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Functional and molecular characterization of B_{sister} genes in the two model species:

Arabidopsis thaliana and *Eschscholzia californica*

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"I see no necessity in the belief that the eye was expressly designed. On the other, I cannot anyhow be contented to view this wonderful universe, and especially the nature of man, and to conclude that everything is the result of brute force. I am inclined to look at everything as resulting from designed laws, with the details, whether good or bad, left to the working out of what we may call chance."

Charles Darwin letter to Asa Gray (a minister)

May 22, 1860

Dedicated to

Getenesh Habtemariam Abayneh (Etet)

Girma Tekleyohans Woldemariam

Meron Biruk Beshewamyeleh
&
My unborn child

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Abbreviations

°C	degree Celsius
3-AT	3-amino-1,2,4-triazole
AGL	AGAMOUS LIKE
AP3-motif	APETALA3 motif
At	Arabidopsis thaliana
bHLH	basic helix-loop-helix
BiFC	bimolecular fluorescence complementation
Bn	Brassica napus
bp	base pairs
bZIP	basic Leucine Zipper
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
ch	chalaza
CDS	coding sequence
CIM	callus induction medium
Col-0	Arabidopsis thaliana ecotype Colombia
DNA	deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DAP	days after pollination
DD	deviant domain

EBS	ESCHSCHOLZIA CALIFORNICA B_{SISTER}
EDTA	ethylene diamine tetra acetic acid
ESca	Eschscholzia californica
EST	expressed sequence tag
ET	EBS transgenic
fm	functional megaspore
fu	funiculus
gy	gynoecium
HD	homeodomain
His	histidine
HOX	homeobox
hr	hour
ii	inner integument
kb	kilo base pairs
l	liter
lacZ	Beta-galactosidase
LB	luria broth
Leu	leucine
MADS-box	MCM1-AGAMOUS-DEFICIENS-SRF'-box transcription factor
max	maximum
mg	milligram
MIKC	MADS, Intervening, Keratin-like and C-terminal domain structure

min	minutes
miRNA	micro RNA
ml	milliliter
mM	millimolar
∫M	micro molar
MMC	megaspore mother cell
mp	micropyle
MS	Murashige and Skoog medium
MYA	million years ago
MYB	myeloblastosis
ng	nanogram
nu	nucellus
OD	optical density
oi	outer integument
ORF	open reading frame
ov	ovule
PA	proanthocyanidin
paleoAP3 motif	paleo APETALA3 motif
PCR	polymerase chain reaction
pe	petal
PI motif	PISTILLATA-motif
PRM	plant regeneration medium

qRT-PCR	quantitative real-time PCR
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolution per minute
RT	room temperature
SD	synthetic dropout
se	sepal
sec	second
SEIM	somatic embryo induction medium
shp1-1 shp2-1	shp1 shp2 double mutant
SHP1 SHP2	SHP1 and SHP2 genes
ssDNA	single strand DNA
st	stamen
TE	tris EDTA
Tris	trisaminomethane
Trp	tryptophan
TRV	tobacco rattle virus
abs shp1-1 shp2-1	abs shp1 shp2 triple mutant
UTR	untranslated region
UV	ultra violet
V	volt

VIGS	virus induced gene silencing
WS-4	Arabidopsis thaliana ecotype Wassilewskija
wt	wild type
Y2H	Yeast two-hybrid
YAPD	Yeast-extract-Adeninehemisulphate-Peptone-Dextros
YFP	yellow fluorescent protein
µl	micro liter

Summary

Ovules are one of the most important organs for seed plants. Understanding their organogenesis has been at the center of research interest for long time that let to the identification of several genes that are involved in ovule development. This includes MADS box transcription factors such as B_{sister} genes. B_{sister} genes are identified more than a decade ago but so far only few species have been used to investigate their function. In this study the *TRANSPARENT TESTA 16 (ABS)* from *Arabidopsis thaliana* and *ESCHSCHOLZIA CALIFORNICA* B_{SISTER} *(EBS)* from *Eschscholzia californica* were chosen to investigate the role of B_{sister} genes in eudicots.

In addition to what was described in previous studies, additional phenotypes which are associated with *ABS* loss-of-function were found that includes defects in seed germination, seed and silique development. In comparison to wild type plants, the number of mature seeds in *abs* mutant is reduced to 33%. Furthermore, seed germination is also affected in *abs* mutant when grown on growth medium supplemented with different salt concentration. In order to investigate the genetic interaction between *ABS* and *SHP* genes, triple mutant lines were generated and characterized. The result shows that the triple mutant produces much more less number of seeds compared to *abs* mutant and these seeds appear to be shrunken in structure. In order to identify de-regulated genes in the triple mutants that resulted in defect in seed structure and plant fertility, expression analysis of selected genes that are known to be involved in either of programmed cell death, lipid metabolism, ovule or seed development was carried out. The expression analysis result shows that *ABS*, *SHP1, and SHP2* directly or indirectly regulate these target genes expression in an independent and redundant manner.

Characterization of *EBS* begins with its expression analysis using *in situ* hybridization and qRT-PCR techniques and the results shows that *EBS* expression starts prior to the ovule primordia formation but becomes restricted to the ovule at later developmental stages specifically strongly expressed in the inner integument and nucellus region of the ovule. Yeast two-hybrid technique was used to identify EBS protein interacting partners

and the result showed that EBS interacts with B-class & C-class floral homeotic proteins. Furthermore, functional analysis of *EBS* via VIGS methods indicates that down-regulation of *EBS* transcripts causes abnormalities in developing seed structure while over-expression of *EBS* in *Arabidopsis* significantly alters the vegetative and floral organ morphogenesis that led to male sterility and also affects seed fatty acid composition.

In order to use *E. californica* for future reverse genetics approach, an efficient stable genetic transformation protocol was optimized using developing seeds as explants. It was possible to produce somatic embryos that regenerate to fully-grown plants. The successful integration of the transgene was verified using molecular technique. This stable transformation protocol will be helpful for the functional characterization of genes which are difficult to analyze using transient gene knock-down methods.

In general, this dissertation provides new findings that can strengthen the current understanding of B_{sister} gene function in angiosperm plant development. The study suggests that for a detailed understanding of B_{sister} genes, it is necessary to consider potential redundantly acting genes (that are mainly MADS-box genes) that have an overlapping expression pattern with B_{sister} genes. Besides that, the conservation and divergence pattern in molecular and functional aspects of B_{sister} genes in these two model species provides insights for further understanding of plant evolution and development.

ZUSAMMENFASSUNG

Die weiblichen Reproduktionsorgane, die Ovulen, gehören zu den wichtigsten Organen der Samenpflanzen. Das Verständnis ihre Organogenese ist seit langem Thema intensiver Forschungen. Wichtige Gene, überwiegend Transkriptionsfaktoren, unter ihnen die B_{sister} Gene, konnten bereits als regulatorische Gene der Ovulenentwicklung identifiziert werden. Bislang wurden aber nur einige wenige Spezies auf ihre B_{sister} Genfunktionen hin untersucht. Als Ziel der Arbeit wurden die eudikotylen B_{sister} Gene *TRANSPARENT TESTA16 (ABS)* von *Arabidopsis thaliana* und *ESCHSCHOLZIA CALIFORNICA BSISTER (EBS) von Eschscholzia californica* für eine funktionelle Charakterisierung ausgewählt.

ABS loss-of-function Mutationen beeinflussen die Samen- und Fruchtentwicklung, was bisher noch nicht gezeigt werden konnte. Im Vergleich mit dem Wildtyp ist die Zahl reifender Samen in *abs* Mutanten um 33% reduziert. Zusätzlich beträgt die Keimungsrate der Samen von *abs* Linien nur 25 % im Vergleich mit 96% für Wildtyp-Samen. Dieser Phänotyp-Komplex wird in der Dreifach-Mutante *absshp1 und shp2* noch deutlich verstärkt. Zusätzlich zeigt die Dreifach-Mutante eine veränderte Samenmorphologie, in der die Samen geschrumpft und zerknittert sind, im Vergleich zu der glatten und ovalen Struktur der Wildtyp-Samen. Die Suche nach von *ABS*, *SHP1*, und *SHP2* reguliertenGenen erbrachte Entwicklungsgene aus unterschiedlichen Kategorien, wie z.B. dem programmierten Zelltod, dem Lipidmetabolismus oder der Samen- und Fruchtentwicklung. Die Untersuchung der Expression dieser potentiellen Zielgene offenbarte dabei eine funktionelle Redundanz von *ABS*, *SHP1* und *SHP2* bei deren Regulierung.

Die Expressionsanalyse von *EBS* mit Hilfe der *in situ* Hybridisierung und der quantitativenreal-time PCR ergibt eine starke Aktivität in den Ovulenprimordien und beschränkt sich später auf die sich entwickelnden Ovulen. Des Weiteren findet sich eine starke Expression in den inneren Integumenten und im Nucellus der Ovulen. Mit Hilfe der *yeast-two-hybrid* Methode konnte gezeigt werden, das das EBS Protein mit floralen homöotischen Proteinen der B-Klasse und C-Klasse interagiert. Die Herunterregulation

der *EBS* Funktion beeinflusste die Samenmorphologie und eine Überexprimierung von *EB*S im heterologen System *Arabidopsis* führte zu einer signifikanten Veränderung der vegetativen und floralen Organogenese, bis hin zur männlichen Sterilität der Pflanzen. Für *E. californica* als Modelorganismus für zukünftige *reverse genetics* Experimente wurde ebenfalls im Rahmen dieser Dissertation Protokolle für eine stabile genetische Transformation optimiert. Dabei wurden sich entwickelnde Samen als Explantate benutzt. Es konnten somatische Embryonen bis hin zu voll entwickelten Pflanzen regeneriert werden. Die erfolgreiche Integration der Transgene wurde mit molekularen Methoden bestätigt. Dieses Protokoll unterstützt die molekulare Analyse von Entwicklungsgenen, was mit transienten *gene knock-down* Methoden allein nur schwer erreicht werden kann.

Generell unterstützen die Ergebnisse dieser Dissertation das Verständnis der B_{sister} Genfunktionen in der Ovulenentwicklung der Blütenpflanzen. Zusätzlich unterstreichen die Ergebnisse, dass es für eine vollständige Erfassung der B_{sister} Genfunktionen notwendig ist, die Proteininteraktionen zu untersuchen. Die mit Hilfe der beiden Modellorganismen erarbeiteten molekularen und funktionalen Aspekte der B_{sister} Gene und deren Konservierung bzw. Diversifizierung werden einen wichtigen Einfluss auf zukünftige Untersuchungen haben, die sich mit der Evolution und der Entwicklung der Pflanzen beschäftigen.

1. Introduction

1.1 Transcription Factors and Plant Development

Plants are among the most diversified living organisms with more than 300,000 species living in various ecological systems [1]. They adapt to various environmental conditions ranging from hot deserts to entirely submerge in water surfaces. Adaptation to such diverse environmental conditions necessitates the presence of complex physiological mechanisms and structures that allow the plants to continue development and propagation. Such architectural diversities are known to be products of chance and necessity [2], and understanding their evolution requires an approach that integrates genetic makeup of the organisms and organ development [3].

Cronk [4] argues that, one of the significant events that result in the formation of novel/complex structures in an organism is genome duplication. Genome duplication provokes modification in the coding sequence and/or regulatory elements that control the expression of a given gene. It has been shown in previous studies that presence or absence of regulatory elements within intragenic sequences of a given gene were able to affect its spatio-temporal expression [5]. Often, such regulatory elements are direct target of transcription factors that recognize motifs and control their expression pattern depending upon external or internal stimuli [6]. In *Arabidopsis thaliana*, more than 1600 transcription factors are implicated in regulating gene expression and these transcription factors are classified into several families based on structures of their protein domain and motifs found in their target DNA sequences recognized by the DNA-binding domains [7,8].

1.2 MADS-box Transcription Factors

The MADS-box transcription factor family is one of the thoroughly investigated gene families. Proteins which belong to this family are characterized by their highly conserved DNA-binding domain of 58 amino acids known as the MADS-domain that specifically binds to $CC(A/T)_6GG$ motifs found in their target genes [9-14]. The name MADS is derived from the initials of the founding members genes: *MCM1* (yeast) [15], *ARG80* (yeast) [16] or

AGAMOUS (plant) [17], *DEFICIENS* (plant) [18] and *SRF* (human) [19]. They are diverse and crucial for many biological functions in eukaryotes [11]. MADS

proteins regulate the expression of genes that are required for vegetative tissue development and reproductive organ identities [20]. Based on their exon-intron and domain structures, MADS-box transcription factors fall into two main categories: Type-I and Type-II MADS-box genes [21].

1.2.1 Type-I MADS-box Transcription Factors

According to Nam et al. [22], type-I MADS-box transcription factors have undergone through a weaker purifying selection and higher frequency of segmental gene duplication that led to a higher rate of birth-and-death evolution. Furthermore, unlike type-II MADS-box genes, type-I MADS-box genes do not have a well conserved Keratin-like domain and usually come with one or two exons [11,23-25]. Nevertheless, based on DNA sequence similarity, the type-I MADS-box genes are further categorized into three different sub-classes namely: Mα, Mβ, and Mγ [26].

Even though the type-I MADS-box genes are less studied in comparison to type-II MADS domain proteins, they are often described to play a key role in female gametophyte development. *AGAMOUS-LIKE 80* (*AGL80/FEM111*), the first type-I MADS-box gene to be characterized from *Arabidopsis,* is involved in regulating the expression of genes required for central cell differentiation during female gametophyte development. In addition to that, it is essential for proper localization of a Mα type-I transcription factor known as *AGL61* (*DIANA*) that controls proper development of central cell, synergid, and egg cell identities [27,28]. *AGL23* is also an Mα type-I transcription factor that is involved in embryo sac development. *agl23* mutant shows an arrest of female gametophyte development and lack of chloroplasts in the developing embryo making homozygous *agl23* lethal [29]. *AGL28* is another type-I MADS-box gene that has a high sequence identity with *AGL23.* It is involved in regulation of flowering time in *Arabidopsis*. Though it is expressed in the developing embryo, its mutant plant does not show any altered phenotype [30-32]. In general, based on these studies conducted so far, the type-I transcription factors are mainly expresses and functions in female gametophyte development in *Arabidopsis*.

1.2.2 Type-II MADS-box Transcription Factors

This lineage of MADS-box transcription factors is comprised of genes where majority of them are well characterized across different species [33]. These genes typically have a conserved MIKC domain structure, named after the initials of their protein domains: MADS, Intervening, Keratin-like and Carboxyl-terminal (MKIC) [9,10]. Each domain is known to have distinct roles: the MADS-domain is required for DNA-binding and protein dimerization, I-domain specifies protein dimerization, the K-domain is decisive for dimeric protein interactions, and C-terminal is transcriptional activation and protein interaction [10,34,35]. The K-domain is comprised of three independent domains: K1, K2 and K3, which are encoded by 3 exons making 70 amino acids long K-domain. It is an indispensable domain since MADS-domain proteins bind to the DNA as a homodimer or heterodimer that is only achievable by means of this domain [33,36]. Studies to find out the minimal protein domains required for the protein-protein interaction between two MADS-domain proteins shows that, the presence of K2 and K3 domains is sufficient enough for heterodimer formation and truncated proteins having only the K1 domain are not able to mediate the protein-protein interaction [37]. In comparison to the other protein domains, the C-terminal is the least conserved region. Even though it has no specific conserved function, studies shows that the C-terminal has other roles such as activation of transcription and stabilizing protein interaction to the formation of DNA-binding [38,39]. Furthermore, it has been recently shown that regulatory motifs, which are present in the C-terminal region, are also crucial for the formation of protein-protein interaction. The *E. californica* B-class protein, SEIRENA, requires the PI-motif present in the c-terminal domain to form a higher order protein complex; and mutants which are truncated for this domain shows an altered floral organ formation due to the absence of a protein complex that is necessary for the floral organ identity [34].

Based on phylogeny reconstructions and exon-intron structure analyses, the type-II lineage of MADS-box genes are further subdivided into two classes: the MIKC^c-type ('c' stands for 'classic') and MIKC*-type [40,41]. The main difference between these two classes is the length of I-and K-domain sequence stretches. MIKC*-type proteins have longer I-domain and K-domain compared to the MIKC^c-type [41]. In spite of the presence of abundant MIKC*-type genes in different species, functional studies are limited to the

model species *Arabidopsis* whereby most studies indicated that MIKC*-type MADS-box genes are mainly expressed during pollen development and regulate the transcription of genes involved in male reproductive organ development; as shown in male infertile *agl66* and *agl104* mutant plants [42,43].

Even though the MIKC^{c}-type genes are involved in several functions, their striking feature is an ability to control identity and development of one of the most remarkable trait of land plants, the flower [36,44]. The following sub-chapters will discuss in detail the role of transcription factors in flowering time control, carpel, ovule and fruit development with more emphasis on the role of MADS-box transcription factors.

1.3 Genetics of Flowering Time Control and Flower Organ Development

As mentioned in the previous section, the MIKC-type MADS-box transcription factors are involved in several regulatory pathways including flower induction and floral organ development. Flowering time is an adaptive trait where its timing heavily depends on the ecology, season and the genetic composition of the plant [45]. Photoperiod, hormone, light quality, temperature, carbohydrate and vernalization falls into either floral-promotion or floral-enabling pathways. These enablers or promoters of flowering conditions control the expression of flower inducing/repressing genes such as, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*), *AGL24*, *FLOWER LOCUS C* (*FLC*), *FRUITFULL* (*FUL*), *MADS AFFECTING FLOWRING 1* (*MAF1*), *SHORT VEGETATIVE PHASE* (*SVP*), *LEAFY* (*LFY*), *FLOWERING LOCUS T* (*FT*), *FRIGIDA* (*FRI*) where majority of belongs to MADS-box transcription factors family [11,26,46-48].

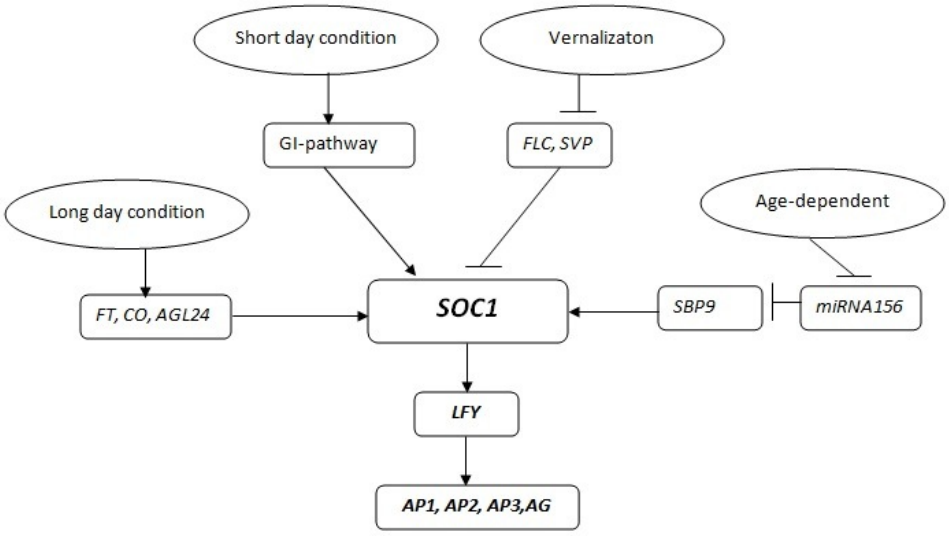

Figure 1: *SOC1* mediated flowering time regulation and floral organ formation pathway

SOC1 is one of the major flowering time regulators in *Arabidopsis* and it codes for a MADS-domain protein mainly localized in leaves and inflorescence meristem. Expression of *SOC1* is regulated in a complex manner that heavily relies on the activation/deactivation of several genes depending on the environmental and internal stimuli as shown in Figure 1 [49]. In a long-day growing condition, *CONSTANCE* (*CO*) and *FT* genes induce *SOC1* expression; while in short-day growing condition, gibberellin acid (GA) pathways favor its expression. *FLC* and *SVP* negatively regulate *SOC1* expression; but upon vernalization their expression becomes reduced favoring *SOC1* expression [47,49,50]. Gradual decrease in the expression of *miRNA156* as the age of the plant advances leads also to the activation of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE9 (SPB9)* expression that subsequently induces *SOC1* expression [51]. In addition, *AGL24* is reported to induce the expression of *SOC1* directly by counteracting the expression of *FLC* and *SVP* in a vernalization independent pathway [52].

Once *SOC1* is expressed, it activates *LFY* and floral organ pattern formation initiated in the course of the induction and maintained expression of floral homeotic genes. Expression of *LFY* is also regulated by several MADS-box transcription factors such as *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), *AGL24* and *FUL* in a redundant manner [53-56]. During flower formation initiation, *LFY* activates the expression of *APETALA1* (*AP1*) and together with *AP1* starts defining pattern formation in the floral meristem via antagonistic

regulation of the gene responsible for shoot apical meristem identity, *TERMINALFLOWER1* (*TFL1*) [57-60]. Furthermore, along with *AP1* and *UNUSUAL FLORAL ORGANS* (*UFO*), *LFY* activates *APETALA3* (*AP3*), a B-class gene, and with *WUSCHEL* (*WUS*) it promotes the expression of *AGAMOUS (AG),* a C-class gene [61,62].

Once the floral meristem is formed, it starts to be partitioned into four concentric whorls that becomes the site for the four floral organ primordial formations. Initiations of these organs primordial is orchestrated by the floral organ identity genes that mainly belongs to the MADS-box transcription factor family. Characterization of floral mutants of *A. thaliana* led to the identification of these floral organ identity genes and formulation of the classical 'ABC model' of flower development [63]. According to this model, the interaction between floral organ identity genes is crucial for the specific expression pattern of each gene within the four concentric whorls. Based on this expression pattern, these genes are categorized into the ABCDE classes where by A-class genes [*AP1* and *APETALA2* (*AP2*)] expressing in the sepal and petal, B-class genes [*PISTILLATA* (*PI*) and *AP3*] expressing in the petal and stamen, C-class gene (*AG*) expressing in the stamen and gynoecium, D-class genes [*SHATTERPROFF1* (*SHP1)*, *SHP2* and *SEEDSTICK* (*STK*)] expressing in the ovule, E-class genes [*SEPALATA 1-4* (*SEP1-4*)] expressing in all floral organs [63]. One of the interesting aspects of some floral organ identity gene is their ability to restrict the expression of another floral organ identity gene by antagonistic repression of the gene within the concentric whorl they are expressed. For example, *AP1* expression is restricted from the third and fourth floral whorl mainly due to the negative regulation of *AP1* by *AG* [64].

