAGA
VIGS-SIR-rev-Xho	VIGS	CTCGAGGCACATCACTTAGTCCCTTG
AG1-pNBV-fw	BiFC (pNBV)	CTATCTAGAATGGATTTCCAAAGTCAAGTA

AG1revBam_dSTOP	BiFC (pNBV)	AGGATCCACCAAGTTGGAGAGTTGTCTG
AGL9-pNBV-fw	BiFC (pNBV)	CTATCTAGAATGGGAAGAGGAAGAGTTGAG
AGL9revBam_dSTOP	BiFC (pNBV)	AGGATCCCCATCCTGGTCCTGGC
DEF3fw-Xba	BiFC (pNBV)	CTATCTAGAATGGGTCGTGGAAAGATTG
DEF3revBAM_dSTOP	BiFC (pNBV)	AGGGATCCTTCAATTTGGAGATTGTA
XbaI_SIR-sir1_fw	BiFC (pNBV)	ATCTAGAATGGGGAGGGGTAAGATAG
SIR_BamHI_rev	BiFC (pNBV)	AGGATCCAAGATATTCGGTATTTTGAGTAG
sir1_BamHI_rev	BiFC (pNBV)	AGGATCCTTTGTTGTTGTTGTTGTTGGTGTTG
XbaI_DEF1_fw	BiFC (pNBV)	ATCTAGAATGGGAAGAGGAAAGATAGAG
DEF1_BamHI_rev	BiFC (pNBV)	AGGATCCTGCAAGGCGTAGATCGTGAG
XbaI_DEF2_fw	BiFC (pNBV)	ATCTAGAATGGGTAGAGGTAAAATTGAGATA
DEF2_BamHI_rev	BiFC (pNBV)	AGGATCCTTCAAAGTGTAGATTGTATGAG
def1FWbiFCXho	TriFC (pART7)	CTCTCGAGATGGGAAGAGGAAAGATAGAGA
def2FWbiFCXho	TriFC (pART7)	CTCTCGAGATGGGTAGAGGTAAAATTG
glo-sirFWbiFCXho	TriFC (pART7)	CTCTCGAGATGGGGAGGGGTAAGATAGAG
sirREVbiFCBamHI	TriFC (pART7)	AGGATCCTCAAAGATATTCGGTATTTT
AG1fwNco	EMSA	CACCATGGCTACGGATTTCCAAAGTCAAGTA
AG1revBam	EMSA	AGGGATCCCTAACCAAGTTGGAGAGTTGTCTG
EcAGL9_fw3	EMSA	AGGATCCATGGGAAGAGGAAGAGTTG
DEF1fw	EMSA	CTCCATGGGAAGAGGAAAGATAGAGA
DEF2fw	EMSA	CTCCATGGGTAGAGGTAAAATTG
SIR-sir1fw	EMSA	TACCATGGGGAGGGGTAAGATAGAG
fwSIRmPIn_1	Site-directed mutagenesis	TCCTAACCAGCACCACCATCAGggGCaGTTCGgC TaCCGAGTGCAGCCAAATCAAC
revSIRmPIn_1	Site-directed mutagenesis	GTTGATTTGGCTGCACTCGGtAGcCGAACtGCccC TGATGGTGGTGCTGGTTAGG

Erklärung gemäß § 11 Abs. 2 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Diese Dissertation wurde wie folgt überarbeitet:

Kapitel 3.1.2.1 *EscaAG1/2* expression is localized in carpels and stamens throughout flower development.
In diesem Kapitel wurden RTq-PCR Ergebnisse des EScaAG1 Gens mit den RTq-PCR Ergebnissen aus einer Veröffentlichung verglichen und diskutiert (Seite 54).

Die *in situ* Hybridisierungsergebnisse wurden mit denen verglichen, die in einer Veröffentlichung publizieret wurden (Seiten 54-55).

Kapitel 3.2.1 *SIR* is expressed in petals and stamens throughout developmental stages
Die RTq-PCR Ergebnisse des *SIR* Gens wurden zusätzlich mit denen verglichen, die in einer Veröffentlichung publiziert wurden (Seite 63).

Manuskript I
"*EcSPT*, the ortholog of the *Arabidopsis SPATULA* gene in *Eschscholzia californica,* is possibly involved in ovule and seed formation"
Das Manuskript ist überarbeitet worden. Eine kurze Beschreibung des *SPT* Gens wurde eingeschlossen und Vorschläge zur Erforschung der Bedeutung des Promoterbereiches für die Funktion des *EcSPT* Gens im Vergleich zu *SPT* aus Arabidopsis thaliana wurden gemacht (Seite 138).
Zusätzlich wurden meine RT-PCR und *in situ* Hybridisierungsergebnisse des *EcSPT* Gens mit Expressionsanalysen aus der Literatur verglichen und ausführlich diskutiert (Seiten 139-140). Zusätzliche Veröffentlichungen wurden eingeschlossen.

Manuskript II
Die neueste Version der Veröffentlichung **"The California poppy (*Eschscholzia californica)* mutant *sirene* sheds light on the function of the C-terminal domain of class B floral homeotic MADS domain proteins"**, die eingereicht wurde, wurde anghängt.

Alle Änderungen wurden im Einvernehmen mit Prof. Dr. Annette Becker (erster Gutachter und Vorsitzender der zuständigen Prüfungskommission) vorgenommen.

Bremen, im Oktober 2011

Svetlana Orashakova

Erklärung

Ich versichere hiermit, dass ich meine Dissertation

"Expression analyses of flower developmental genes in *Eschscholzia californica*"

selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Svetlana Orashakova,
Bremen, Oktober 2011