Among these classes of genes, the E-class is comprised of genes that act highly in a redundant manner throughout the specification of each floral organ. The E-class genes are very essential in the floral organ morphogenesis mainly due to their important role in mediating high order protein complexes. According to the "floral quartet" model, higher order protein complexes formed between floral organ identity proteins mediated by the E-class genes binds to the two CArG box DNA motifs present in the promoter region of target genes to initiate their expression [36].

1.3.1 Role MADS-box Transcription Factors in Gynoecium Development

A proper synchronized development of gynoecium building blocks (stigma, carpel and style) is essential for the success of producing a viable seed. In *Arabidopsis*, gynoecium development commences during stage 6 of flower development and it becomes ready for fertilization by stage 13 [65]. By stage 13, the gynoecium is comprised of an elongated epidermal cells on the top known as the stigma which are essential for the reception of the pollen grain [66]. The style gives the gynoecium a cylindrical shape and it is comprised of epidermal cells and stomata tissue [66]. Within the style is the transmitting tract where the pollen germinates and travel through towards ovules. Two valves and replum makes the ovary outer parts while the inner sides is comprised of a septum that divides the ovary into two compartment and serve as site of ovule formation [67].

In *Arabidopsis*, *AG* plays an important role in the carpel and stamen morphogenesis [68]. It represents the C-class genes and its expression in *Arabidopsis* begins when *LFY* and *WUS* bind to the regulatory sequences present in *AG* intragenic sequence at early stage of third and fourth whorl primordia formation [62,68], whereas its expression is restricted from the first and second floral organ whorls by *AP2* [69,70]. *AG* loss-of-function causes defects in the two central reproductive organs, stamen and gynoecium; replacing them with petal- and sepal-like structure and continue the formation of floral meristem [71]. Gain-of-function plants, ectopically expressing *AG* gene, were able to convert the first and second whorl organs into carpel like and stamen like structures respectively indicating its essential role in formation of reproductive parts of the flower [35].

Paralogs of *AG*-like genes are found in other species showing similar pattern of expression and function at least for one copy of the *AG* orthologs [72-83]. In *E. californica*, there are two *AG* orthologues (*EScaAG1* and *EScaAG2*) that redundantly function in specifying the stamen and carpel identity. Down-regulation of both genes causes a homeotic conversion of stamen into petal and loss of carpel identity along with floral meristem indeterminacy, indicating the functional conservation of *AG* across different lineages [83].

Monocots homologs of the *Arabidopsis AG* were also well characterized using model species *Oryza sativa*, *Zea mays*, and *Triticum aestivum* [84-86]. Maize also has two *AG* homologs, *ZAG1* and *ZAG2*, that are highly identical in amino acid sequence but differ in expression pattern. Expression of *ZAG1* starts during stamen and carpel primordial formation while *ZAG2* starts to accumulate during late stage of developing carpel [85]. Three copies of *Arabidopsis AG* homolog known as *WAG* are reported to be present in wheat. One copy of *WAG* is reported to be expressed in the reproductive and non-reproductive organs of the spikes while another copy of *WAG* is found out to be expressed in the pistil organ [77]. Four *AG* homologs are present in rice which act in a redundant manner in the regulation of stamen and carpel identity along with floral meristem determinacy [84]. These findings imply that the ancestral function of *AG* gene (stamen and gynoecium development) is strongly conserved in both monocots and dicots.

Due to genome duplication events, genes that belong to the *AG* subfamily have gone through subfunctionalization, neofunctionalization or becomes non-functional [87]. In some species, a variation in degree of expression and functional role of the paralog genes were documented implying that after duplication event, one of the paralog has become a pseudo gene or becomes sub-functionalized [74,78,83]. Such variation in function and sequence information within the *AG* subfamily lead to the formation of two distinct lineage, where some of these genes function in a redundant manner with *AG* or can undergo neofunctionalization and caught up in a new role of plant development [87]. For example, presence of functional redundancy was shown in *ap2 ag* double mutants that were able to form carpel like structure in their first whorl; indicating the presence of other genes responsible for such morphogenesis [88]. *SHP1* and *SHP2,* previously known as *AGL1* and *AGL5*, have been shown to be sufficient enough to redundantly act to form the carpeloid like structure present in the *ap2 ag* double mutants since plants which are quadruple mutants for *shp1 shp2 ag ap2* completely lack a carpel like structure. Further, when transgenic *ag* mutants were transformed with a construct that constitutively express *SHP2,* the carpel and stamen like structures were formed that proves again the functional redundancy between *AG* and *SHP2* [89]. Moreover, carpel like structure is absent on the first floral organ whorl of those plants that are triple mutant for

ap2 ag spatula (*spt*) or *ap2 ag crabs claw* (*crc*). This further indicates regulation of carpel formation extends beyond *AG* or *AGL* genes but instead can be independently promoted by other genes that belong to the YABBY and basic-helix-lop-helix transcription factors family [90].

1.3.2 Role of Transcription Factors in *Arabidopsis* Ovule Development

As mentioned in the previous section, the gynoecium organ of most angiosperm flower is comprised of one or more carpel (either individual or fused), pistils with stigma on the tip and the ovary at the base. The ovary contains single or several ovules that develops into seed. In comparison to other floral organs, the morphogenesis and evolutionary origin of ovule is quite different and complex. Origin of ovules is estimated to date back to 400 million years and since they are originated prior to most of the floral organs, they can also be considered as a separate organ in flowering plants [91,92].

Arabidopsis ovule is mainly composed of a nucellus tissue enclosing the megasporocytes, two integuments that enclose the nucellus and becomes the seed coat after fertilization, and a funiculus that establishes ovule attachment to the placenta. *Arabidopsis* ovule development take place during stage 8-14 of floral organ development and it is further sub-divided into four main ovule developmental stages [65,93].

Stage-1 ovule development

During this early development phase, ovule formation is initiated from the primordia found on the placenta surface of the carpel tissue. And with subsequent cell division and expansion, a finger like protrusion comprised of an epidermal cell layer and a sub-epidermal tissue is formed [93]. Auxin and cytokinin synthesis and transport were reported to play a key role in providing the platform for the establishment of ovule primordium as shown in mutations that led to increase in cytokinin degradation promoting ovule formation [94,95].

So far, a particular gene that is entirely responsible for *Arabidopsis* ovule identity has not been identified. Several genes have been implicated to be involved in the ovule identity and development despite none of their mutation causes a complete loss of ovule formation [96]. Nevertheless, studies shows that transcription factors, mainly MADS-

box transcription factors, play a significant role in specifying the formation of ovule primordia [95]. It has been mentioned in section 1.3.1 that *SHP1, SHP2* and *AG* act in a redundant manner in carpel formation. Nevertheless, the *SHP1 and SHP2* genes are reported to be involved in the ovule identity formation in redundant manner along with *SEEDSTICK* (*STK*): previously known as *AGL11*. Homeotic transformations of the ovule into carpel like and leaf like structures was observed in the *stk shp1 shp2* triple mutants [89]. Similar defects in ovule development was observed when *STK* orthologues from *Petunia hybrida*, *FLORAL BINDING PROTEIN7* (*FBP7*) and *FBP11*, were knocked-down indicating the necessity of *STK* for ovule development and its functional conservation across dicots [97].

In addition to MADS-domain proteins, transcription factors that contain the *AP2*-domain were also reported to be involved in the ovule development including *AP2* [98]. *AP2* is involved in the organ identity formation of each four whorls including ovule. *AP2* Loss-of-function mutation causes a homeotic conversion of ovules into carpel like structures and other homeotic conversion of floral organs together with reduction in floral organ number [98,99].

Stage-2 ovule development

This is a developmental stage where the nucellar cells undergo cell elongation and proliferation to differentiate into the nucellus that harbors the megaspore mother cell (MMC) [100]. Furthermore the inner and outer integuments formation is initiated from the chalaza region and progress towards the distal region in order to completely cover the nucellus and by the end of this stage the MMC becomes enlarged and undergoes meiosis to produce four megaspores [93].

According to Endress et al. [101], the integuments plays a significant role in the morphogenesis of the ovule by giving it several functional parts such as nucellus, chalaza, micropyle and funiculus. Besides to giving a defined feature to the ovule, proper development of ovule sporophytic cells determine the female gametophyte development [102]. Therefore strict control of proper cell division and cell elongation must take place to ensure plant fertility [103].

The inner and outer integuments are comprised of cells which are structurally different. Cells of the outer integuments are relatively large and consist of larger vacuoles than to the inner integument cells indicating their development are controlled separately by different genes [104]. Several genes have been implicated in the initiation and development of either of ovule integuments. Among them is a homeodomain transcription factor known as *BELL1*. It is the first gene identified to be essential for proper integument development in *Arabidopsis*. *bel1* mutants have a thick funiculus attaching the ovule with the placenta and lack the inner integument while their outer integument is replaced with an abnormal carpel-like structure that gives them a bell-shape structure. Orientation of the nucellus within the ovule also differs and is not completely covered by this modified integument. Furthermore, due to a defect in the embryo sac formation and subsequent abnormal gamete formation, *bel1* mutant plants are female sterile [105]. According to Ray et al. [106], some of these homeotic conversions of the ovule integuments into a carpel like structure are enriched with higher expression of *AG* indicating *BELL1* counteract the expression of *AG* in the ovule integument region. While *BELL1* autonomously control the integument initiation and embryo sac development, a regulatory network between *BELL1* and *AG* is implicated in the ovule primordium identity [107]. The degree of organ alteration becomes even much more pronounced when plants quadruple mutants for *bel1 shp1-1 shp2-1 stk* showed absence of functional megasporangia along with sever defects in ovule integument development. Based on the mutant phenotypes and a yeast-two hybrid data it is suggested that formation of higher order protein complex between *AG, STK, SHP1, SHP2* and *BELL1*, is essential for proper ovule integument initiation and development in *Arabidopsis*[108]. Even though these genes regulate the ovule development to a large extent, mutant plants lacking all these genes were still able to form few wild type like ovule primordial outgrowth and integument initiation, indicating that ovule development is controlled by several redundantly acting genes [96,99,107,109].

Another gene required for proper ovule integument development is known as *AINTEGUMENTA* (*ANT*). It is an AP2-domain protein and essential for the initiation of the integument growth. Similar to *bel1* mutants, the ovule primordia in *ant* mutants remains unaffected and mutants are female sterile. However, unlike *bel1* mutants, *ant*

mutants fail to make both integuments at all [110]. Similarly an altered ovule integument formation via increase cell number and cell arrangements was observed in the *Arabidopsis short integuments1* (*sin1*) mutants. *sin1* mutation causes the lack of complete nucellus coverage accompanied with a defect in embryo sac development that becomes apparent after fertilization [105].

In *Arabidopsis*, the growth pattern of inner integument differs from the outer integument. The inner integument grows in a symmetrical manner surrounding the nucellus while the outer integuments have an asymmetric pattern with large number of cell proliferation occurring on the abaxial side of the ovule [102]. Maintenance of such polarity of cell division is guided by several genes. Among them is a member of YABBY gene family coding for a zinc-finger and HMG-like protein known as *INNER NO OUTER* (*INO*). *INO* is required for the proper growth of the outer integument as shown in *ino* mutants that fail to initiation the outer integument completely. On the other hand, the outer integument development does not entirely rely only on *INO* expression. Despite the presence of *INO* expression, integument development fails to develop in *ant* mutants indicating the presence of redundantly acting genes [111].

aberrant testa shape (*ats*) is another ovule mutation resulting in a defect in seed structure due to the presence of a modified single integument resulted from the fusion of the two integuments. *ATS* codes for a protein that belongs to the *KANADI* (*KAN*) family, where two of its other members: *KAN1* and *KAN2*, also redundantly control ovule outer integument development [104]. According to Villanueva et al. [111] and McAbee et al. [104], *INO* and *ATS* are required to determine the polarity of outer and inner integuments respectively. Both promote the integuments growth on the abaxial side of the ovule. Such restriction of *INO* expression from the adaxial side of the ovule outer integument is achieved by *SUPERMAN* (*SUP*) gene. This is shown in *sup* mutants that reveals unrestricted expression of *INO* to the adaxial side of the ovule causing a symmetrical outer integument growth which leads to altered seed structure [112,113]. In addition to that, the protein complex formed between AG-SEP-BELL1 is found out to be essential to activate the expression of *INO* and promote the integument formation by restricting the expression of *AG* and *WUS* in the integument [109].

As shown in the previous paragraphs, most of the time, there is a direct relationship between the proper developments of the sporophytic tissue and the subsequent gametogenesis process. The megasporocyte or MMC is the product of an active differentiation of archesporial cells that arises from hypodermal cells of the nucellus [114]. In *Arabidopsis*, this sporogenesis process is controlled by a gene known as *SPOROCYTELESS* (*SPL*). *SPL* (also known as *NOZZLE* (*NZZ*)) is a transcription factor expressed mainly in the megasporocyte and microsporocyte of *Arabidopsis*. *spl* mutants have ovules that fails to form the MMC due to the absence of archesporial cell differentiation [115]. Besides to that, the MMC formation is crucial for the expression of genes required for the subsequent meiosis step vital for the production of four megaspores [102,115]. Yang et al. [114] stated that the presence of ethylene is one of the crucial elements that triggers the onset of meiosis in tobacco and promote the gametophyte development in *Arabidopsis*.

Stage-3 ovule development

During this developmental stage, three out of the four megaspores degenerates leaving only one megaspore that is closest to the chalazal region known as functional megaspore. This megaspore develops into a mono-nuclear embryo sac, also known as the female gametophyte (megagametophyte). This is the stage where all the pre-fertilization events of the ovule specifically the gametogenesis completed [93,96,105]. According to Reiser et al. [102], the functional megaspore enlarges in size and undergoes a three successive karyokinesis to form a syncytium. After cellularization takes place, the embryo sac contains seven cells with eight nuclei; that prior to fertilization migrate to the chalazal end, central and micropylar region of the embryo sac to become involved in different function. Three cells that migrate to the chalazal end become the antipodal cells and become involved in nutrient transport to the embryo sac [102,116]. The two synergid cell along with the egg cell migrate to micropylar region and function in pollen tube guidance and release of the sperm cells [117-119]. The central and the egg cell becomes fertilized with the sperm cells and becomes the endosperm and zygote respectively [120].

Regulation of this intricate double fertilization process requires proper cell identity formation during the female gametophyte development. Several genes specifically expressed in the female gametophyte have been identified and characterized mainly using the model species *Arabidopsis*[121]. As shown in section 1.2.1, gene that belongs to the type-I MADS-box transcription factors, such as *FEM111*, *DIANA* and *AGL23*, are involved in the female gametophyte development. In addition to that, type-II MADS-box genes such as *SHP1, SHP2* and *STK* have an additional role in indirectly regulating ovule development by controlling the tissue specific expression of *VERDANDI* (*VDD*), a gene which is important for female gametophyte cell identity formation. *VDD* loss-of-functions affects the synergid and antipodal cell identity and subsequently affects the fertilization process [122].

Stage-4 ovule development

This developmental stage is characterized as a post-fertilization stage where the double fertilization process takes place after the pollen tube delivers two sperms to fertilize the central cell and the egg cell that develop into an endosperm and zygote respectively [102]. After successful fertilization process, development of the zygote into an embryo takes place and during this time the nucellus tissue which initially surrounds the female gametophyte undergoes a programmed cell death (PCD) to facilitate the transport of nutrients to the endosperm and embryo [123]. Several mechanism of PCD had been proposed in plants including the production of proteases and nucleases that stimulate the disruption of vacuole, protein and DNA degradation [124-126]. According to Chen et al. [127], degradation of the nucellar cells coincides with the expression of a gene coding for an aspartic protease-like protein indicating timely expression of proteases coding genes to be crucial for proper seed development. For example, *PROTEIN DISULFID ISOMERASE5* (*PDI5*) is involved in the timely expression of genes coding for Cys-proteases. In *PDI5* loss-of-function mutants early onset of PCD occurs which affects the seed viability [124]. *OSMADS29*, rice B_{sister} gene, is also expressed in the nucellus and nucellar projection region where it is crucial for the activation of genes coding for Cys-proteases and several other PCD-related genes. Absence of on time activation of PCD facilitating genes alter the viability and seed grain filling as shown in *OSMAD29* knock-down lines [128-130].

1.3.3 The Role of B_{sister} Genes in Plant Development

The B_{sister} gene clade, named due to their close relationship to B-class genes based on phylogenetic reconstruction, are the latest to be identified as $MIKC^c$-type MADS-box genes. Members of this clade are present in all angiosperms and gymnosperms investigated so far with highly conserved expression pattern across these species [131].

ARABIDOPSIS B_{SISTER} protein (ABS) encoded by the *TRANSPARENT TESTA 16* (*TT16 or ABS*) is the first B_{sister} protein to be functionally characterized [132]. *ABS* expression is restricted to the ovule and the protein is localized in the nucleus [132,133]. Ovules from *abs* mutant lines have an altered inner integument development and a straw color, except in their chalaza-micropyle area, due to the inactivation of the *BAN* gene [132]. Despite these defects, plant morphology and fertility was reported to remains unaffected [132]. Ectopic expression of *ABS* using a constitutive promoter (*CaMV35S*) was shown to affect plant development, specifically leaf and floral organ development [37]. Yeast-based protein-protein interaction assay indicated that, ABS protein is able to form a SEP3 protein mediated higher order protein complex with D- and E-class proteins such as STK, SHP1, and SHP2 [37]. Based on these molecular interaction assay results, mutant plants that lacked the wild type allele for *ABS* and *STK* were functionally characterized to find out if there is also a genetic interaction and the result showed that *abs stk* double mutants have phenotypes which were not observed in their single mutant genotypes. The *abs stk* double mutant genotypes completely lacks the ovule inner integument and has an altered female gametophyte development that leads to low fertility and poor seed setting [133]. *Arabidopsis* also has another B_{sister} gene that is paralogue to *ABS, GORDITA* (*GOA*) previously known as *AGL63* [37]. Based on phylogenetic tree analyses, *GOA* is reported to come into existence during the genome duplication event that occurred only in the Brassicaceae family [134]. *In silico* analyses showed that *GOA* has 59% nucleotide sequence identity with *ABS* until their first 3 exons but differ significantly from that onwards giving *GOA* a domain structure that deviates from the highly conserved MIKC domain structure present in all B_{sister} genes. Deletions of nucleotides in the 4^{th} and 6^{th} exon together with a premature stop codon, prior to the 28 codon unlike to *ABS,* led to a translation frame shift resulting in a new domain structure called Deviant-Domian (DD) in *GOA* [134]. In addition to that, unlike

ABS, the expression of *GOA* is not only restricted to the female reproductive organ, but also present in leaves and sepals. *GOA* Loss-of-function mutation was reported to increase fruit size due to an increase in cell size of the valve mesophyll layers showing the functional diversification of paralog B_{sister} genes in *Arabidopsis* [135,136].

Another B_{sister} genes characterized from Brassicaceae family is isolated from *Brassica napus* (Canola). Canola has seven B_{sister} genes where majority of them are involved in proper development of the endothelium and proantocianidine (PA) accumulation [137]. Down-regulation of *Bntt16s* showed several phenotypes such as: late flowering, dwarfed plants, wrinkled leaf, larger floral organs, decrease in number of inflorescence and total flowers, shorter siliques, fewer seeds, and defect in the pollen tube guidance. In addition, down-regulation of *BnTT161-4* leads to seeds with abnormal shapes (flattened and wrinkled) and seeds with defective embryo to no embryo[137]. The reduction in the accumulation of fatty acid and altered expression of genes involved in different physiological processes in *BnTT161-4* knock-down lines indicates the vast physiological importance of *BnTT16s* in canola plant development [137]. In addition to the functional similarity between *BnTT161-4* and *ABS* with regard to endothelial development and PA biosynthesis, complementation of the *Arabidopsis abs-6* mutant by *BnTT16s* and restoring the wild type seed appearance indicates the functional conservation of B_{sister} genes in these two species [138].

Petunia hybrida B_{sister} gene, *FLORAL BINDING PROTEIN 24* (*FBP24*), is expressed exclusively to the ovary[139]. Even though a transposon insertion mutant does not reveal any alteration in the plant development, with a co-suppression approach it was reported that down-regulation of *FBP24* affects the plant fertility and seed pigmentation. Despite having similar function, expression pattern and protein interacting partners with *ABS*, *FBP24* is not able to complement the *Arabidopsis abs* mutant [139].

Unlike eudicots, a monocot B_{sister} gene acts differently during seed development. *OsMADS29,* one of the three B_{sister} homologs in rice, regulate the expression of genes which are required for PCD in the nucellar region of developing seeds. Expressed mainly in the ovule, down-regulation of *OSMADS29* resulted in shrunken seeds with poor grain-filling rate. Auxin regulated expression of *OSMADS29* binds to the promoters

of a Cys protease to stimulate the degradation of nucellus and nucellar projection via PCD which ensures proper development of the endosperm [129,130]. Another rice B_{sister} Loss-of-function mutant, *fst* (ORF is identical to *OSMADS29*), also showed a strong phenotype where the plant is completely female sterile due to the absence of embryo and endosperm development [128].

1.3.4 *Arabidopsis thaliana* Fruit Development

Fruit development is considered as the last stage of sexual reproduction, where all the complex floral organ formation comes step by step to a successful end with a fruit containing viable seed. Plants with dehiscent fruit, such as *Arabidopsis* and *E. californica*, open up their fruit and shatter their seeds upon maturation. In *Arabidopsis*, fruit development starts at stage 14 (flower opens and fertilization already took place) [140]. The subsequent developmental stages are mainly described as the elongation of the ovary tissue. This signifies that, proper pattern formation and tissue organization across the apical-basal axis of the gynoecium prior to fertilization event to be essential for the subsequent fruit development [120].

Arabidopsis fruit consists of four major parts: the septum (divides the fruit into half and forms a structure that enables pollen tube growth), the valve (the silique wall), the replum (the abaxial side of the fruit that remains attached to the plant after the fruit opened) and the valve margins (form the separation layer where the fruit opens) [141]. After fertilization took place, hormones released from the fertilized ovule trigger the elongation and division of cell layers of the valve and the septum. Such apical-basal distribution of auxin on the gynoecium dictates the pattern formation [120,142]. Late in silique development, the cell layers that form the marginal valve expand slowly creating a constriction, which will develop into a dehiscence zone. Formation of a constricted marginal valve together with lignifications of the endocarp cells of the valve enable the opening of the fruit and the shattering of the seeds upon maturation [143].

Since fruit is completely or partially derived from the carpel tissue, its proper development is directly or indirectly under the control of most of the genes that are also crucial for carpel and/or ovule development, like *AG*, *FUL*, *SHP1, and SHP2* [141]. As mentioned in the previous paragraph, a typical characteristic of fruit development is its longitudinal growth that allows the developing seed to get more space as their size

increases. Such morphogenesis of the fruit valve is controlled by the *FUL* gene in *Arabidopsis*. *FUL* loss-of-function mutation was reported to cause absence of cell expansion and alters cell division in the valve tissue giving rise to a short silique, which opens prematurely due to a conversion of valve cells into valve margin-like cells [144]. The valve margin cells are important for the formation of a dehiscence zone and their development is regulated by the *SHP1* and *SHP2*. Transcripts of *SHP1* and *SHP2* are localized in the carpel and ovule tissue, and during the later stages of flower development a specific localization is detected in the area where valve margin and the replum meets [145]. Such restriction of *SHP1* and *SHP2* genes expression is accomplished by negative regulators, such as *FUL* and *REPLUMLESS* (*RPL*), in the valve and replum region to maintain cell identity [140,144,146]. *SHP1* and *SHP2* share 87% amino acid sequence similarity and with similar expression pattern they act in redundant manner with regard to dehiscence zone formation and subsequent lignification of the valve cell layer. *SHP1 SHP2* loss-of-function mutation causes a defect in the formation of the dehiscence zone upon which matured silique fails to open [58]. Despite the fact that *SHP1* and *SHP2* are required for valve margin lignification and formation of separation layer, the presence of valve-margin like structures in the *ful shp* double mutant plants point out the presence of other genes sufficient enough to make such structures on the valve [140]. These genes, which are also involved in the dehiscence zone formation belongs to the basic-helix–loop–helix transcription factor family and are called *ALCATRAZ* (*ALC*) and *INDEHISCENT* (*IND*). *IND* plays a major role in formation of valve-margin like structure as shown in *ful ind* mutant plants that fail to form such ectopic structure. Furthermore, normal fruit elongation is observed in plants quintuple mutant for *shp1-1 shp2-1 ful ind alc* indicating the major role of *FUL* as the controller of spatio-temporal expression of genes required for valve margin formation and differentiation [120,147,148].

Another MADS-box gene required for proper fruit development is the *Arabidopsis* B_{sister} gene *GOA*. It is required for fruit valve cells expansion regulation that is shown in *GOA* loss-of-function mutants, which give rise to short & thick fruit compared to wild type plants. Down regulation of genes favoring cell proliferation and expansion were also documented in the *GOA* over-expression lines supporting the idea of *GOA* as cell expansion regulator during fruit development [136].

1.4 History of B_{sister} Genes Evolution in Seed Plants

Floral organ identity genes crucial for the morphogenesis of the reproductive parts are highly conserved in sequence and function compared to those involved in bearing non-reproductive organs [14]. It has been suggested, for example, that members of the B-class subfamily are strongly conserved and linked with stamen development in both gymnosperms and angiosperms, except in the latter case they are also involved in petal identity formation indicating genes forming the perianth organs are comparatively recent to the reproductive organs [9,14].

Novel flower structures, like petals or sepals, are chiefly the result of gene duplication events occurred on the ancestral genes, mainly due to whole genome duplication [60,87,149,150]. This is mainly due to the fact that the fate of a duplicated gene in the subsequent generation falls into one of the following categories: loss, subfunctionalization or neofunctionalization [151]. Within the MADS-box genes, it has been suggested that whole genome duplication events contribute for the birth of new subclades comprised of genes that have sequence similarity with the ancestral gene while differing in function [152,153]. Case in point is the duplication of ancestral B-class genes that led to the origin of two sub-clades known as AP3-like and PI-like genes. The corresponding classification is based on phylogenetic analyses and presence of specific motifs found in their protein C-terminal [PI-motif (in PI homologues) and PI Motif-Derived/euAP3-motif/paleoAP3 motif in AP3-like genes], essential for proper functioning of the protein, signifying major gene duplication event in their ancestral gene [34,154].

B_{sister} genes, sister clade of B-class genes, are found in all seed bearing plants studied so far indicating their importance for plant development [155]. Signature motifs present in B-Class genes, such as the paleoAP3 motif, are as well present in similar fashion in B_{sister} genes [131]. It has been suggested that such motif is further present in the fern (*Ceratopteris richardis*) B-class like gene, indicating the presence of a common ancestor for B-class and B_{sister} genes (Figure 2) [149,156].

Due to polyploidization and genome duplication events, copy number of B_{sister} genes varies from species to species ranging from a single copy, for example in *Gnetum gnemon,* up to seven copies in *Brassica napus.* Proteins coded by B_{sister} genes share

similar domain structure and expression pattern, even though their way of regulating ovule development differs from one another as described in Table 1. Moreover recent gene duplication event has led to the birth of a structurally and functionally new B_{sister} gene only specific for the Brassicaceae family [128,129,131,132,134,136-139,157].

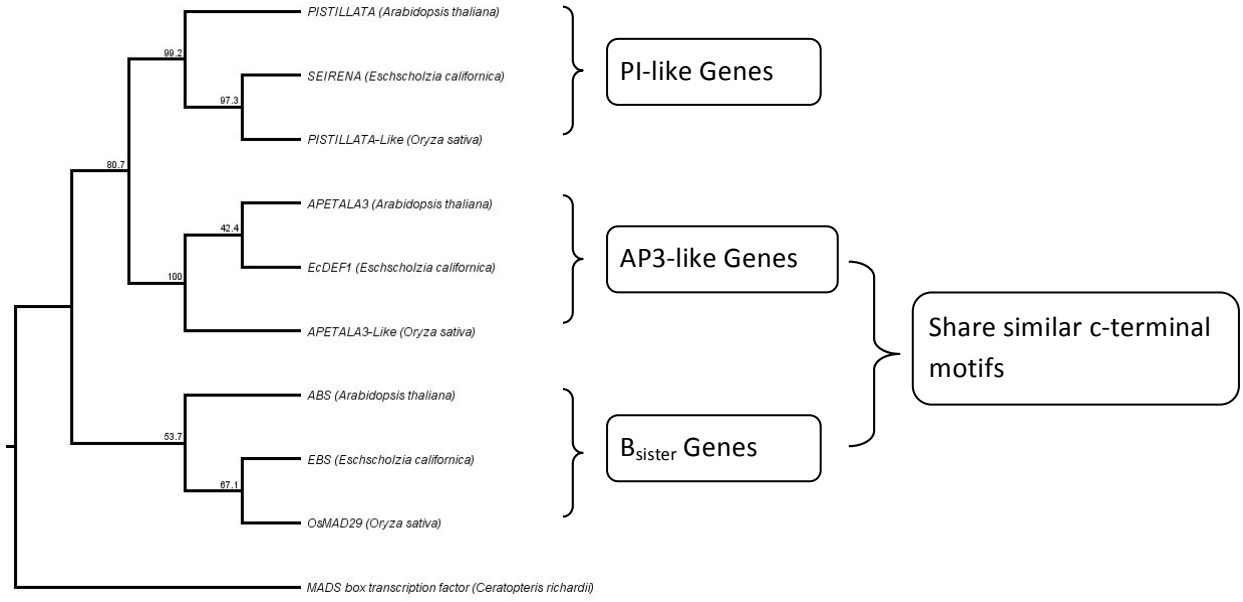

Figure 2: Amino acid alignment based Maximum likelihood phylogenetic tree construction showing the evolution of B_{sister} and B-Class Genes. MADS-box gene from the fern *Ceratopteris richardis* is used as an outgroup. The B_{sister} and AP3-like genes share similar C-terminal motifs such as the paleo-AP3 motif, euAP3 motif and PI-Derived motif where as the PI-like genes contain only the PI-motif. Modified from [152]

One significantly different gene among B_{sister} gene clade is the *ABS* paralog *GOA*. Protein domain structure in *GOA*-like genes deviates from the conserved MIKC type structure which is a characteristic of in most B_{sister} genes. As mentioned in previous sections, a frame shift caused by nucleotide deletions on the 4th and 6th exon plus a premature stop codon 28 codons earlier than its paralog *TT16* led to origin of a new domain called Deviant-domain (DD). Based on sequence homology, so far GOA-like genes were identified from *Capsella rubella*, *Arabis alpina*, *Arabidopsis lyrata, Brassica napus*, *Thellungiella parvula* and *Brassica rapa* (Amey Bhide and Olesia Gavryliuk, Personal communication) indicating their presence in Brassicaceae only, which perhaps is due to a specific single gene duplication event as described by Erdmann et al. [134].

Table 1 Characteristic of B_{sister} genes from different lineages [128,129,131,132,134,136-139,157]

Species	Gene name	Expressed in	Function
G. gnemon	*GGM13*	Nucellus, inner envelope	N/A
Arabidopsis	*ABS (ABS)*	Inner Integument	Involved in the regulation of Inner integument development of the ovule and seed pigmentation
	GORDITA (GOA)	Flower bud, Silique, Ovule	Regulates fruit growth via cell expansion control
P. hybrida	*FBP24*	Ovule primordia, Nucellus and integument	Inner integument development of the ovule
B. napus	*BnTT161-4*	Silique, Seed	Regulate development of floral organs, fertility and seed oil content
	BnGOA	N/A	N/A
O. sativa	*OsMADS29-31*	Nucellus and nucellar projection	Regulate the expression of genes required for the onset of PCD in ovule
Z. mays	*ZMM17*	Silk	N/A
A. majus	*DEFH21*	Inner integument	N/A
T. aestivum	*TaAGL35*	Inner Integument, Nucellus	N/A

(N/A: data not available)

Among these *GOA*-like genes, *Arabidopsis GOA* is the only one functionally characterized so far. Unlike its paralog *ABS*, expression of *GOA* is not restricted to the ovule; instead, transcripts are also detected in flower bud, mature flower, silique, and leaf. Functionally, *GOA* further differs from *ABS*, its role not being limited to the ovule integument development instead controlling the cell elongation of a developing fruit valve [135,136]. It is pointed out that, the difference in function might be attributed to the presence of a D-Domain that plays a significant role in a higher order protein complex formation in GOA with proteins that do not interact with ABS. *ABS* and *GOA*, moreover, differ in their cis-regulatory elements which are present along the promoter regions, that might affect their expression pattern [135]. Based on the above significant difference between the two paralog genes and the absence of redundant function, it is suggested that post gene duplication, *GOA* has undergone neofunctionalization [134].

1.5 Stable Genetic Transformation of *E. californica*

For a thorough characterization of a given gene, analyses of its loss-of-function or gain-of-function via genetic manipulation of the host plant is vital and among the methods is stable genetic transformation where it comes as a preferable choice in reverse genetics approach. For this purpose, a number of stable genetic transformation protocols have been published for plant species, mainly for cash crops [158-162]. Nevertheless, several protocols are also optimized for species that are used for different basic research purposes such as *Arabidopsis* and *Petunia* [163,164]. However, efficient optimized stable transformation protocols are still lacking for species that are important in evolutionary developmental studies such as *E. californica* (California poppy). California poppy has become a chosen model species in the evolutionary developmental genetic and plant secondary metabolites studies and several important findings have been reported that proved the importance and significance of using it as a model species [34,83,165]. California poppy is known for its high amount of plant secondary metabolites production and several studies have been done in the isolation and characterization of pharmacologically useful compounds from it [166-168]. So far, numerous optimized protocols and other resources are available for molecular and phenotypical characterization of California poppy. A detailed description of the floral and vegetative morphogenesis [169], optimized *In situ* hybridization protocol [165] and microRNA chip for

expression analyses [170], available fast neutron irradiated mutant population for forward genetics experiments, a BAC library covering majority of the genome [171], a virus induced gene silencing (VIGS) protocol for transient down-regulation of gene [172], and sequence database from floral and vegetative organs are also available [170,173]. Until now, most of the studies conducted to characterize the function of a given gene from California poppy rely on the use of transient down-regulation methods such as VIGS or by transforming cell culture suspensions. The down-side of such transient infection is that it can only provide information for a single generation assessment of the gene of interest, and the efficiency of the method varies across seasons and developmental stage of the plant. Even though cell culture suspensions facilitate the analyses of secondary metabolites, it cannot contribute any significance in obtaining an insight to studies that require analyses of the plant morphology since whole plant regeneration is not accomplished. Furthermore, in order to obtain the complete understanding of a given gene, analyses of subsequent generation is required which can only be achieved by integration of the transgene into the plant genome. Previous studies has shown the possibility of obtaining a stable transgenic plant using excised cotyledons and embryonic callus as an explants and different *Agrobacterium* strains for inoculation [174-176]. However some of the protocols were not reproducible in our lab (Aravinda Yellina, Personal communication).

1.6 Aims of the Projects

The fact that *ABS* loss-of-function shows a weak phenotype and recent evidences indicating the possibility for the presence of redundantly acting genes, were the driving force to further investigate in detail the functional relevance of *Arabidopsis* B_{sister} genes. In this study, attempts are made to study further the importance of *ABS* in *Arabidopsis* ovule/seed development and find out the presence of functional redundancy with other MADS-box transcription factors. Phenotypical and molecular investigations of plants mutant for these genes are supposed to provide substantial data that can strengthen the understanding of ovule development in angiosperms.

In addition to the weak phenotype associated with *abs* mutant, functional characterization of *FBP24* loss-of-function mutants fails to show any alteration in the plant development. Possible hypothesis for the lack of strong phenotype in these mutant genotypes possibly might be;

i) Presence of redundantly acting gene, or

ii) Loss of function in higher eudicots.

Thus, in order to find out unknown function of B_{sister} genes and to understand the functional diversification and/or conservation of B_{sister} genes so as to fill the information gap that existed between the monocots and core eudicots, analyses of a basal eudicot B_{sister} gene is crucial.

Based on these assumptions, further investigation of a B_{sister} gene is carried out using another model eudicot species, *E. californic. E. californica* belongs to the earliest branching eudicot lineage, Ranunclales [177,178]. Due to its phylogenetic position, it helps us to understand the evolutionary history of flowering plants and furthermore it has become a preferred model species in the study of alkaloid biosynthesis and also developmental biology [34,83,166,179-181]. This dissertation contains the analyses of *E. californica* B_{sister} gene (*EBS*) using molecular and genetics methods so as to provide further understanding of B_{sister} genes in angiosperms. The findings obtained from these investigations are believed to provide informations regarding the role of B_{sister} genes in angiosperms. Furthermore, in order to use *E. californica* as a model species efficiently for other developmental studies, a stable genetic transformation protocol is optimized in order to facilitate the functional characterization of genes at different developmental stages and generations.

2. Materials

2.1 Plant Material

Arabidopsis wild type (ecotype Columbia and Wassilewskija) and the mutant alleles *abs*, *shp1-1 shp2-1*, *goa-1* and *agl16-1* described in [89,132,136,182] are obtained from European *Arabidopsis* Stock Center (NASC). California poppy (*E. californica*'Aurantiaca Orange King') seeds were obtained from B&T world Seeds Sarl, Paguignan, France.

2.2 Bacterial Strains

Chemical competent *Escherichia coli* strain DH5α was used for cloning purposes. Electro competent *Agrobacterium tumefaciens* strain GV3101 was used for plant transformation.

2.3 Yeast Strain

Saccharomyces cerevisiae strain AH109 was used for the Yeast-two hybrid assay.

2.4 Growth Media

2.4.1 Bacteria growth media

LB medium (for plates, 1 l)

1% (w/v) Peptone

0.5% (w/v) Yeast extracts

1% (w/v) NaCl

1.5% (w/v) Agar

Adjust pH to 7.2

2-YT medium

1.6% (w/v) Peptone

1% (w/v) Yeast Extract

0.5% (w/v) NaCl

Adjust pH to 7.0

SOC medium

20 g/l Peptone

5 g/l Yeast extract

10 mM NaCl

2.5 mM KCl

10 mM $MgSO_4$ x $7H_2O$

2.033 g/l $MgCl_2$ x 6 H_2O

20 mM glucose (filter sterilized added after autoclaving)

2.4.2 Yeast growth media

Solid YAPD medium

1% (w/v) Yeast extracts

2% (w/v) Peptone

2% (w/v) Dextrose

0.01% adeninehemisulfate

1.8 % (w/v) Agar

SolidSD-Leu-Trp (SD-LW) medium

2.67 % (w/v) Minimal SD Base (Clonetech, France)

0.062 % (w/v) DO Supplement (-Leu/-Trp) (Clonetech, France)

Adjust pH to 5.8 with NaOH

1.8 % (w/v) Agar

Autoclave

Solid SD-Leu-Trp-His (SD-LWH) medium

2.67 % (w/v) Minimal SD Base (Clonetech)

0.062 % (w/v) DO Supplement (-Leu/-Trp/-His) (Clonetech)

Adjust pH to 5.8 with 1 M NaOH

1.8 % (w/v) Agar

Autoclave

Add 3mM 3-AT before plating.

2.4.3 Plant and tissue culture growth media

Solid MS Medium

2.21 g/l Murashige & Skoog medium including B5 vitamins (Duchefa Biochemie, Haarlem, The Netherlands)

7 g Phytoagar

Fill up to 1 l with sterile ddH_2O; adjust pH to 5.8 and autoclave

Solid Co-cultivation medium

3.16 g/l B5 Vitamins

30 g/l Sucrose

8.4 g/l Phytoagar

Fill up to 1 l with sterile ddH_2O; adjust pH to 5.8 and Autoclave

After cooling add filter sterile 2 mg/l α-Naphthalene acetic acid (NAA) and 0.1 mg/l 6-Benzylaminopurine (BAP)

Solid Callus induction medium

3.16 g/l B5 vitamins

30 g/l Sucrose

8.4 g/l Phytoagar

Fill up to 1 l with sterile ddH_2O; adjust pH to 5.8 and Autoclave

After cooling add filter sterile 2 mg/l NAA, 0.1 mg/l BAP, 300 mg/l Ticarcillin potassiumclavulanate and 1 mg/l Phosphinothricin (PPT)

Solid Somatic embryo induction medium

3.16 g/l B5 Vitamins

30 g/l Sucrose

8.4 g/l Phytoagar

Fill up to 1 l with sterile ddH_2O; adjust pH to 5.8 and Autoclave

After cooling add filter sterile 1 mg/l NAA, 0.5 mg/l BAP, 300 mg/l Ticarcillin potassiumclavulanate and 1 mg/l PPT

Solid Root regeneration medium

3.16 g/l B5 Vitamins

30 g/l Sucrose

5 g/l Gelrite

Fill up to 1 l with sterile ddH_2O; adjust pH to 5.8 and Autoclave

2.5 Solutions and Buffers

2.5.1 General purpose

TE-buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA, pH 8.0

50X TAE

40 mM Tris-acetate, pH 7.5

1mM EDTA

Rapid DNA extraction buffer

50 mM Tris-HCl, pH 8.0

25 mM EDTA

250 mM NaCl

0.5% SDS

Bacteria Plasmid DNA extraction (STET) buffer

8% sucrose

5% Triton X-100

50 mM EDTA

50 mM Tris pH 8.0

10X MOPS buffer

0.2 M (N-morpholino) propanesulfonic acid (MOPS)

50 mM sodium acetate

RNA denaturation buffer

10ml 100% deionized formamid

3.5ml 40% formaldehyde

1.5ml 10 x MOPS buffer

RNA loading buffer

25% (w/v) Ficoll type 400

0.1 M EDTA solution (pH 8.0)

25% (w/v) bromophenol blue

2.5.2 Yeast assay

Potassium-phosphate buffer

61.5 ml 1 M K_2HPO_4

38.5 ml 1 M KH_2PO_4

PEG MW 3350 (50% w/v)

100 g PEG 3350 (Sigma-Aldrich, Hamburg, Germany)

200 ml ddH_2O

Single-stranded Carrier DNA (2.0 mg/ml)

200 mg Salmon Sperm DNA (Sigma-Aldrich, Hamburg, Germany)

100 ml TE buffer

Mix at 4°C for 2 hr

Store in 1 ml samples in -20°C

Frozen competent cell (FCC) solution

5% (v/v) glycerol

10% (v/v) DMSO

2.5.3 Histology and *In situ* hybridization

Fixation solution (FAE)

10% (v/v) Formaldehyde

5% (v/v) Acetic acid

50 % (v/v) Ethanol

Triethanolamine solution

0.1 M Triethanolamine

Adjust the pH to 8.0

0.6% (v/v) Acetic anhydride was added just before use

Dextran Sulfate

50% (w/v) Dextransulfate

Dissolve at 60°C.

Store at -20°C.

1 M Na Phosphate (pH 6.8)

46.3 ml of 1M $Na_2HPO_4.2H_2O$

53.7 ml of 1 M $NaH_2PO_4.H_2O$

10x *in situ* salts

3 M NaCl

0.1 M Tris pH 8.0

0.1 M Na Phosphate pH 6.8

0.05 M EDTA

Autoclaved and stored at -20°C

4 M $LiCl_2$

4 M $LiCl_2$

Autoclave and store at +4°C.

1 M $MgCl_2.H_2O$

1 M $MgCl_2.H_2O$

Autoclave

$MgCl_2$ is very hygroscopic. Do not store opened bottles for long period of time!

10 X Phosphate Buffer Saline (PBS)

1.3 M NaCl

70 mM $Na_2HPO_4.2H_2O$

30 mM $NaH_2PO4.H_2O$

Adjust the pH to 7.0 with HCl

20 X SSC

3 M NaCl

0.3 M Na Citrate

Adjust the pH to 7.0 with HCl

1 M Tris pH 7.5 –9.5

1 M Tris

Add HCl to adjust pH 7.5 and 8.0. Use NaOH for pH 9.5 (Three different 1 M Tris solution with different pH)

Proteinase K buffer

20 mM Tris-HCl pH 7.5

2 mM $CaCl_2$

Autoclave

Add 5 µg/ml Proteinase-K before use

Paraformaldehyde solution

8 g Paraformaldehyde

Up to 200 ml 1X PBS solution

Dissolve on heat plate and work under chemical hood.

Buffer 1 (for *In situ*)

100 mM Tris-HCl pH 7.5

150 mM NaCl

Buffer 2 (for *In situ*)

100 mM Tris-HCl pH 9.5

100 mM NaCl

50 mM $MgCl_2$, pH 9.5

0.5 % Blocking Solution

0.5% Roche, Mannheim, Germany Blocking powder in Buffer 1 solution

Autoclave and store at +4°C.

Washing solution-A

1 % Bovine serum Albumin (BSA) in Buffer 1

Add 0.3 % Triton X

Antibody solution

1% Bovine Serum Albumin in Buffer 1 solution

Add anti-dig antibodies in 1:1250 ratios (i.e. 8 µl antibodies for 10 ml solution)

NBT-BCIP Staining solution (Should be prepared fresh)

100 mM Tris pH 9.5

100 mM NaCl

50 mM $MgCl_2$

10% polyvinyl alcohol

It should be prepared on heated plate at 60°C. After cooling add 2mM NBT and 2mM BCIP

Glycine buffer

2 mg/ml Glycine in 1x PBS Buffer

Prepare a day in advance.

2.5.4 Southern blot

Denaturation buffer

0.5 M NaOH

1.5 M NaCl

Autoclave

Neutralization buffer

0.5 M Tris-HCl

1.5 M NaCl

Adjust the pH to 7.5 and autoclave

20 X SSC

3 M NaCl

0.3 M Na Citrate

Adjust the pH to 7.0 with HCl

Autoclave

Maleic acid buffer

0.1 M Maleic acid

0.15 M NaCl

Adjust the pH to 7.5

Autoclave

10% SDS

Blocking solution

0.5% blocking reagent (Roche, Mannheim, Germany, Mannheim) in Maleic acid solution

Autoclave

Washing buffer B

0.3% Tween 20 in Maleic acid buffer

Detection buffer

100 mM Tris-Base, pH 9.5

100 mM NaCl

Autoclave

Stripping buffer

0.2 M NaOH

0.1% SDS

Antibody solution

0.05% Anti-Digoxigenin-AP (Roche, Mannheim, Germany, Mannheim) in blocking solution

2.5.5 Transient assay

Injection buffer (For VIGS)

10 mM $MgCl_2$

10 mM Acetosyringone

0.1 mM 2-(N-morpholino) ethanesulfonic acid MES

Re-suspension solution (For BiFC)

10 mM $MgCl_2$

0.15 mg/ml Acetosyringone

2.6 Enzymes

Products are purchased from these companies based on their ease of availability, quality and price tag.

Name	Purchased from
Reverse Transcriptase	Fisher scientific, Schwerte, Germany
T4 DNA Ligase	New England Biolabs, Frankfurt am Main, Germany
T4 DNA Polymerase	Fisher scientific, Schwerte, Germany
Restriction enzymes	New England Biolabs, Frankfurt am Main, Germany
DNase I	Roche, Mannheim, Germany
SYBR Green mix	Roche, Mannheim, Germany
RNaseA	New England Biolabs, Frankfurt am Main, Germany
Lysozyme	Sigma-Aldrich, Hamburg, Germany
T7 polymerase	Roche, Mannheim, Germany
Proteinase K	Sigma-Aldrich, Hamburg, Germany

2.7 Cloning vectors

Vector	Purpose	Resistance	Source
pGEM-T Easy	Cloning PCR product	Ampicillin	Promega, Mannheim, Germany
pJET 2.0	Cloning PCR product	Ampicillin	Fisher scientific, Schwerte, Germany

Vector	Purpose	Resistance	Source
pGBKT7	Yeast two-hybrid assay	Kanamycin	Clontech, St-Germain-en-Laye, France
pGADT7	Yeast two-hybrid assay	Ampicillin	Clontech, St-Germain-en-Laye, France
pART7	Shuttle vector for over-expression construct	Ampicillin	Glycerol stock
pHANNIBAL	Shuttle vector for hpRNAi construct	Ampicillin	Glycerol stock
pTRV2	VIGS	Kanamycin	Glycerol stock
pMLBART	Transient and stable transformation of plants	Spectinomycin	Glycerol stock
pNBV-GENE-YC	BiFCassay	Ampicillin	Glycerol stock
pNBV-GENE-YN	BiFCassay	Ampicillin	Glycerol stock

2.8 Solvents

Substance	Company
Dimethyl Sufoxide (DMSO)	Serva Electrophoresis, Heidelberg, Germany
KOH	Carl Roth, Karlsruhe, Germany
NaOCl	Riedel-de Haen, Seelze, Germany

2.9 Staining substances

Substance	Purpose	Company
FastGreen G	Histology	Carl Roth, Karlsruhe, Germany
Safrarin-O	Histology	Sigma-Aldrich, Hamburg, Germany
DNA stain-G	DNA stain	Serva Electrophoresis, Heidelberg, Germany

3. Methods

3.1 Bacteria Manipulation

In this study, two main bacteria species are used for two main purposes. *Escherichia coli* cells are used for cloning works and *Agrobacterium tumefaciens* are used for the purpose of infecting plants or explants.

3.1.1 Preparation of Competent *E. coli* Cells

The *E. coli* strain DH5α was grown overnight in 5 ml of LB medium at 37°C. The overnight culture was diluted with fresh LB medium in the ration of 1:200 and re-grown at 37°C until the early log phase OD_{600} reached 0.2-0.4. Afterwards, the cells were harvested by centrifugation at 4°C at a speed 5000 rpm for 10 min. From this step onwards, the work was done on ice. After discarding the supernatant, the cells were resuspended in ½ culture volume of ice-cold 0 .1 M $CaCl_2$ and kept on ice for 1 hour .The cells were again centrifuged again at 5000 rpm for 10 min at 4°C and the pellet was resuspended with a 1/10 cultured volume of 0.1 M $CaCl_2$. Ice-cold sterile glycerol to a final concentration of 10% (v/v) was added and kept immediately in liquid nitrogen until it is transferred to -80°C freezer for long term storage.

3.1.2 Heat Shock/Calcium Chloride Method for *E.coli* Transformation [183]

Chemical competent *E. coli* cells were taken from -80°C freezer and kept on ice until they get thawed. Water bath was set at 42°C and 50 ng of plasmid DNA was added to the competent cells. The mixture was gently mixed and kept on ice for 20 min. Then the tube was placed in the pre-warmed water bath (heated to 42°C) for 90 sec followed by placing it immediately on ice for 2 min to reduce the damage that occurs to the cells. 950 µl of pre-warmed SOC medium was added to the tubes which serve as a source of carbon and other nutrients for the bacteria growth. The tubes were then placed in a 37°C shaker set at 250 rpm and kept for 60 minutes. 100 µl of the final culture was used to spread on LB plate containing the appropriate antibiotic concentration for selection. The plate was kept in 37°C incubator overnight.

3.1.3 Preparation of Glycerol Stocks of Bacteria

Glycerol stocks of bacteria were prepared in a ratio of 1:3, where one colony was picked from the master plate, dissolved in 2 ml YEP or LB medium and inoculated for 2-3 hours

on a shaker at 250 rpm, then transferred to 25 ml YEP or LB media containing the necessary amounts of antibiotics and incubated on a shaker at 200 rpm at 28°C or 37°C for 15 h. The stock solution was prepared using 150 µl of glycerol (86%) and 850 µl of growing bacterial suspension in 2 ml tube and immediately stored at -80°C for long term storage.

3.1.4 Preparation of Competent *A. tumefaciens* Cells

The *A. tumefaciens* strain GV3101 was grown overnight on LB medium supplemented with 10 µg/ml gentamicin sulphate and 100 µg/ml rifampicin antibiotic. The following day, several colonies were transferred into 100 ml liquid LB medium supplemented with the same antibiotic concentrations and grown overnight at a temperature of 28°C on a shaker at 200 rpm. 50 ml of the overnight culture was transferred to a fresh 500 ml LB media in 2.5 l flask and allowed to grow overnight at a temperature of 28°C at 200 rpm until the OD_{600} reaches 1-1.5. The cell culture was then transferred into two 250 ml pre cooled centrifuge bottles and kept on ice for 20 minutes. Then the cells were centrifuged at a speed of 4,000 x g at 4°C for 15 minutes. The pellet was again resuspended with 250 ml of cold sterile water and again centrifuged at a speed of 4,000 x g at 4°C for 15 minutes. The last two steps were repeated four times in order to make the cells free from salt. Then the cells were resuspended with 25 ml of cold sterile 10% glycerol and all the suspended cells were combined together into 50 ml Falcon centrifuge tube and centrifuged at a speed of 3500 rpm at 4°C for 10 minutes. Finally the supernatant was removed and the cells were resuspended with 2 ml of ice cold 10% glycerol. 50 µl of the resuspended cells were distributed in PCR tubes and quick frozen using liquid nitrogen. Cells were kept at -80°C for long-term storage.

3.1.5 Transformation of *A. tumefaciens* through Electroporation [184]

Competent *A. tumefaciens* (strain GV3101) cells were taken from -80°C freezer and kept on ice to thaw. 50 ng of a plasmid solution was gently mixed with the 50 µl of competent cells in a 200 µl PCR tube and the mix was transferred to a pre-cooled cuvette (gap 0.2 cm) and electroporated in a electroporator at: 25 µF capacitor, 200 Ω (ohm) resistance and 2.5 KV, field strength between 6.25 –12 kV/cm for 4-8 msec. 1000 µl of pre-warmed (28°C) SOC medium was added immediately and the mixture was transferred to a new 2 ml tube followed by incubating the tubes for 1 hour at 28°C with

shaking (200 rpm). 20 µl of the resulting culture was spread on LB plates (containing the appropriate antibiotics) and grown for 2-3 days at 28°C.

3. 2 Yeast Manipulations

In this study, yeast cells are used for the purpose of analyzing protein-protein interaction following the method known as Yeast Two-Hybrid. The method utilized the two domains of *GAL4* transcription factor which is fragmented and cloned into two vectors. These chimeric proteins becomes functional only if there is an interaction between proteins encoded by the gene of interests cloned upstream of these domains. Such interactions will enables the reassembly of the transcription factor and restore its functionality that leads to the expression of downstream reporter genes[185].

3.2.1 Preparation of Competent Yeast Cells [186]

Yeast cells were grown in YPAD medium to a titer of $2X10^7$ cells/ml. Overnight culture was repeated with fresh YPAD medium and grown until it reach the same titer. The following day cells were centrifuged at 3000 x g for 5 min, washed with 0.5 volumes of sterile water and then resuspended with 0.01 volumes of sterile water. Samples were then centrifuged to pellet the cells at 3000 x g for 5 min. Pellet was resuspended with 0.01 volumes of Frozen Competent Cell (FCC) solution and dispensed (50 µl) into 1.5 ml eppendorf tubes. Cells were then transferred to -80°C freezer for long term storage.

3.2.2 Rapid Yeast Transformation [186]

Competent yeast cells (strain AH109) were spread on YPAD medium and grown for four days at 30°C. Carrier DNA was kept in boiling water for 5 min in order to denature the DNA. Freshly grown yeast cells were scrapped (approximately 50 µl blob) and resuspended in 1 ml sterile ddH_2O followed by centrifugation for 30 sec at maximum speed. The supernatant was removed the pellet is resuspended with a solution comprised of 240 µl PEG 3350 50% (w/v), 36 µl 1 M LiOAc, 50 µl boiled carrier ssDNA (2 mg/ml), 1 µg plasmid DNA, and filled up to 360 µl with sterile ddH_2O. The components were thoroughly mixed until the cells were fully resuspended followed by incubating the tubes in pre warmed water bath (42°C) for 2 hr. Samples were then centrifuged for 30 sec at maximum speed followed by removing the supernatant with micropipette. Cells were then resuspended with 0.5 ml sterile water by stirring with

micropipette tip and mixed thoroughly. 200 µl of the sample mix was added on appropriate selection medium and incubated at 30°C for 3-4 days.

3.2.3 Yeast Two-Hybrid Plate Assay [187]

Representative yeast colonies were picked from selection plate and analyzed for the presence of plasmids of interest by following the standard colony PCR method. Positive colonies were then resuspended in sterile ddH_2O and a dilution series was prepared in the following manner: 1:10, 1:100, 1:1000, and 1:10000. 2 µl from each dilution series was added on two different selective mediums: SD-LW (for Beta-galactosidase assay) and SD-LWH (for growth assay). Plating was done in duplicates where one replica grows at 30°C and the other one kept at RT both for 5 days.

For Beta-galactosidase assay (*lacZ* assay), 5 ml of chloroform was poured on aluminum covered surface under the chemical hood and yeast plates were kept upside down on it for 5 min. Once the chloroform is completely evaporated, additional 1 ml of chloroform was added directly to the yeast cells on the plates and left until the chloroform is completely evaporated. Meanwhile, 0.08 g of low melting agarose was dissolved in 8.5 ml of water by heating in microwave and once it is cooled down to 40°C, 1 ml of potassium-phosphate buffer and 0.5 ml of X-Gal solution (20 mg/ml) was added and mixed thoroughly. The mix was directly poured on the yeast plates to completely cover the yeast cells and left to solidify. Finally the yeast plates were kept overnight in the dark at room temperature. Images were taken and documented using a digital camera.

3.3 Plant manipulation

3.3.1 Plant growth on soil

*Arabidopsis*and *E. californica* seeds were sown and watered on fertilizer enriched soil and stratified at +4°C in dark for 5 days. Subsequently they were transferred to long-day (16:8 hr light:dark cycle) growing condition at 23°C. *Nicotiana benthamiana* seeds were treated with 1 ml bleach by agitation for 10 min prior to sowing. The seeds were washed 5 times with sterile H_2O and then planted in similar pattern to the other plants.

3.3.2 Plant growth on plates

Arabidopsis seeds were surface sterilized with ethanol for 1 min followed by washing with 3% NaOCl for 5 minutes. Afterwards seeds were rinsed 5 times with sterile double-

distilled water and transferred to a plate containing MS-medium and stratified for three days at 4°C in dark. The plates were then transferred to a long-day (16:8 hr light:dark cycle) growing condition at 23°C. For germination assay cotyledon expansion and greening is used to define seeds as germinated. For salt stress assay, three different concentrations (50, 100 and 200 mM) of Sodium chloride (NaCl) were added in the growing media. As a negative control, growing media without NaCl was used.

3.3.3 Floral-dip Transformation of *Arabidopsis*[164]

A. tumefaciens strain GV3101 carrying the binary vector *pMLBART_35::EBS* was grown in 5 ml LB medium at 28°C with shaking at 200 rpm. When the culture was well grown, OD_{600}=1.0, it was transferred to a 1 l Erlenmeyer flask containing 500 ml LB supplemented with 100 µg/ml Spectinomycin, 10 µg/ml Gentamicin sulphate and 100µg/ml Rifampicin. The culture was incubated over night under agitation at 28°C followed by transferring the cell culture into 250 ml plastic centrifugation tubes and centrifuged for 15 min at 5000 rpm at RT. The supernatant was discarded and the bacteria pellet was resuspended with 500 ml of 5% sucrose solution supplemented with 0.02% Silwet L-77 (Lehle Seeds®). *Arabidopsis*plants that started forming inflorescence were selected and dipped into the bacterial solution for 30 sec. The plants were immediately transferred into a plastic bag, sealed and kept overnight at RT. The following day, the plants were rinsed with water and transferred into the growth chamber. Floral dip was repeated twice in a week interval. Seed from inoculated plants were harvested and replanted on soil. For selection of transgenic lines, germinated seeds were sprayed with 200 µM BASTA® (glufosinate ammonium) 5 times every other day. Plantlets which survived the herbicide were transferred into a new pot for further analyses.

3.3.4 Crossing of *Arabidopsis* Plants

Plants that are chosen to be the maternal plant were subjected to emasculation of their floral organs except for the gynoecium using forceps. The pollen donor plant stamen was removed with forceps and brought in contact with the stigma of the maternal plant. The siliques were then marked for follow-up and seeds were collected after maturity.

3.3.5 Plant Genotyping

Screening for homozygous mutant lines and transgenic lines was carried out following a PCR based genotyping method. A set of three primers were designed where by two of them anneals to the T-DNA flanking sequence and the third primer is anneals to the T-DNA sequence. Allele specific primers are listed in the appendix 1.

3.3.6 Virus-Induced Gene Silencing (VIGS) [188]

Virus-induced gene silencing is a technique that employs the natural plant RNA defense mechanism. It is well known that infection of plant tissue by viruses activates the RNA-based defense mechanism known as RNA interferances (RNAi). In RNAi mechanism, a double stranded RNA is recognized and processed in to single stranded RNA that becomes integrated in theRNA-induced silencing complex to initiated binding and degradation of specific viral mRNA sequences. The VIGS method also follows similar manner however, along with the virus structural and functional components, short nucleotide sequence of the plant gene of interest is cloned into the viral genome which later also becomes target of the RISC-complex causing loss of function in the encoded protein.

A. tumefaciens strain GV3101 containing the vector constructs *pTRV2_EBS*, *pTRV2_Empty*, *pTRV2_EScaPDS* and *pTRV1* were grown (OD_{550} 0.8-1.2) overnight at 28°C in 4 ml LB medium supplemented with 50 µg/ml kanamycin sulphate, 10 µg/ml gentamicin sulphate and 100 µg/ml rifampicin. Well grown culture was then transferred into 100 ml Erlenmeyer flask which contains 40 ml LB media supplemented with similar antibiotic concentrations. The following day, 5 days old *E. californica* plants were watered in the morning prior to infiltration. Once the overnight bacteria culture was well grown (OD_{550} 0.8-1.2), they were transferred into 50 ml Falcon™ tube and centrifuged at 4000 rpm for 20 min at RT. The supernatant was discarded and the pellet was resuspended with 20 ml of Injection buffer by vortexing thoroughly and kept for 2 hr at RT. Prior to infiltration, a 1:1 ratio mix was prepared between the *pTRV2-X* carrying bacteria culture with *pTRV1* culture and homogenized by inversion. Mixed culture was transferred into injection syringe and 100-150 µl of the mix was directly injected into the plant shoot apical meristem (SAM). The plants were then kept overnight at 4°C in the dark to facilitate the infection and the following day they were transferred to

greenhouse. First floral bud was used for RNA extraction and the subsequent floral buds were kept for phenotypical characterization.

3.3.7 Bimolecular fluorescence complementation (BiFC) assay [189]

BiFC is a technicque mainly used to study protein-protein interaction. Proteins of interests are fused with fragment of a non-fluorescent protein which becomes only fluorescent in the presence of physical interaction between the two proteins which lateroncan be visualized by an epifluorescence microscope.

3.. *tumefaciens* strain GV3101 containing the appropriate BiFC vector constructs, p19 and 35S::YFP were grown in 10 ml 2-YT medium supplemented with the appropriate antibiotics. On the day of infiltration, 3 to 4 weeks old *Nicotiana benthamiana* plants were chosen and watered early in the morning. Well grown overnight bacterial culture (OD_{600}=1.2) were centrifuged for 10 min at 4000 rpm at RT. Cells were then resuspended with 500 µl of re-suspension solution. The resuspended cells were kept for 2 hrs at RT. The appropriate mixes of bacteria were then carried out in 1.5 ml eppendorf tubes which contain:

- 250 µl P19 culture of OD_{600}=2.4
- 500 µl BiFC construct #1 of OD_{600}=1.2
- 500 µl BiFC construct #2 of OD_{600}=1.2

The bacteria suspension was then injected into a fully expanded *N. benthamiana* leaves. Three replica for each construct tested was made using different plants. Five days after infiltration, leaf discs were then excised from the site where the injection was carried out and kept into a 2 ml eppendorf tube containing 300 nM DAPI solutions plus a drop of Silwet L-77. The eppendorf tubes were then transferred to desiccators to remove the gas from the tissue and to facilitate the penetration of stain into the spongy mesophyll cells. Leaf discs were then placed on a microscope slides and mounted with ddH_2O which contains also few drops of Silwet L-77. A slide cover slip was placed on top of the tissue and image was visualized and taken using fluorescence microscope (Leica DM5500 B, Germany) equipped with digital camera (Leica DFC450)

3.4 Histology [190,191]

3.4.1 Tissue Fixation

Plant tissue was harvested and placed quickly into ice-cold fixation solution (FAE). For floral buds the tips of the tissues were removed in order to facilitate the penetration of the fixative solution. The tissues were then transferred to vacuum desiccators and degassed for 30-60 min. Once the gas is completely removed from the tissues, the FAE solution was replaced with fresh FAE solution and samples were then kept overnight at 4°C.

3.4.2 Tissue Embedding

The fixed tissue was washed twice with 70% ethanol for 5 min on ice followed by dehydration step with 85% and 95% ethanol solution each for 90 min at RT. The tissue was then incubated for 90 min in 100% absolute ethanol followed by incubation in ethanol: Limonene mix in different ratio (3:1, 2:1, 1:1, and 1:2) each for 60 min at RT. Finally the tissue was kept in 100% Limonene twice for 60 min at RT followed by replacing the Limonene with solid paraplast and samples were kept at 60°C in oven for 3-5 days. Fresh liquid paraplast was changed twice per day and finally the tissue together with the paraplast were poured on a pre-heated Petridish and kept at RT to solidify. The samples were then kept at 4°C until sectioning.

3.4.3 Tissue Sectioning

The wax blocks were prepared by removing unnecessary wax substance. The wax block were glued on the mounting block and placed into a microtome adaptor. Tissue was then sectioned to a size of 10 µM thick using a rotary microtome (Leica RM2235, Germany) and place on a superfrost microscope slide (Fischer Scientific, USA) that contains ddH_2O useful for stretching the sections. Finally the water was removed using paper towels and slides were kept overnight at 42°C on a heating plate. The next day, slides were transferred to 4°C for longer storage.

3.4.4 Dewaxing

The slides were incubated in Limonene twice for 10 min and with 100% ethanol twice for 2 min. Final dehydration was carried out by incubating the slides in 95%, 85%, 70%,

50%, and 30% ethanol series each for 5 min. The slides were then used immediately for staining.

3.4.5. Staining

3.4.5.1 Safrarin-O and Fast Green Based Staining

Slides were stained for 30 min at RT with 0.1% Safranin-O solution (made with 30% ethanol) and then rinsed with 30% ethanol. Following, the slides were kept for 5 min at RT in 1% NaHCO3 (prepared in 30% ethanol) and then incubated in 30% and 50% ethanol each for 5 min. Final staining was done by incubating the slides in 0.2% alcoholic Fast Green solution (made with 100% ethanol) followed by washing twice in 100% ethanol at RT each for 5 min. Slides were then left to dry, mounted with Entellan (Merck Millipore, Germany) and covered with cover slip. Stained tissue sections image was taken by using binocular microscope under bright field condition (Leica M165 C, Germany).

3.5 Nucleic acid analyses

3.5.1 Plasmid DNA Isolation

2 ml of overnight grown bacterial culture was placed into 2 ml eppendorf tube and centrifuged at maximum speed for 1 min. The supernatant was discarded and additional 2 ml was added and centrifuge in similar setting. After removing the supernatant, the pellet was resuspended with 400 µl of STET buffer plus 40 µl of 10 mg/ml Lysozyme. The mix was placed in boiling water for 40 seconds followed by 5 min centrifuge at maximum speed. The clot formed was removed using a toothpick and equal volume of pre-cooled 2-propanol was added in the samples followed by 5 min centrifugation at maximum speed. After removing the supernatant, the pellet was washed with 70% ethanol by centrifuging for 5 minutes at maximum speed. After removing the ethanol, the pellet was air-dried and 50 µl of TE buffer supplemented with 10 mg/ml Rnase A was added on the pellet and samples were kept at 37°C for 1 hr to dissolve the DNA. DNA concentration was determined using the Spectrophotometer and samples were then stored at -20°C.

3.5.2 Agarose Gel Electrophoresis

Standard agarose gel was prepared using 1% agarose (Sigma-Aldrich, Hamburg, Germany) in 1X TAE buffer and dissolved by heating in microwave. 0.05% of DNA STAIN-G was added after it cooled down and was poured on a gel casting equipment. DNA sample was mixed with 1X DNA loading dye and loaded on the solidified agarose gel. Standard electrophoresis was carried out using 120 V for 40 min. Image from the gel was taken using a gel-doc instrument equipped with UV light and digital camera.

3.5.3 RNA Gel

RNA gel was prepared by mixing 36.1 ml Milli-Q H_2O, 5 ml 10xMOPS, and 0.75 g standard agarose in a 100 ml Erlenmeyer flask. The agarose gel was dissolved by heating in microwave. Once the agarose is melted, the mixture was cooled down and transferred to clean chemical hood where by 8.9 ml of Formaldehyde (35%) was added to the mix followed by casting the gel immediately. Solidified gel was placed in electrophoresis apparatus filled with 1X MOPS buffer. Once samples are loaded, standard electrophoresis was carried out using 85 V for 45 min. Image from the gel was taken using a gel-doc instrument equipped with UV light and digital camera.

3.5.4 Restriction Digest of Plasmid DNA

Restriction digest of plasmid DNA was carried out for the purpose of cloning and also for checking the exact integration of the gene of interest. The master mix for a double digest was set as per the recommendation of the manufacturer (New England Biolabs, Frankfurt am Main, Germany). Mix for a 10 µl total volume is comprised of 1 µl Buffer 4, 1 unit restriction enzyme, 1 µg plasmid DNA and ddH_2O up to 10 µl was prepared and thoroughly mixed and incubated at 37°C for 1 hr followed by 65°C for 20 min. DNA was analyzed with standard gel electrophoresis as described in section 3.5.2.

3.5.5 Polymerase Chain Reaction (PCR)

The standard PCR master mix was prepared by mixing 2.5 µl 10X Buffer (Fisher scientific, Schwerte, Germany), 0.5 µl 10 µM dNTP mix (Fisher scientific, Schwerte, Germany), 0.5 µl 10 µM forward Primer, 0.5 µl 10 µM reverse primer, 10-50 ng DNA template, 0.15 µl Taq DNA polymerase (Fisher scientific, Schwerte, Germany), and filled up to 25 µl with sterile ddH_2O.Samples were mixed well and short spin centrifuged. PCR was carried out in PCR thermo cycler and a program starting with 95°C for 5 min

and 94°C for 30 sec followed by 35 cycles at Tm-5°C for 30 sec, primer extension at 72°C for n min (n = length of amplicon in kb) and a final incubation at 72°C for 5 min. DNA was analyzed with standard gel electrophoresis as described in section 3.5.2.

3.5.6 Colony PCR

Independent colonies were picked from overnight grown bacteria/yeast cells and resuspended with 10 µl sterile water. 2 µl of resuspended cells were used as DNA template for standard PCR reaction.

3.5.7 PCR Product Clean Up

PCR product cleanup was carried out using NucleoSpin® Gel and PCR Clean-up kit following the manufacturing protocol (MACHEREY-NAGEL, Düren, Germany).

3.5.8 DNA Extraction from Agarose Gel

DNA fragments separated by agarose gel electrophoresis were excised from the gel un der UV-trans illuminator and purified using NucleoSpin® Gel and PCR Clean-up kit following the manufacturing protocol (MACHEREY-NAGEL, Düren, Germany).

3.5.9 DNA Sequencing

DNA cloned in vectors was sequenced using vector specific primers. A total volume of 7 µl reaction mix comprised of 200 ng of plasmid DNA, 1 µl OF 10 µM primer, and sterile ddH_2O is prepared and mixed thoroughly. The reaction mix was sent to sequencing facility. The AB1-files were then processed and analyzed by using Geneious software (Geneious)

3.5.10 Rapid Plant DNA Extraction [192]

One or two 1 cm diameter leaf discs were collected from *Arabidopsis* plants and kept in 2 ml cryogenic vials that contain 600 µl Rapid DNA extraction buffer and ball mill. Tissue was then homogenized twice for 15 seconds using a homogenizer followed by 1 minute maximum centrifugation step to precipitate junk tissue. 400 µl of the supernatant was transferred into a clean 1.5 tube and equal volume of pre-cooled 2-propanol was added and mixed slowly. Samples were then centrifuged for 5 minutes at maximum speed. Supernatant was removed and the pellet was washed with 70% Ethanol by centrifuging for 5 min at maximum speed. Pellet was air-dried and 100 µl of TE buffer supplemented

with 10 mg/ml Rnase A was added for each sample. DNA concentration was determined using the Spectrophotometer. Samples were then stored at -20°C.

3.5.11 Plant RNA Extraction

RNA extraction was performed using the plant-RNA-OLS®Kit following the manufacturer instruction (Omni Life Science, Bremen, Germany).

3.5.12 Complementary DNA (cDNA) Synthesis

First strand cDNA was synthesized by using the RevertAid™H-Minus First Strand cDNA Synthesis Kit (Fisher scientific, Schwerte, Germany) following the manufacturer protocol.

3.5.13 Reverse transcriptase (RT) PCR

RT-PCR was carried out in similar way to the standard PCR. However, cDNA was used as template for the reaction. Reference gene *AtACTIN* and *EScaACTIN* were used as cDNA synthesis quality control. PCR products were analyzed with standard agarose gel electrophoresis as described in 3.5.2.

3.5.14 Quantitative Real-time (qRT) PCR[193]

Quantitative real-time PCR is a polymerase chain reaction based technique mainly used to quantify the amount of amplified product generated in every cycle of PCR by using fluorescence reporter molecule. PerlPrimer [194] software was used to design an exon-intron spanning primers. The primer pairs were tested for their amplification efficiency and their product melting temperature. Once their primer efficiency is between the range 1.9-2 and when the melting curve analyses shows a single amplicon, primers were then approved to be used for the qRT-PCR assay. The Roche LightCycler®480 system (Roche, Mannheim, Germany) platform was used to run the qRT-PCR where each sample was made from a total reaction volume of 20 µl comprised of 5 µl of1:50 diluted cDNA template, 1 µl of each primer (10 µM), 3 µl double distilled sterile water and10 µl SYBR Green I Master (Roche, Mannheim, Germany). The assay was carried out with conditions starting with 95°C for 5 min and 95°C for 10 sec followed by 45 cycle at 60°C for 10 sec and 72°C for 10 sec. Primer test was carried out in similar manner where by standard cDNA dilution series (Undiluted, 1:10, 1:100, and 1:100) were used as templates. *ACTIN* and *EF1-α* were used as reference genes for *Arabidopsis*;

whereas *EScaGAPDH* and *EScaACTIN* genes were used as reference genes for *E. californica*. Two biological replicates with two technical replicates were used for each data point and analysis of data was carried out following the $\Delta\Delta^{CT}$ (cycle threshold) method and geometric averaging [195].

3.5.15 *In situ* Hybridization (According to [190] and [191] with some modification)

In situ hybridization is a technique mainly used to identify the localization of gene transcripts with in a tissue. Application of a Fluorochrome labeled Complementary single stranded oligo nucleotide in a fixed histologic specimen, enables to detect the precise localization of the target oligos within the sample tissue.

Tissue preparation

Tissue fixation, embedding and sectioning (7 µM) was carried out in similar manner mentioned in section 3.4. However, the last dehydration step with 100% ethanol was carried out with the addition of 0.1% eosin to ease the visualization of tissue sections.

In situ RNA Probe preparation

Cleaned up PCR products which were amplified with primers designed for *In situ* experiment were used as a template for RNA probe synthesis. The in vitro transcription was carried out by mixing 2 µl 10 x NTP labeling mixture, 2 µl 10 x Transcription buffer, 1 µl (20U) Protector RNase inhibitor, 2 µl RNA T7 Polymerase, 1µg Template DNA, and finally RNase-free MilliQ-water was added to a final volume of 18 µl. The tubes were then incubated for 6 hrs at 37°C followed by additional 15 min incubation at 37°C after adding 2 µl DNase I. The reaction is stopped by adding 0.2 M EDTA (pH 8.0). The RNA was precipitated by adding 2.5 µl of 4 M LiCl and 75 µl of 100% ethanol into the samples and mixed well followed by overnight incubation at -20°C. The next day, the samples were centrifuged for 10 min at 13,000 rpm at RT followed by washing the pellet with 70% ethanol. The ethanol was removed using micropipette and the pellet was allowed to dry. Final re-suspension of the RNA probe was done by adding 30 µl of RNase-free water and samples were kept on ice for 2 hr. The RNA probe quality was checked in standard agarose gel and comparison was made between the final RNA probe and the RNA probe prior to DNase I treatment. The RNA concentration of the

probe was then measured by using Spectrophotometer. The RNA probe was then kept in -80°C until it is used.

Dot Blot

Dot blot assay was carried out to find out the optimum RNA concentration required for detection. Serial dilutions of the RNA probe (1:10, 1:100, and 1:1000) were prepared and 1 µl of it was loaded on a positively charged nylon membrane. In addition 1 µl of pure water was also added on the membrane as a negative control. The nucleic acid spots were fixed by UV cross-linking for 3 min. The membrane was then transferred to a plastic container that contains a Buffer-1 and incubated for 2 min with shaking at RT followed by incubating the membrane in 0.5% blocking solution for 30 min at RT. The blocking solution was then replaced by Antibody solution and membrane was incubated for additional 30 min at RT. The membrane was then washed twice for 15 min with the Buffer-2 at RT followed by incubating in 5 ml Buffer-2 which contains 5 µl NBT (Nitro-blue tetrazolium chloride) and BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) mix for 10 min at RT. When color signals appeared on the membrane, the reaction was inactivated by washing the membrane with water. Membrane was air-dried and image was taken using a digital camera.

Tissue Section Pre-treatment

Dewaxing and dehydration was carried out by incubating slides twice in Limonene for 10 min followed by incubation in ethanol series: 100%, 95%, 90%, 80%, 60%, 30% and ddH_2O each for 2 min at RT temperature. The slides were then kept in 2 X SSC solutions for 20 min and then incubated with Proteinase K buffer for 30 min at 37°C with gently agitation. Following the incubation the sections were kept in the Glycine buffer for 2 min at RT and transferred to in 1X PBS solution twice for 5 min. The sections were then kept in a chemical hood and incubated in a glass container filled with paraformaldehyde solution for 10 min at RT followed by incubating twice in 1X PBS solution for 5 min at RT. The slides were then transferred into Triethanolamine solution and incubated for 10 min at RT followed by incubating twice in 1X PBS solution for 5 min at RT. The slides were then treated with ethanol series: 30%, 60%, 80%, 90%, 95%

and 100% each for 30 sec at RT. For overnight storage, slides were kept in a container filled with small amount of 100% ethanol.

RNA Hybridization

Hybridization solution was prepared by mixing 200 µl 10x in situ salts, 800 µl Deionized Formamid, 400 µl pre-warmed (60°C) 50% Dextran sulfate, 40 µl 50x Denhardt's solution, 20 µl tRNA (100 mg/ml) and 140 µl H_2O. This mix is used for 10 slides. The hybridization solution was preheated at 60°C and mixed thoroughly. The desired RNA probe concentration was prepared to a final volume of 20 µl and additional 20 µl of deionized formamid was added to the probe to make the final volume of 40 µl. The RNA probe was then incubated on heating block at 80°C for 2 minutes and immediately kept on ice for 2 min. 160 µl of the hybridization solution was mixed with the 40 µl RNA probe mix and applied directly on the tissue sections. The slides were covered with a cover slip and kept elevated above a wet paper towels using pasture pipettes in plastic container. The plastic container was sealed properly and hybridization took place in hybridization oven at 47°C for 16 hrs.

Post-hybridization Treatment

Each slide was dipped into a pre-warmed (55°C) 0.2X SSC solution and then washed with freshly prepared 0.2X SSC solution twice for 60 min at 55°C followed by incubating for 5 min in 1X PBS at RT. The slides were then transferred into a plastic box with sample side up and incubated with 0.5% Roche, Mannheim, Germany blocking solution for 45 min at RT. The blocking solution was replaced with washing solution-A and incubated for 45 min at RT followed by incubating the slides in antibody solution for 1 hr at RT. The slides were further washed with washing solution-A four times each for 15 min and then washed with Buffer 2 for 10 min at RT. Finally the slides were kept vertically in NBT-BCIP Staining solution for 1-5 days at RT in the dark. When enough staining is observed, the reaction was inactivated by washing the slides with water followed by 70% and 95% ethanol each for 5 min. The slides were then dried and mounted with Entellan and covered with a glass cover slip. Image was taken using binocular microscope equipped with digital camera under bright field condition.

3.5.16 Southern Blot [196]

Southern blotting is a technique used to identify the presence of specific DNA sequences within a given DNA sample. Unlike *in situ* hybridization which utilizes detection within tissue sections, DNA samples are transferred and fixed onto a nylon membrane for detection. Nevertheless, similar to *in situ* hybridization, Fluorochrome labeled complementary single stranded oligo nucleotide are used to identify target. This method is mainly used to analyze copy number of a transgene or for identification of unknown gene within a genome.

Probe Synthesis

DNA probe was synthesized using the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) by mixing 5 µl 10X PCR Reaction Buffer (Fisher scientific, Schwerte, Germany), 5 µl 10X PCR DIG mix (Roche, Mannheim, Germany), 2 µl 10 µM Forward Primer, 2 µl 10 µM Reverse Primer, 0.75 µl High Fidelity Taq Polymerase (NEB), 10 ng/µl Template, and with sterile ddH_2O to make the total volume 50 µl. An additional control reagent mix was also prepared by replacing the PCR DIG mix with unlabeled10 µM dNTP mix. The reagents were mixed well and centrifuged briefly followed by transferring the tubes in PCR thermal cycler. The PCR product was then analysed in standard agarose gel whether the labeled probe appears to have larger molecular weight compared to the unlabeled PCR product or not. Dot blot was carried out in similar manner as mentioned in the *In situ* hybridization section.

DNA Extraction and Restriction Digest of DNA

Genomic DNA from *E. californica* was extracted by using a DNA extraction kit (DNA-Cloning service). 10 µg of DNA was fragmented by using restriction enzymes. The restriction digest was carried out overnight at 37°C followed by DNA precipitation by adding 1/10 volume 3 M sodium acetate pH 5.2 and 3 volumes of absolute ethanol. The tube was then incubated overnight in -20°C and after centrifugation for 20 min at maximum speed; the pellet was washed with 500 µl 70% (v/v) ethanol. The DNA was then resuspended in 30 µl TE buffer and incubated on ice for 2 hr to dissolve the pellet.

Gel Electrophoresis and Blotting

Restrict digested DNA samples were mixed with 1X loading dye and loaded on 0.8% agarose gel and run overnight at 30 V. Gel Image was taken using UV-trans illuminator and documented. The gel was then submerged in 250 ml 0.25 M HCl for 10 min followed by incubation in denaturation solution twice for 15 min at RT. The gel was then transferred into neutralization solution and incubated twice for 15 min at RT. A tray filled with 20X SSC buffer was prepared and a glass plate was kept on top of the container. A whatman filter paper bridge was constructed on top of a glass plate where the ends of the filter paper were submerged in the 20X SSC buffer. Three filter papers in the size of the gel were soaked in 20X SSC buffer and placed on top of the bridge. Capillary southern transfer was carried out by placing the agarose gel on top of the three filter papers. Plastic wrap was placed around the gel to avoid the direct contact of the tissue towels with the bottom whatman filter paper. The positively charged nylon membrane was first rinsed with water and soaked with 20X SSC buffer and placed on top of the gel. Additional 20X SCC soaked 4 filter papers were placed on top of the nylon membrane. Finally tissue papers were stacked on top of the filter papers and 500 ml water bottle was place on top of them to distribute the weight evenly. The capillary transfer was carried out overnight at RT.

DNA Hybridization

After capillary transfer, the nylon membrane was rinsed with 2X SSC and left to dry. The nucleic acids were then fixed by UV-cross linking for 5 min. The membrane was then transferred into a hybridization tube and incubated with 35 ml DIG Easy-Hyb solution (Roche, Mannheim, Germany) for 3 hrs at 42°C. In the mean time, the DNA probe was incubated at 100°C for 5 min and placed on ice immediately to keep it denatured. The DNA probe was then added to a new DIG Easy Hyb solution (Roche, Mannheim, Germany) that replaced the previous pre-hybridization solution. The hybridization was carried out in hybridization oven overnight at 42°C.

Detection

The membrane was washed twice for 5 min in 2X SSC + 0.1% SDS solution at 42°C followed by washing with 0.5X SSC + 0.1% SDS and 0.1X SSC + 0.1% SDS each for 15 min at 65°C. The membrane was then transferred to a plastic container and submerged in maleic acid buffer for 1 min at RT followed by submerging it in 0.5% blocking solution for 30 min. The blocking solution was replaced with antibody solution and incubation was done for 30 min at RT and final washing was carried out by treating the membrane with the washing buffer-B twice for 15 min. The membrane was submerged in detection buffer for 2 min at RT. 1% CDP-star (Roche, Mannheim, Germany), chemiluminescent substrate, was made with detection buffer and drops were made on a plastic foil using a pipette. The membrane was then placed upside down on the substrate and covered with another plastic foil and incubated for 5 min at RT. Finally the substrate was then removed by smoothening with filter paper and the membrane was sealed by the two plastic foils. The chemiluminescence signal was documented by using a chemiluminescence camera with 10 min exposure time. For long term storage, the membrane was rinsed with sterile water and incubated twice for 20 min in stripping solution at 37°C followed by storing in hybridization tube filled with 2X SSC solution at 4°C.

3.6 *E. californica Agrobacterium*-mediated Stable Transformation Procedure

a. Bacterial strains and plasmid constructs

Two different constructs were made in order to down-regulate and over-express *E. californica* B_{sister} gene (*EBS*). Restriction enzyme recognition site containing primers (EBS-XbaI+Xho: 5'-ATC TAG ACT CGA GGA GTT CAA TAA TCA G-3'and EBS-ClaI+KpnI: 5'-AAT CGA TGG TAC CTA GAT ATC CAT ATC CA-3') were used to amplify 450 base pair long partial sequence of *EBS* using proof reading Taq polymerase. A binary vector, *pHANNIBAL*, was chosen to generate an intron containing hairpin RNA (hpRNAi) construct. The PCR fragment and *pHANNIBAL* vector were digested with restriction enzymes and ligated to form an inverse repeat construct (*pHANNIBAL_hpRNAi-EBS*) that transcribed into a self-complimentary 'hairpin'RNA to initiate the silencing of *EBS* expression. NotI restriction enzyme was used to cut out the *hpRNAi-EBS* fragment from the *pHANNIBAL* vector and ligated it into a binary vector

pMLBART to construct *pMLBART_hpRNAi-EBS* construct. The *pMLBART* vector was chosen since it contains BAR gene as selection marker in its T-DNA expression cassette.

For over-expression constructs, the complete coding sequence of *EBS* was extracted from primary cloning vector, *pGEM-T-EBS* by using the EcoRI restriction enzyme and cloned into a shuttle vector that contains the *CaMV35S* promoter known as *pART7*. The 35S::*EBS* fragment was extracted from the *pART7* vector using the NotI restriction enzyme and ligated into the binary vector *pMLBART* to make *pMLBART_35S::EBS* construct. Both plasmid constructs were sequences to verify the integration of correct coding sequence.

A. tumefaciens strain GV3101 was transformed with the recombinant plasmid vectors *pMLBART_hpRNAi-EBS* and *pMLBART_35S::EBS* and a single colony was taken from the plate and grown in 5 ml liquid LB media supplemented with 50 µg/ml Gentamicin, 50 µg/ml Spectinomycin, and 50 µg/ml Rifampicin at 28°C on a gyratory shaker (130 rpm) until they reach OD_{600}=0.5-0.8. 1 ml of this culture was used to inoculate 100 ml of LB liquid media supplemented with similar antibiotic concentration and incubated in similar settings mentioned above. The next day, cells were centrifuged at 3000 rpm for 15 minute at room temperature in Falcon tubes. Supernatant was removed and the pellate was resuspended with B5 liquid media (3.16 g/l B5 powder, pH 5.8) to OD_{600}=1.0 and cells were incubated for 1-2 hours at 28°C shaking at 130 rpm.

b. Explant preparation

Fruits were harvested 22 days after anthesis and surface sterilized with 70% ethanol for 1 minute followed by rinsing with sterile water 1-2 times. Critical sterilization was carried out using 3% sodium hypochlorite solution containing a drop of Tween-20 or Triton X-100. The fruit pods were kept for 20 min in the solution while shaking at 130 rpm followed by a thorough washing with sterile water until the remnant of bleach was removed completely. Finally pods were air dried under a clean-bench and opened using sterile scalpel to remove developing seeds. Seeds were wounded with a sterile needle in order to facilitate the penetration of the *Agrobacterium*.

c. Seed inoculation and co-cultivation

Wounded seed explants were immersed in the *A. tumefaciens* suspension for 20 min in a sterile Petri dish. After the inoculation, seeds were blot dry on a sterile filter paper and transferred into the callus induction media (CIMS) that contains no antibiotic and selection agent (3.16 g/l B5 powder, 30 g/l sucrose, 8.4 g/l Phytoagar, and pH 5.8. After sterilization, 2 mg/l and 0.1 mg/lα-Naphthalene acetic acid (NAA) and 6-Benzylaminopurine (BAP) respectively were added. Co-cultivation was carried out for two days in the dark at 22°C.

d. Callus induction and somatic embryogenesis

After co-cultivation, seeds were washed with sterile water twice and then resuspended in sterile water containing 20 mg/l Ticarcillin potassium clavulanate and kept on shaking for 20 min at RT. Seeds were blotted dry and transferred to callus induction media (CIMS) supplemented with antibiotics and BASTA for selection. The explants were kept on CIMS medium for approximately 4 weeks in the dark at 22°C until callus was formed while fresh media was used in 7 day interval.

Once calli formation was achieved, white calli were transferred to a somatic embryo induction media, SEIM. Calli were kept for 3-4 weeks on SEIM medium at 22°C in the 16-hr light/8-hr dark growth condition until somatic embryogenesis took place. Fresh SEIM media was used every week.

e. Plant regeneration and transplantation

After somatic embryos were formed, they were transferred on plant regeneration medium, (PRM) to induce root formation and they were kept in growth chamber at 22°C in 16-h light/8-h dark at a light intensity of approximately 140-180µmol$m^{-2}s^{-1}$ from cool-white florescent lamp. Fresh PRM media was used on a weekly interval until plantlets were regenerated with a good root system. Regenerated plants with approximately 3-4 inches of shoots were removed from the PRM media and the Gelrite was washed with sterile water. Next, the plantlets were blotted dry on sterile filter paper and carefully transferred without destroying the root system to a transparent plastic container partially

filled with sterile moist perlite/vermiculite. The regenerated plantlets were kept in growth chamber with similar setting as mentioned above. After one week, plantlets were transferred to sterile soil (70% Soil and 30% sand) and were acclimatized for 2-3 weeks at controlled environment and subsequently transferred to the greenhouse.

4. Results

4.1 Analyses of *Arabidopsis* B_{sister} genes

4.1.1 Generation of Double and Triple Mutant Genotypes

Homozygous double and triple mutant lines were generated to investigate the presence or absence of functional redundancy between the genes. Crossing of the following single and/or double mutant lines was carried out:

- *abs* X *goa-1*
- *abs* X *shp1-1 shp2-1*
- *agl16-1* X *goa-4*
- *agl16-1* X *goa-1*
- *abs* X *goa-1* X *agl16-1*

Verification for the presence of mutant allele was carried out by PCR based technique. Two different wild type ecotypes, Colombia (Col-0) and Wassilewskija (WS-4), were used since the *abs* genotype is in the WS-4 background, the remaining mutant lines are in the Col-0 background.

4.1.2 *ABS* Loss-of-Function Mutation Affects Plant Fertility

Wild type plants with WS-4 background are known to flower much earlier than the Col-0 ecotypes. Except for these flowering time difference, no visible alteration in vegetative and reproductive organ development was observed in the homozygous double and triple mutant lines compared to the parental and wild type lines. However, the number of seeds per silique count analyses showed a significant difference between Col-0 and genotypes with *abs* allele when grown in controlled growth chamber (Figure 3A).

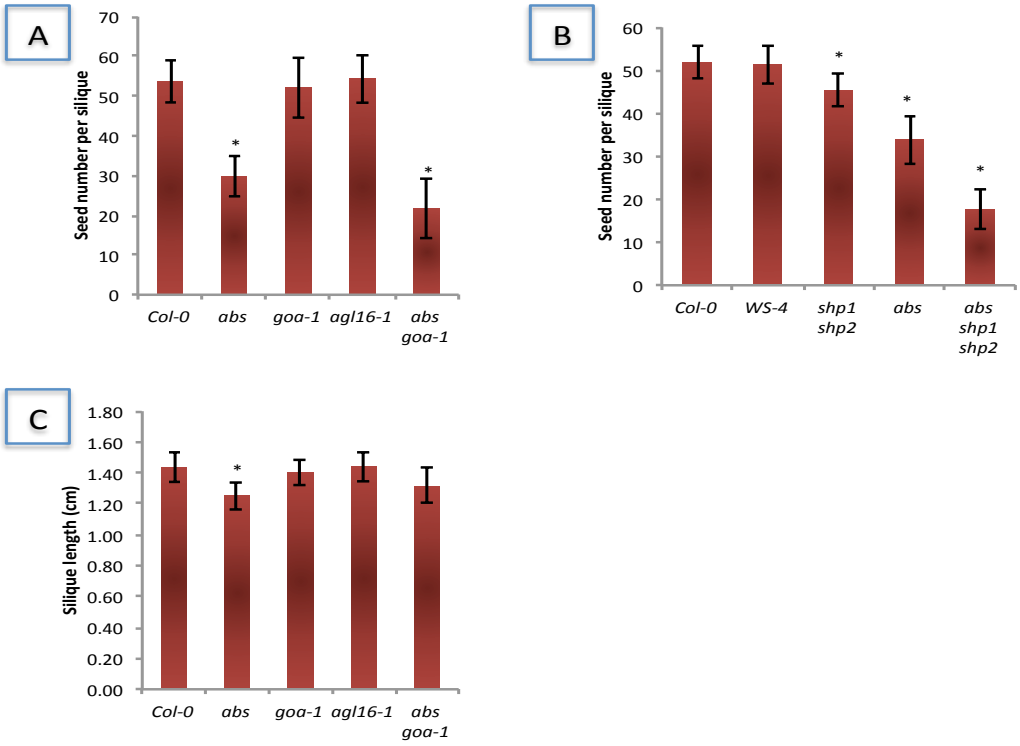

Figure 3: Comparison of seed number between wild type and mutant genotypes. (A) Comparison of seed number per silique between wild type Col-0 and mutant genotypes grown in a growth chamber. (B) Comparison of seed number per silique between two wild types and three mutant lines. Genotypes carrying *abs* allele show a strong reduction in seed number in comparison to the others. (C) There is a significant difference ($P<0.01$) in silique length between *abs* genotype and the wild type. Asterisks above the bar indicates significance difference compared to wild type genotypes as inferred by two-way T-test statistical analyses ($p<0.05$, $n=30$).

The experiment was also repeated under green house condition, using the two wild type ecotypes (Col-0 and WS-4), *abs*, *shp1-1 shp2-1,* and *abs shp1-1 shp2-1* genotypes. Similarly, the result shows that genotypes homozygous for the mutant allele *abs* have a reduced seed number compared to the other genotypes (Figure 3B). Compared to the wild type plants, the seed number is reduced by 33% and 66% in the *abs* and *abs shp1-1 shp2-1* genotypes respectively. The reduction in seed count becomes much more prominent in those genotypes which lack the wild type allele for *ABS* and its protein interacting partners, either *GOA* or *SHP1 SHP2,* indicating the presence of functional redundancy between them. In addition, the silique length in *abs* mutants is significantly reduced compared to the Col-0 silique (Figure 3C). These results indicate that *ABS* is required for proper seed setting and silique development in *Arabidopsis*.

4.1.3 *ABS* Loss-of-Function Mutation Affects Seed Germination

In order to investigate the effect of altered seed testa [132,136] in seed germination, seeds from *abs*, *goa-1*, and wild type plants were subjected to surface sterilization and grown on MS medium supplemented with different salt concentration. As shown in Figure 4, with an increase in salt concentration, the number of seeds germinated decreases in all genotypes while no seed was able to germinate on growth media supplemented with 200 mM NaCl concentration.

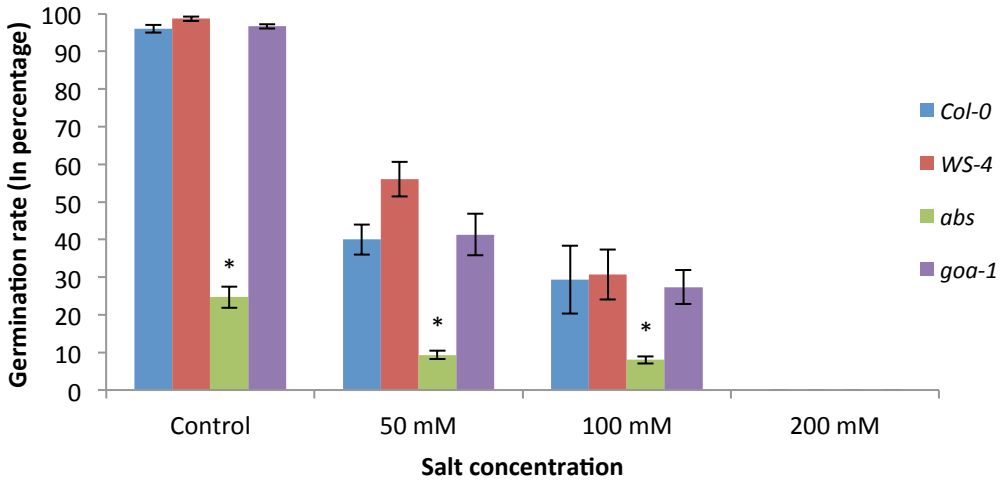

Figure 4: Seed germination assay on growth media containing different salt concentration. Seeds from genotypes homozygous for the mutant allele *abs* shows a reduced germination compared to the other genotypes (n=50). Asterisks above the bar indicates significance difference compared to wild type genotypes as inferred by two-way T-test statistical analyses ($p<0.05$)

However the numbers of seeds germinated from *abs* mutant was significantly lower in comparison to the other genotypes in all growth medium. In the control growth media (no NaCl added), more than 96% of seeds from wild type and the *goa-1* mutant lines were able to germinate where as less than 25% of seeds from *abs* genotype were able to germinate under the same growth condition. On growth media supplemented with 50 mM NaCl, more than 40% of seeds from wild type plants and the *goa-1* mutant line were able to germinate whereas less than 10% of *abs* seeds were able to germinate. And at 100 mM NaCl concentration, only 8% of seeds were able to germinate from the *abs* seeds while at least 30% of wild type and *goa-1* seeds were able to germinate. In order to find out if the ecotype background has an effect on the seed germination, the WS-4 ecotype was included in the germination assay. The result shows the WS-4 ecotype even germinates considerably better than the Col-0 ecotype signifying the poor

germination of *abs* seeds is due to the absence of functional *ABS* protein that leads to deregulation of *BAN* gene that is essential for PA synthesis and deposition [132]. This result agrees with previous studies which indicated that flavonoids play a crucial role in providing protection for the seed against biotic and abiotic factors [197].

4.1.4 *ABS, SHP1* and *SHP2* are Required for Proper Seed Development

Despite the absence of visible phenotypic defects with respect to vegetative and reproductive organ development in wild type, *abs*, and *shp1-1 shp2-1* genotypes, the number of seeds per silique of *abs shp1-1 shp2-1* triple mutants was significantly lower in comparison to the wild type and also to the parental genotypes (Figure 3B). Furthermore, seeds from the triple mutant lines have a morphology that is different from both the wild type and mutant parental lines (Fig. 5A-D). The *abs shp1-1 shp2-1* triple mutant produces seeds which are shrunken and wrinkled in shape as shown in Figure 5D. Despite the presence of very few shrunken seeds in the *abs* and *shp1-1 shp2-1*, the seeds from the triple mutant lines are completely shrunken and wrinkled. Moreover, numerous aborted ovules are observed in the triple mutant lines as shown in Figure 5I. Based on reciprocal cross analyses, all phenotypes observed were found out to be associated with maternal effect (Lucia Colombo, Personal communication).

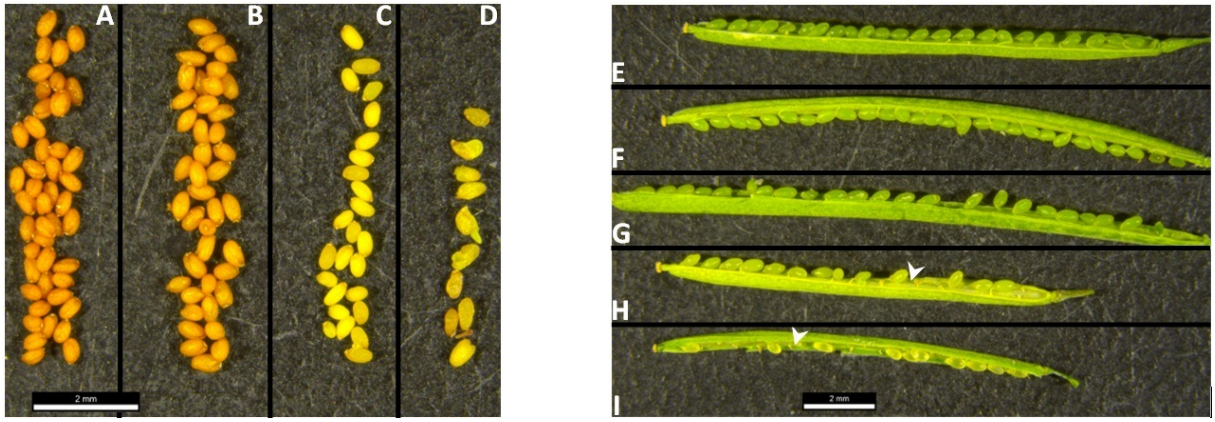

Figure 5: Comparison of developing and mature seed between wild type and mutant genotypes. *Arabidopsis* matured seeds from a single silique of: (A) Wild type, (B) *shp1-1 shp2-1* double mutant (C) *abs* mutant, and (D) *abs shp1-1 shp2-1* triple mutant seeds. Seeds from the triple mutant line are few in number and have abnormal seed shape. *Arabidopsis* developing seeds in opened silique of: (E) Wild type Wassilewskija-4, (F) Wild type Colombia-0, (G) *shp1-1 shp2-1* double mutant, (H) *abs* mutant, (I) *abs shp1-1 shp2-1* triple mutant. Numerous aborted seeds (indicated with white arrow) are observed in *abs* and *abs shp1-1 shp2-1* triple mutant genotypes. Scale bar is 2 mm.

4.1.5 *ABS, SHP1* and *SHP2* Together and Independently Regulate Expression of Several Genes

In order to understand the shrunken seed phenotype and reduced seed setting observed in the triple mutant genotypes, expression analyses was carried out for candidate genes using qRT-PCR. Candidate genes were selected based on either their previously described loss-of-function mutation phenotype that is similar to what is observed in *abs shp1 shp2* triple mutant or their overlapping expression pattern with *ABS*, *SHP1,* and *SHP2.* For example, similar shrunken seed phenotype and/or reduced fertility was reported previously in loss-of-function mutation of genes such as *PROMOTION OF CELL SURVIVAL 1 (PCS1)* and *SULFUR TRANSFERASE1 (STR1)*, *SHRUNKEN SEED1 (SSE1)*, *WRINKLED1 (WRI1)* and *MOB1-LIKE (MOB1)* [198-201]. Shrunken seed phenotype is also reported to be associated with down-regulation of genes involved in programmed cell death pathway in rice. Auxin regulated expression of *OsMADS29* binds to the promoters of a Cys protease to stimulate the degradation of nucellus and nucellar projection via PCD which ensures proper development of the endosperm [130]. Therefore candidate genes, which are also involved in apoptosis were incorporated in the analyses. Furthermore, MADS-box transcription factors involved in ovule development such as *ABS*, *SHP1*, *SHP2*, *AGL15*, *FUL,* and *STK* were also included in the expression analyses in order to understand expression regulation in different developmental stages [202,203].

Expression analyses were carried out using tissues taken from three different developmental stages: floral bud, stage 16 silique and stage 17B silique [65]. The results from the qRT-PCR assay show the presence of a developmental stage and genotype specific variation in gene expression. Moreover, expression of several genes was found out to vary significantly between the two wild type ecotypes. Hence, a special approach was followed to investigate individual genotypes and their gene expression network in both developmental stages. Expression of candidate genes in the *abs* genotypes was compared to the wild type WS-4 ecotype background while for *SHP1 and SHP2* target genes, the wild type Col-0 ecotype was used for analyses. Comparison of gene expression in the triple mutant lines was carried out only if the candidate genes relative expression is equivalent between the two ecotypes.

4.1.5.1 Pre-fertilization Expression Analyses of Candidate Genes

Prior to fertilization, the expressions of *SHP1*, *FUL*, *At1G27170*, *At1G53350*, *At3G14460,* and *PCS1* are significantly up regulated in *abs* mutant lines compared to wild type (WS-4) genotype (Figure 6A-C). This indicates that, *ABS* negatively regulates the expression of these genes prior to fertilization.

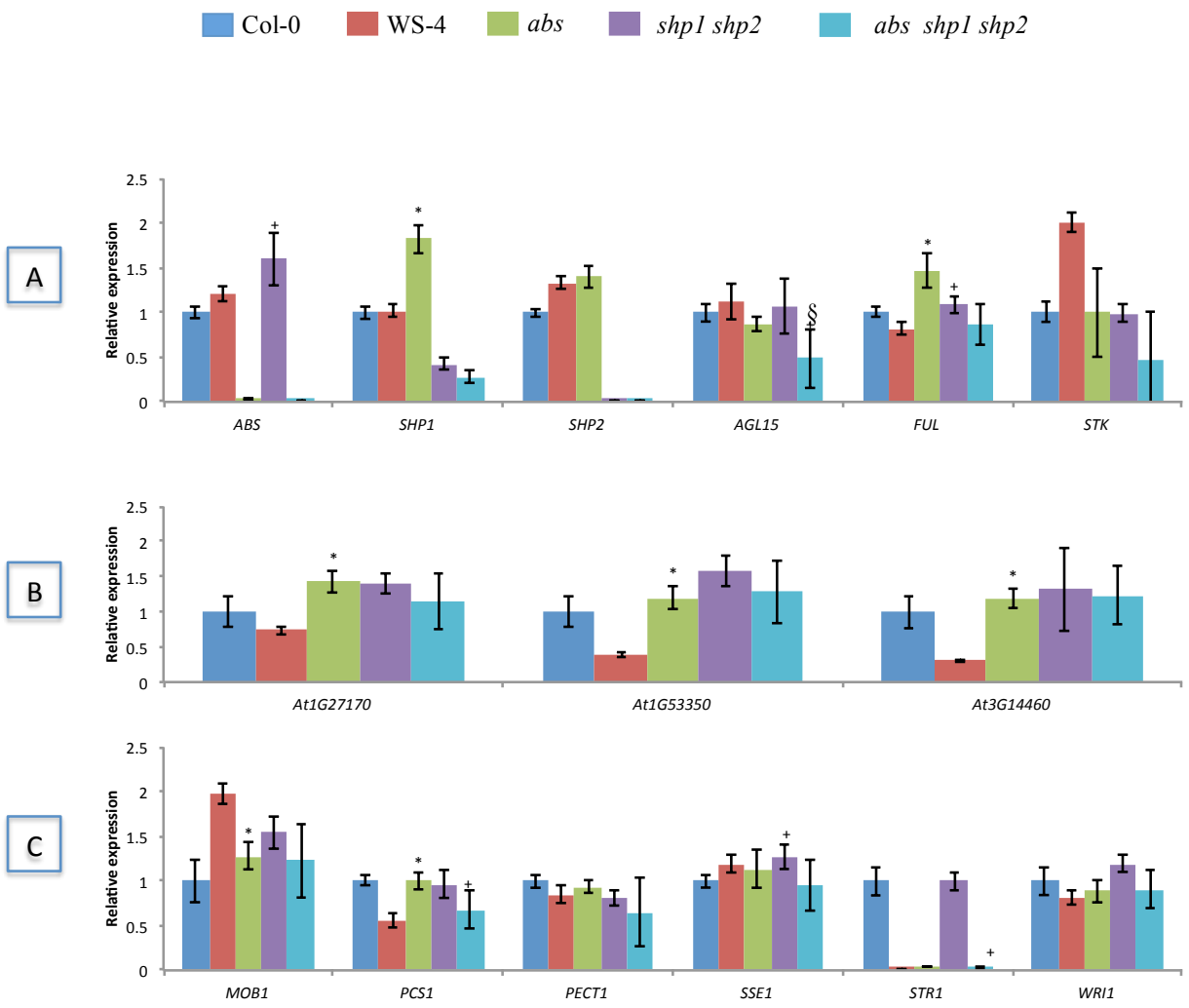

Figure 6: Pre-fertilization expression analyses of candidate genes by quantitative RT-PCR. (A) MADS-box transcription factors. (B) Apoptosis-related genes. (C) Essential genes for ovule and seed development. '*' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in WS-4. '+' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in Col-0. '§' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in both wild types.

In *shp1 shp2* double mutant, expression of *ABS*, *FUL*, and *SSE1* is significantly upregulated in comparision to Col-o indicating that prior to fertilization, SHP1 and/or SHP2 negatively regulate expression of *ABS*, *FUL* and *SSE1*.

Between the two ecotypes, equivalent expression value was obtained for some genes such as *AGL15*, *PECT1*, *SSE1*, *STK*, and *WRI1*. Nevertheless for most of these genes except for *AGL15*, no statistical difference in expression was observed in the *abs shp1 shp2* triple mutant. *AGL15* is significantly down-regulated in the triple mutant genotype in comparison to both wild type plants (Figure 6A).

4.1.5.2 Post-fertilization Expression Analyses of Candidate Genes

Post-fertilization expression of these candidate genes was carried out using siliques from two developmental stages, stage 16 and stage 17B [65]. Even though only one biological replica of WS-4 was used for the analysis of stage 16 silique samples, the result is somewhat similar to what is observed in the floral bud for the expression of *SHP1*, *FUL At1G27170*, *At1G53350*, and *At3G14460* which appears to be significantly up regulated in *abs* mutant as compared to WS-4 (Fig 7A-B).

In addition to that, expression of *SSE1* and *AGL15* are also up regulated in *abs* mutant. However, none of the analyzed genes were significantly up regulated in *shp1 shp2* mutants. Instead, expression of *MOB1*, *PCS1*, *PECT1*, and *WRI1* is significantly down regulated in *shp1 shp2* mutants as compared to Col-0. Despite the absence of equivalent expression of most of the genes in the two wild type ecotypes, a significant expression de regulation was observed for *PCS1* and *SSE1* where they are down regulated and up regulated respectively in the triple mutant as compared to both wild type genotypes.

Expression analysis of the genes at developmental stage 17B silique shows a different outcome as compared to the previous two stages used for analysis (Fig. 8A-C). Only expression of *SHP1* and *SHP2* are significantly up regulated while expressions of *PCS1*, *At1G27170*, *At1G53350*, and *At3G14460* are down regulated in *abs* mutant as compared to WS-4. Expressions of *ABS*, *FUL*, *PCS1* and *STK* are up regulated while expression of *AGL15* is down regulated in *shp1 shp2* mutant as compared to Col-0.

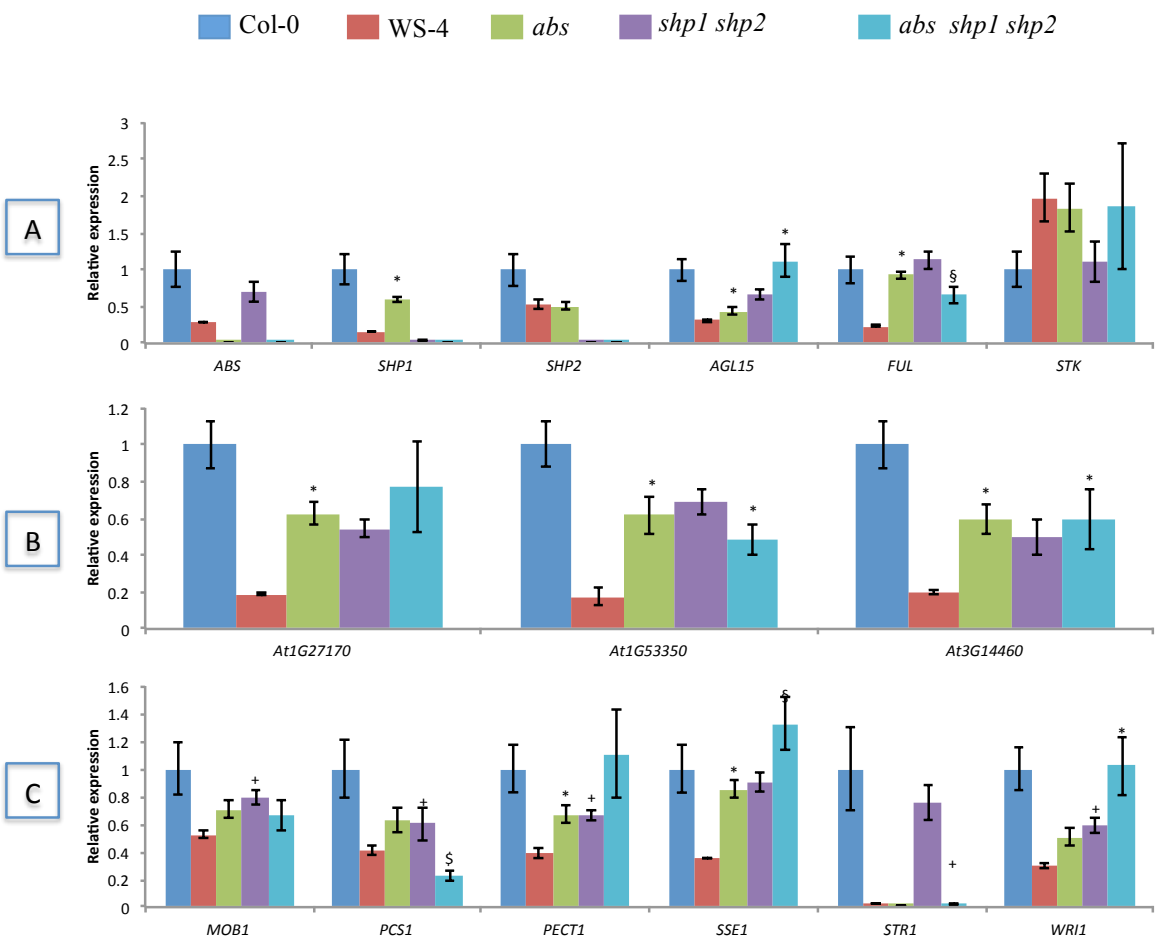

Figure 7: Post-fertilization (stage 16) expression analyses of candidate genes by quantitative RT-PCR. (A) MADS-box transcription factors. (B) Apoptosis-related genes. (C) Essential genes for ovule and seed development. '*' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in WS-4. '+' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in Col-0. '§' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in both wild types.

These results shows that *ABS* and *SHP* regulates together or independently the expression of several genes prior and post fertilization. Interestingly, most of the result shows that *ABS* and *SHP* negatively regulate the expression of one another. Besides the result also shows that *ABS* instead of *SHP* to be involved mainly in the regulation of genes which are involved in apoptosis. Furthermore, both *SHP* and *ABS* genes do significantly affect the expression of MADS-box genes as shown mainly in the de regulation of *FUL* and *STK* in *abs* and *shp1 shp2* mutant. Despite the absence of statistical difference in some of the data sets, expression of *STK* is mainly affected in *abs* mutant indicating the presence of a strong molecular interaction between the two genes supporting the genetics interaction reported before [133].

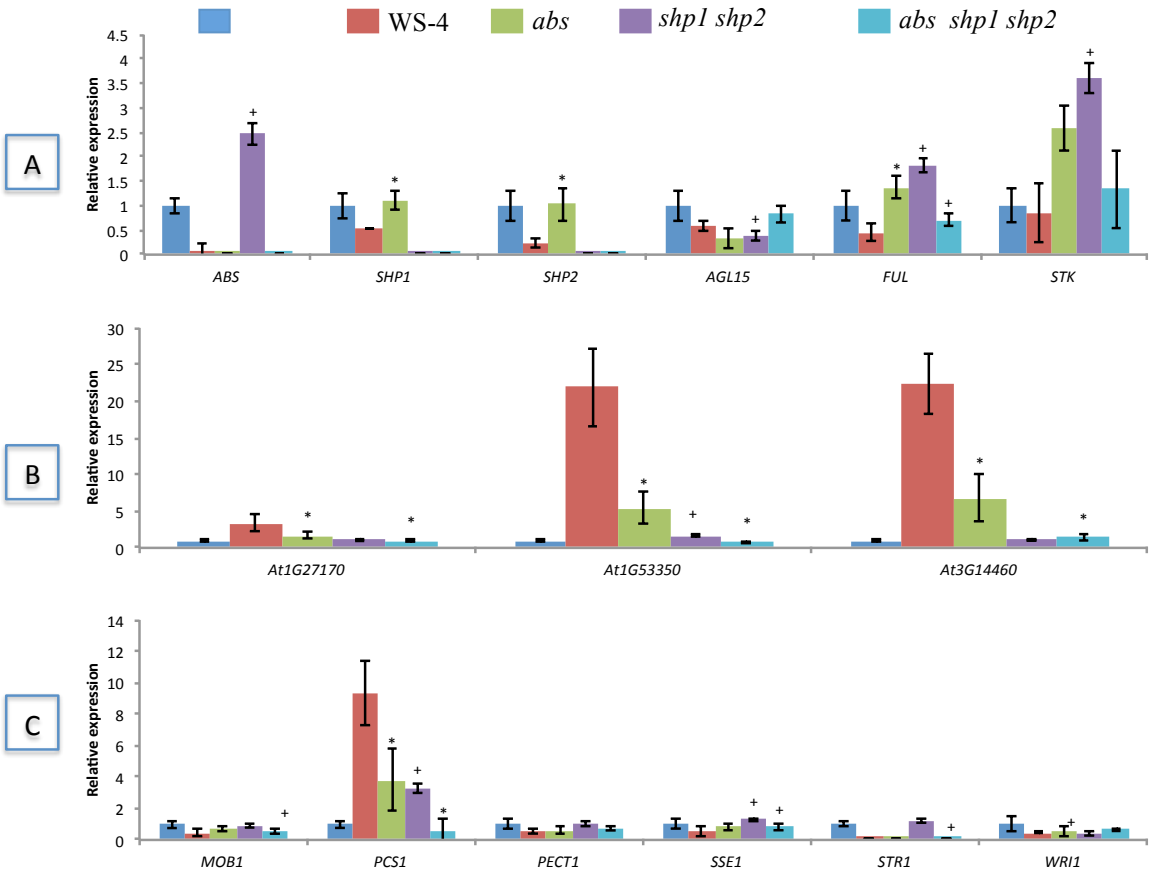

Figure 8: Post-fertilization (stage 17B) expression analyses of candidate genes by quantitative RT-PCR. (A) MADS-box transcription factors. (B) Apoptosis-related genes. (C) Essential genes for ovule and seed development. '*' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in WS-4. '+' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in Col-0. '§' above the bar indicates statistical difference ($p < 0.05$) in comparison to expression in both wild types.

4.2 Characterization of *E. californica* B_{sister} Gene (*EBS*)

4.2.1 Identification, *In Silico* and Copy Number Analyses of *EBS*

Since *E. californica* whole genome sequence is not available, a *B_{sister}-like* mRNA sequence from *Papaver setigerum* was used as a query sequence for a blast search analyses on an EST database of *E. californica* (generously obtained from Jim Leebens-Mack Lab). A 906 bp long contig that includes the 5'UTR and partial coding sequence was identified from the database and the whole ORF was completed following 3'RACE method. The *E. californica* B_{sister} gene (*EBS*) complete CDS is 888 bp nucleotides long that codes for a 296 amino acids long protein. In order to understand further the evolutionary relationship of B_{sister} genes with in land plants, available homologous amino acid sequence of B_{sister} gene were used from several species that includes gymnosperm, monocots, basal eudicot and core eudicots and a phylogenetic tress was reconstructed (carried out by our collaborator Dr. Lydia Gramzow from Jena University). The result shows that the B_{sister} genes are grouped in four clades (gymnosperm, monocot, basal eudicot and core eudicots) which fits to the previously known relationship among the species analyzed (Figure 9A).

In silico analyses of the *EBS* amino acid sequence was also carried to characterize and predict some features of EBS protein. According to the Motif Scan web tool [204] EBS protein is comprised of domains that gives it the well conserved MIKC structure. Prediction of sumoylation site using the SUMOsp 2.0 software [202] shows the presence of a type-I (Ψ-K-X-E) sumoylation site at the amino acid position 95. In addition, a type-II non-consensus sumoylation site was also predicted at the amino acid position 139. This indicates that EBS protein might be targeted and modified by SUMO proteins after translation. Furthermore, the probability of the amino acid sequence to form a coil-coil conformation was analyzed by using the COILS server [205]. The prediction indicates that the I-domain and K-Domain regions of the protein have a high probability to form a coil-coil structure as shown in Figure 9B. Presence of a coiled coiled structure has been previously described to be an essential structure for the formation of protein-protein dimer and this result shows that EBS has a potential to form a protein dimer with other MADS-domain protein.

A


GGM13 [Gnetum gnemon]
AY436726 [Drimys winteri]
117362565 [Ricinus communis]
5692cassava [Manihot esculenta]
9889cassava [Manihot esculenta]
AC202591 [Medicago truncatula]
Glyma20g00400.1 [Glycine max]
PtMADS30 [Populus trichocarpa]
164256569 [Gossypium hirsutum]
11207821 [Gossypium arboreum]
78347457 [Gossypium hirsutum]
212145294 [Theobroma cacao]
GOA [Arabidopsis thaliana]
ABS [Arabidopsis thaliana]
ABS [Arabidopsis lyrata]
TT16 [Capsella rubella]
TT16.1 [Brassica napus]
TT16.2 [Brassica napus]
209939452 [Citrus_sinensis]
BS1 [Vitis vinifera]
FBP24 [Petunia x hybrida]
LBS [Solanum lycopersicon]
DEFH21 [Antirrhinum majus]
PtMADS38 [Populus trichocarpa]
PtMADS45 [Populus trichocarpa]
56537716 [Citrus sinensis]
TT16 [Carica papaya]
BS2 [Vitis vinifera]
AY436713 [Aquilegia alpina]
EBS [Eschscholzia californica]
AF230698 [Asarum europaeum]
OsMADS31 [Oryza sativa]
ZMM17 [Zea mays]
OsMADS29 [Oryza sativa]
TaAGL35 [Triticum aestivum]
OsMADS30 [Oryza sativa]
Gymnosperms
Core eudicots
Basal eudicots
Monocots

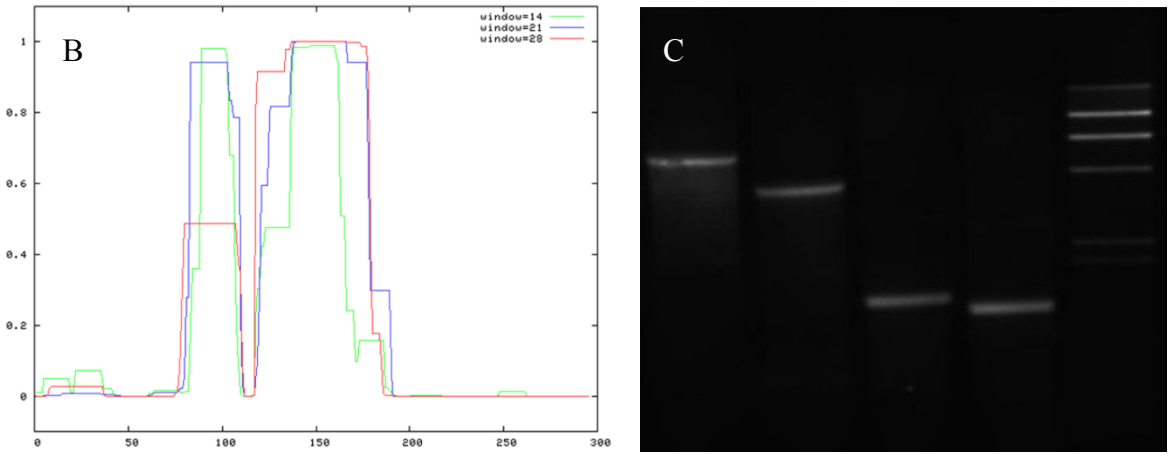

Figure 9: *In silico* analyses and copy number analyses of *EBS*. (A) Phylogeny of representative B_{sister} genes from several species. Phylogeny is determined using MrBayes on protein-guided nucleotide alignments and using *GGM13* from *G. gnemon* as an outgroup representative (B) Prediction of coil-coil conformation using the amino acid sequence of *EBS*. High probability of coil-coil formation in the I-domain and K-domain is predicted. (C) Low stringent Southern blot using four different restriction enzymes (from left to right HindIII, EcoRI, ClaI, BamHI, and DIG labeled DNA ladder) shows the presence of a single copy of *EBS*.

Since copy number of B_{sister} genes varies from species to species and since *E. californica* has undergoes through a whole genome duplication event, investigation of *EBS* copy number was carried out following a low stringency Southern blot protocol using genomic DNA of *E. californica* treated with four different restriction enzymes. A single hybridization signal was detected in each sample indicating the presence of a single copy of B_{sister} gene in *E. californica* (Figure 9C), which is important information to know in advance before proceeding with functional characterization.

4.2.2 *EBS* is Predominantly Expressed in Developing Ovules

To target the specific localization of *EBS* transcripts, RNA *in situ* hybridization was carried out using tissue sections from different floral developmental stages (Stage 6, 7, and 9). Floral stage 6 is characterized by a developmental landmark where the stamen primordial gives rise to the microsporangia. Followed by is the commencement of ovule initiation categorized into stage 7. Stage 9 is the critical developmental stage for the female gametophyte development where by female meiosis starts and the embryo sac development initiated [169].

In situ hybridization analysis result shows that during early stage of floral organ development (Stage 6), strong expression of *EBS* was detected in the developing stamen (Figure 10A). And at developmental stage 7, *EBS* transcript was detected in overall the floral organs but strong expression is still detected in the stamen,

gynoecium, ovules, vascular tissues and apical region of the petal primordium (Figure 10B).

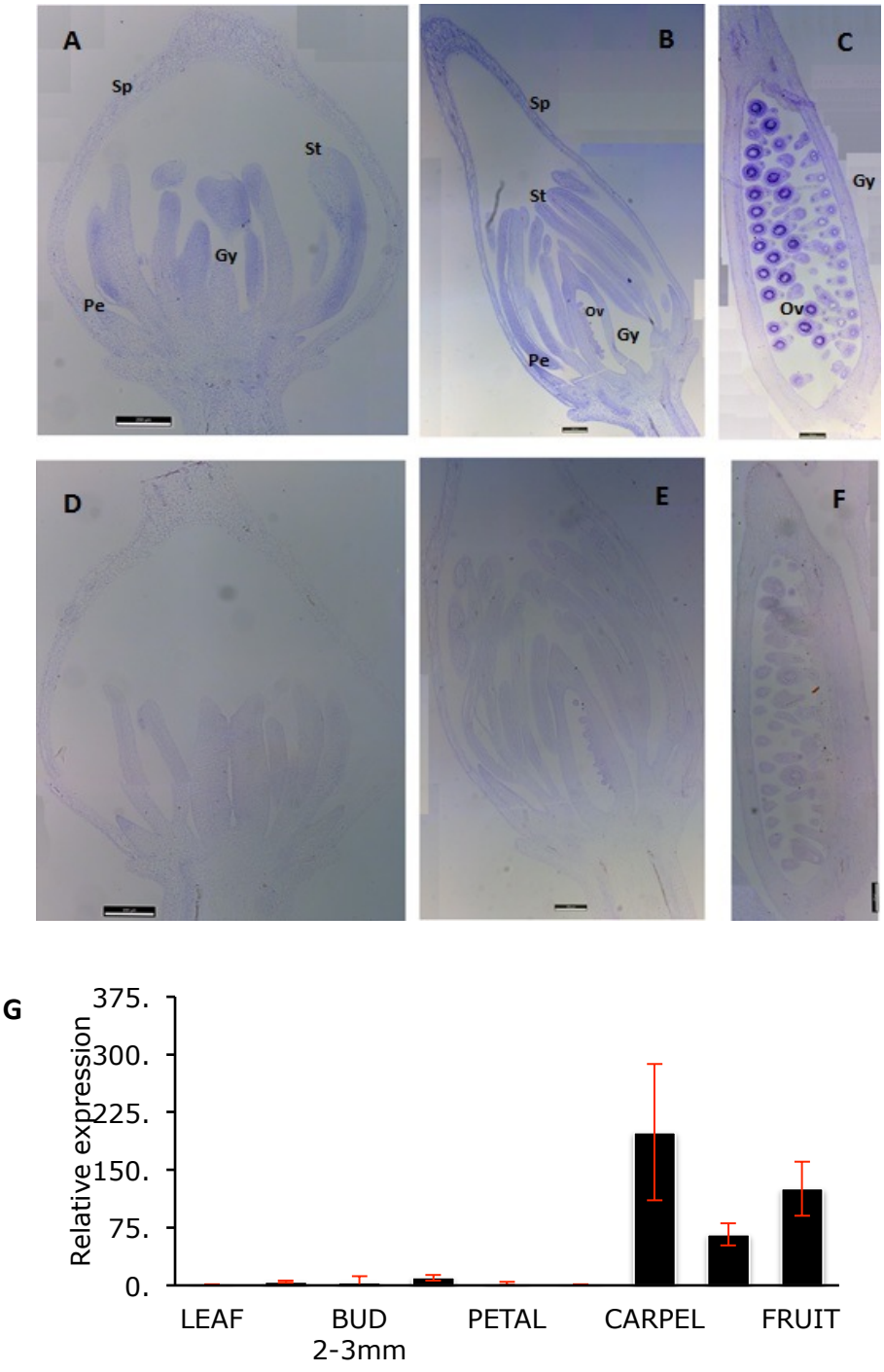

Figure 10: Expression analyses of *EBS* by *in situ* hybridization (A-F) and quantitative real-time PCR (G). (A-C) *In situ* hybridization using anti-sense RNA probe on sections from three developmental stages; (A) Stage 6, (B) Stage 7, (C) stage 9. (D-F) *In situ* hybridization using sense RNA probe on sections from three developmental stages; (D) Stage 6, (E) Stage 7, (F) Stage 9. Scale bar: 200 µm. (G) qRT-PCR analysis of *EBS* transcripts using vegetative and reproductive tissues (stage 9). Bars indicate standard deviation. Sp, Sepal; Gy, Gynoecium; Ov, Ovule; Pe, Petal; St, Stamen

However, during developmental stage 9 a specific and strong localization of *EBS* transcripts was detected in the nucellus and inner integument region of the ovule (Figure 10C) along with a weak hybridization in the vascular tissue of the funiculus.

Quantitative relative expression of *EBS* in different plant organ was carried out to obtain a quantitative data and to compare the relative expression across these organs. The results shows that *EBS* is strongly expressed in tissues that are part of the female reproductive organ such as carpel, fruit and seed. Very low expression was detected in stamens, sepal, petal, different size of floral buds, and the leaf (Figure 10G). *EBS* is expressed in the carpel approximately 200 fold times in comparison to expression in leaf. The *in situ* result agrees with the qRT-PCR results suggesting the higher expression observed in carpel, seed and fruit is possibly associated with the strong expression of *EBS* in the ovules.

4.2.3 *EBS* Protein Interacts with B-class and C-class Floral Homeotic Proteins in Yeast System

Analysis of protein interaction partners is essential to predict or explain the gene function. In this study, a yeast two-hybrid experiment was carried out to investigate the protein interaction between EBS protein and candidate floral organ identity proteins. Truncated EBS containing the M-domain, I-domain and K-domain was used for the assay in order to avoid the auto-activation behavior of the C-terminal domain. *Lac-Z* assay and growth assay on synthetic drop out media lacking three amino acids (leucine, tryptophane and histidine) were used to screen for interacting partners at 30°C and room temperature. The candidate proteins were tested in advance for their ability to auto-activate the reporter gene and the result shows that no auto-activation of the reporter gene by the candidate proteins [34].

The yeast two-hybrid assay result shows that EBS protein is able to form a homodimer with itself and heterodimer with other floral organ proteins. Strong protein interaction was observed between EBS and EScaDEF1, EScaDEF2, EScaAG2 and EBS (Figure 11). These interactions are positive both at RT and 30°C indicating their strong interaction. Furthermore, EBS is able to form a weak interaction with EScaDEF3 and it is not able to form a heterodimer with EScaAG1, EScaAGL6, EScaAGL9 (EcSEP3), and SIR. Both positive homodimer and heterodimer interaction results are consistent

when EBS is fused with the activation domain or binding domain of the *GAL4* transcription factor.

Biflourecence complementation assay (BiFC) was carried out to verify the yeast based protein interaction result in planta by infiltrating the leaves of *Nicotiana benthamiana* with *an A. tumefaciens* bacteria culture containing the candidate genes fused with YFP fragment. Only a one way positive interaction was observed between EBS-YN and EcSEP3-YC but none of the proteins which were able interact in the yeast system were not able to form a protein dimer with EBS in tobacco transient assay (data not shown). In order to validate the yeast two-hybrid result in the plant system, the BiFC experiment needs to be repeated.

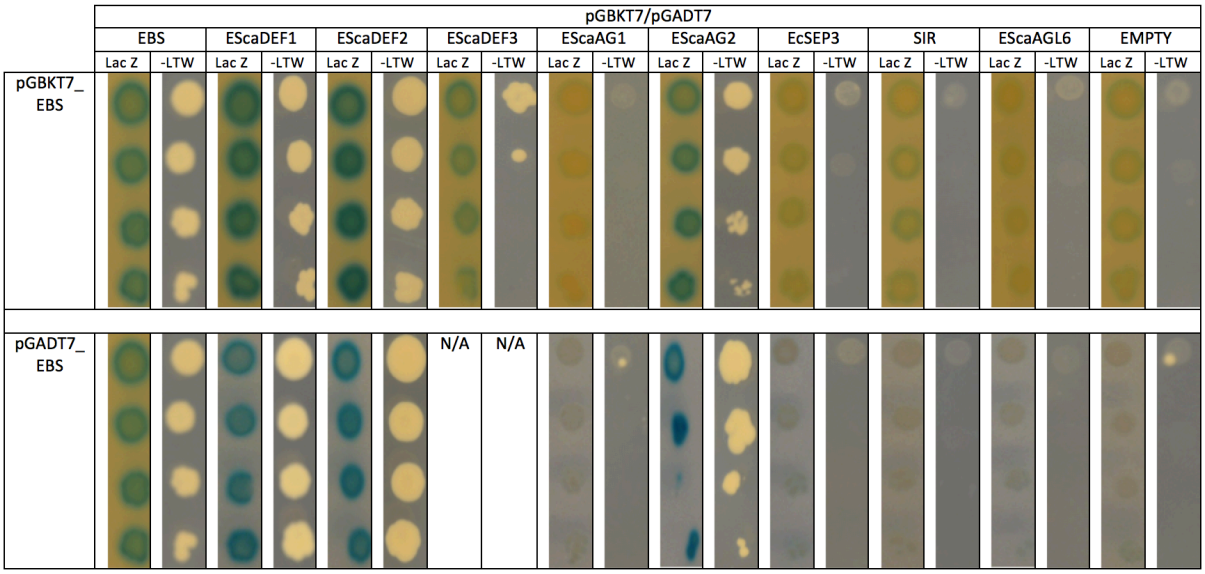

Figure 11: Yeast two-hybrid Protein-Protein Interaction analyses for EBS and selected floral homeotic proteins. EBS is able to form protein complex with EBS, EScaAG2, EScaDEF1, and EScaDEF2. Protein interaction was verified in both *lacZ* assay and growth assay on –LTW medium. (N/A), data not available.

4.2.4 Down-regulation of *EBS* Affects Seeds Morphology

To learn more about the function of *EBS*, Virus Induced Gene Silencing (VIGS) technique was used utilizing the Tobacco Rattle Virus (TRV) vector system to specifically silence *EBS* transcripts. A Week old seedlings were infiltrated with a mix of *pTRV1* and the following constructs: *pTRV2_EBS*, *pTRV2_EcPDS* and *pTRV2_Empty*. Total of 52 plants per mix were used for this experiment and the first flower from each

plant was used for phenotypic analyses while the second floral bud was used for expression analyses following qRT-PCR method. The down-regulation of *PHYTOENE DESATURASE* (*PDS*) gene causes a discoloration of the leaf due to absence of carotenoid biosynthesis [188]. This serves as a positive control for the experiment. The result showed that, except for those infiltrated with *pTRV2_EcPDS* construct, the remaining plants showed no deviation from untreated plants in their vegetative and floral organ growth and development. From the 52 plants treated with *pTRV2_EBS*, only 11 were able to show more than 30% reduction of *EBS* transcripts in comparison to the wild type plants (Figure 12A).

From these 11 plants, VIGS-8 and VIGS-23 shows more than 75% of *EBS* transcript down-regulation compared to the wild type. Developing seeds taken from fruits 20 days after pollination (DAP) showed that seeds from VIGS-8 and VIGS-23 have an irregular shape compared to the wild type plants (Figure 12B). However, developing seeds from the remaining lines do not show any altered seed structure. This indicates that, down-regulation of *EBS* affect the seed grain filling and the phenotype seems to be dosage dependent.

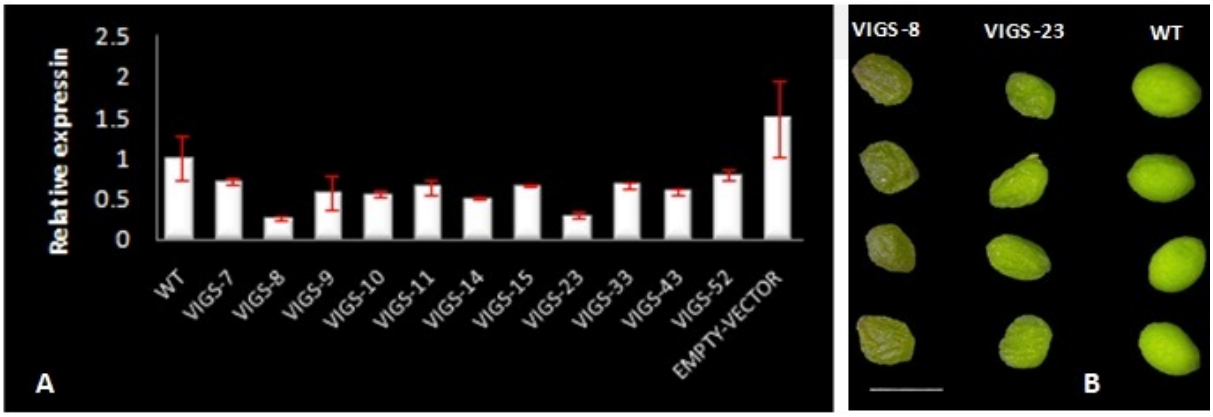

Figure 12: Molecular and phenotype analysis of VIGS treated plants (A) Relative expression of *EBS* transcript in selected treated and wild type plants. VIGS-8 and VIGS-23 plants showed around 80% reduction in *EBS* transcript. (B) Developing seeds from VIGS-8 and VIGS-23 appears to have a wrinkled phenotype compared to the wild type seeds .

4.2.5 Heterologous Expression of *EBS* in *Arabidopsis* Affects Plant Development

ABS gain-of-function in *Arabidopsis* was reported to alter the vegetative and reproductive organ development. Curling of rosette leaves, late flowering, inflorescences with reduced floral sizes, partial sterility, and silique with shrunken phenotype were some of the phenotypes reported in plants over-expressing *ABS* [132]. In order to investigate if *EBS* is also involved in similar developmental processes to that of *ABS*, *Arabidopsis* Col-0 flowers were dipped in an *Agrobacterium* suspension containing pMLBART binary vector constructed with coding region of *EBS* sequence driven by the constitutive *CaMV 35S* promoter. 16 independently BASTA resistant lines were obtained and their phenotype was analyzed. Verification for the presence of the transgene was additionally carried out by PCR and RT-PCR analysis. A number of morphological defects were observed in the transgenic lines including curling of rosette leaves, shrinking of silique, wrinkled seeds and dwarfed plants (Figure 13A, D, E). Out of the total 16 transgenic lines, 5 independent lines show much stronger phenotypes that includes very dwarf and infertile plants. In addition, inflorescence meristem determinates bearing a very small flowers that are male sterile with no or reduced petal formation (Figure 13B-C).

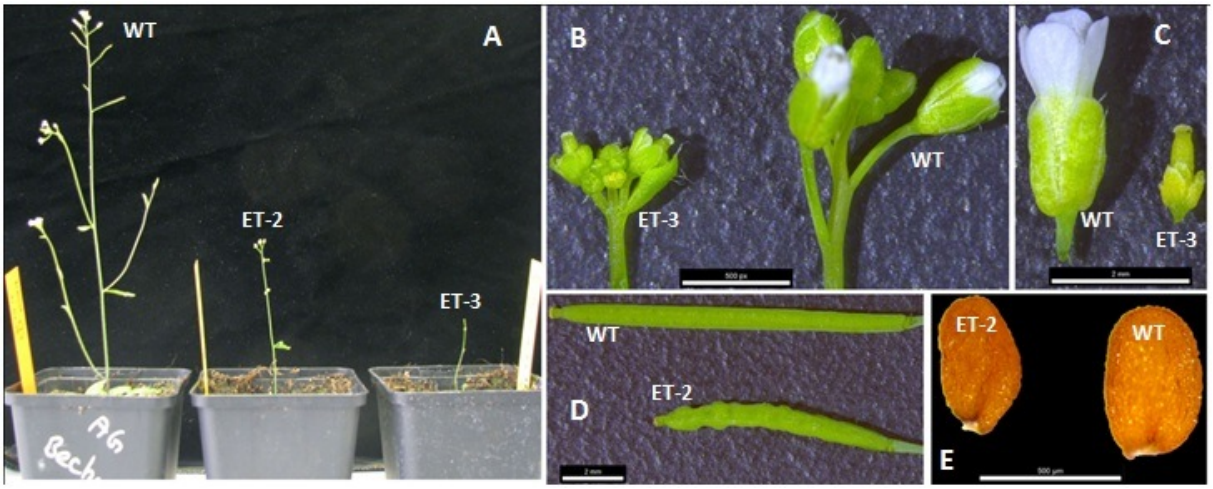

Figure 13: Heterologous expression of *EBS* in *Arabidopsis*. (A) Comparison of plant height between wild type and transgenic lines. (B-C) Sever phenotype was observed in some lines where the size of floral organ is significantly smaller compared to the wild type flowers. Petal is absent and the stamens are short and sterile. (D) Silique size is reduced and wrinkled in the transgenic lines compared to the wild types. (E) Seeds from transgenic line showing a wrinkled structure compared to the wild type. WT, wild type; ET-2 & ET-3, Independent transgenic lines.

4.2.6 Heterologous Expression of *EBS* in *Arabidopsis* Affects Seed Oil Content

Down-regulation of *Brassica napus* B_{sister} transcripts (*BnTT16s*) has been reported to cause several defects in plant development and composition of seed oil content [138]. In order to investigate if ectopic expression of *EBS* in *Arabidopsis* influence the seed oil content, seeds from transgenic *Arabidopsis* over-expressing *EBS* (ET-2) and Col-0 wild type were used for the analyses of seed oil content (Experiment was carried out by our collaborator Prof. Rod Snowdon from plant breeding department, Universität Giessen). The result showed that there is a significant change in the seed fatty acid composition between the two genotypes. The *EBS* over-expressing line has a high content of myristic acid, oleic acid and linoleic acid compared to the wild type plants (Figure 14). However low amount of stearic acid and 11-eicosenoic acid is present in the over-expression line seed compared to the wild type seed. No significant difference was observed between the two genotypes for the remaining of fatty acids compositions. This indicates that, EBS might be involved in the synthesis of some fatty acids and probably the shrunken seed phenotype observed in the VIGS-8 and VIGS-23 lines is due to the decrease production of storage oil.

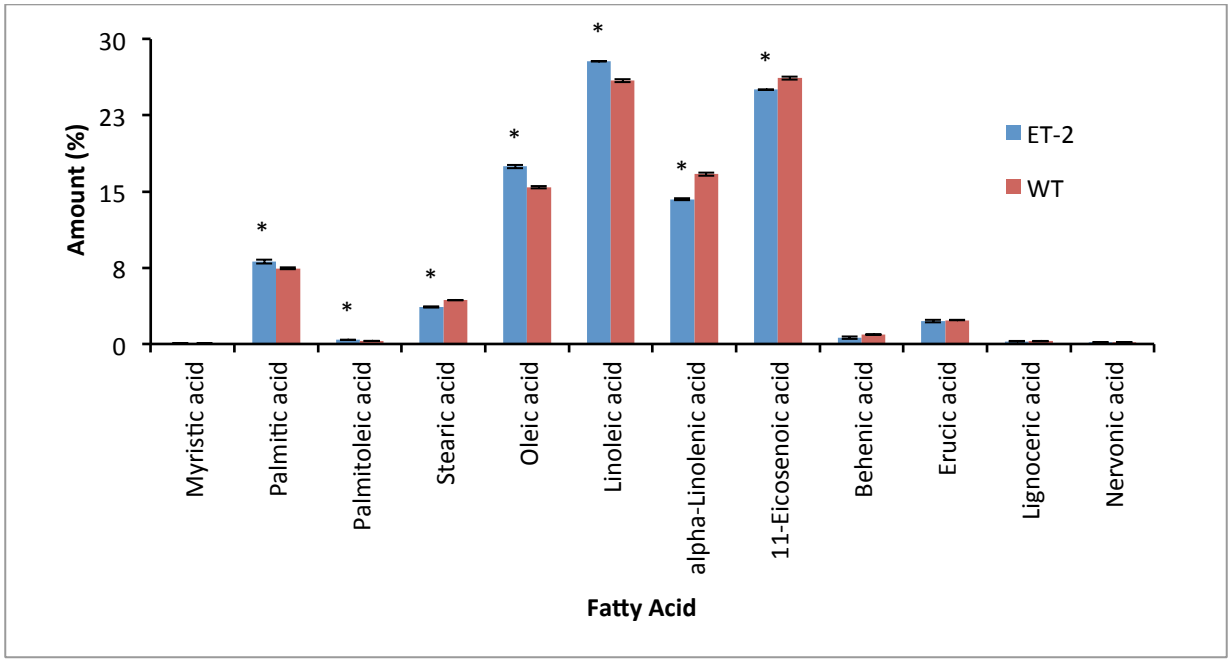

Figure 14: Analyses of seed Fatty acid composition in *Arabidopsis* Col-0 and 35S::EBS transgenic (ET-2) seeds. Aestrix above the bars indicated significance difference inferred by two-way T-test statistical analyses ($p<0.05$) and bars indicates standard deviation.

4.2.7 *EBS* don't complement *ABS* in *abs* Mutant

As mentioned in section 4.2.5, there is a similarity in gain-of-function phenotype in both *EBS* and *ABS*. Even though both have different protein interacting partners, mutant complementation experiment was carried out to investigate if *EBS* is able to restore the wild type phenotype when introgressed into the *abs* mutant. Crossing was carried out between a line overexpressing *EBS* and *abs* genotypes and a PCR based genotyping was carried out to screen for homozygous *abs* mutant that carry the transgene. The result shows that T_2 genotyped plants retain the pigmentation mutation indicating the absence of complementation of *ABS* by *EBS.*

4.3 Stable Genetic Transformation of *E. californica*

Here we report an efficient stable genetic transformation protocol for California poppy using developing seed as explants. Callus induction medium and plant regeneration medium along with optimized selection agent concentration to maximize the efficiency of transformation and regeneration are reported. This protocol is optimized in collaboration with previous colleagues of the research work group namely Aravinda Yellina and Sabrina Lange. (Aravinda Yellina and Sabrina Lange were able to identify suitable explant for infection and plant regeneration. In addition they optimized the callus induction and regeneration media. I optimized optimum BASTA concentration to be used for selection of transgenic callus and was able to reproduce the optimized protocol).

4.3.1 Analyses of Regenerated Plants

A total of 361 developing seeds (22 DAP) were inoculated with an *Agrobacterium* culture containing the binary vector construct *pMLBART_35S::hpRNAi-EBS* and *pMLBART_35S::EBS* respectively. 100 untreated developing seeds (22 DAP) were used as negative control.

Four different phases were identified throughout the regeneration process; callus formation, shoot formation, root formation, and complete plant regeneration (Figure 15). When callus formation starts around two weeks after the inoculation, it emerges from the seed cover and comes in direct contact with the selection mediium (Figure 15A) and over the following weeks putative transgenic calli is formed with cell proliferation (Figure 15B) whereas the non-transgenic ones becomes darken and stop proliferating.

Out of the total explants treated, 82% and 51% of them were able to form callus four week after inoculation with *Agrobacterium* carrying the *pMLBART_35S::hpRNAi-EBS* and *pMLBART_35S::EBS* binary vectors respectively. However, 72% of the untreated explants were also able to form callus. This indicates the concentration of BASTA that was used (1 mg/l) was not strong enough for selection. Therefore, the concentration was increased to 5 mg/l to increase the selection pressure in the subsequent growth mediums. Those explants that were able to form a healthy callus were transferred to shoot regeneration medium and exposed to long day growth condition. The callus

became greenish in 2-3 days after exposure to light and leaf like structures emerges from the calli indicating somatic embryogenesis (Figure 15C) and shoot formation was completed four weeks after moving to the new growing condition. From those explants that were able to produce callus, only 27% (treated with *35S::hpRNAi-EBS* construct) and 21% (treated with *35S::EBS* construct) were able to form shoot like structures respectively.

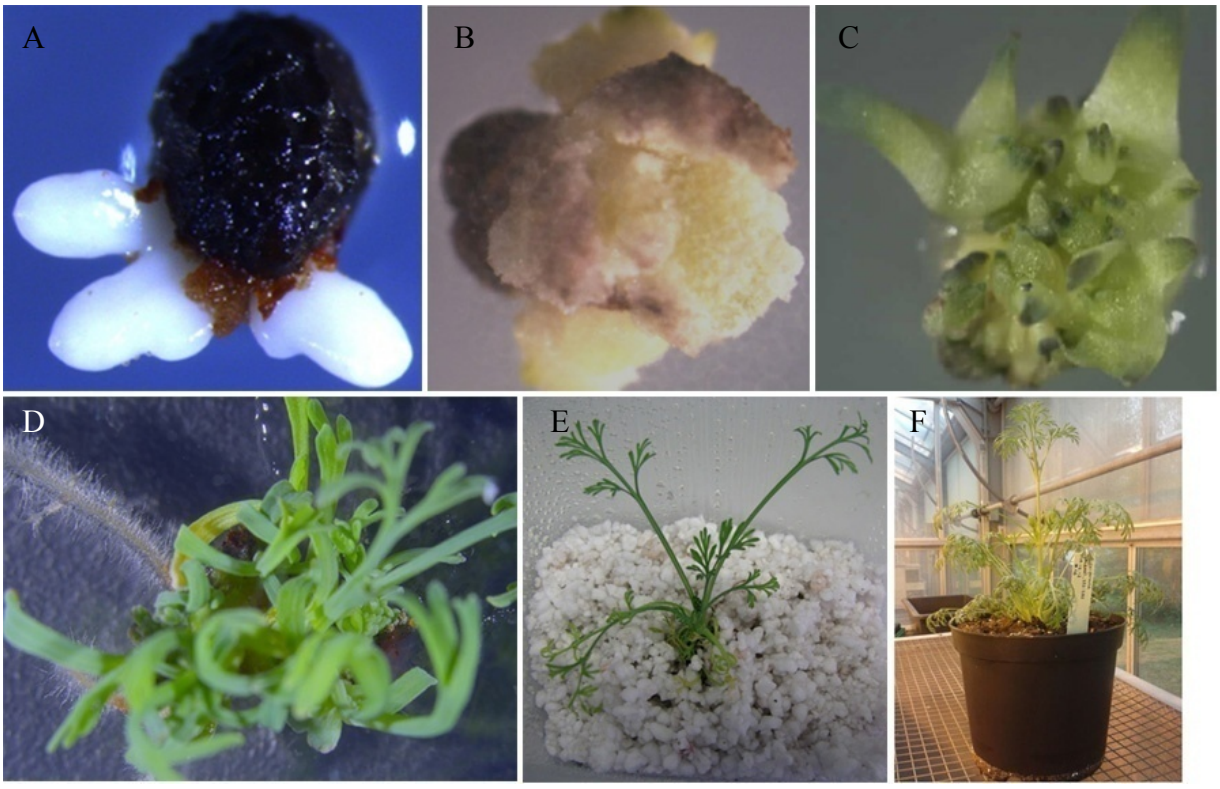

Figure 15: Regeneration of transgenic Californica poppy plant from developing seeds via somatic embryogenesis. (A), Initiation of callus formation; (B), callus formation completed four weeks after inoculation; (C), somatic embryogenesis; (D), root regeneration; (E), transplanting of regenerated plant in perlite; (F), transplanting of regenerated plant in soil.

No explants were able to survive from the untreated control indicating the efficiency of 5 mg/l BASTA concentration for selection. In order to initiate root formation, the explants were transferred on a media containing sucrose but lack additional hormone supplements. Four weeks after growing on the plant regeneration medium, only 7% and 15.4% of the explants treated with *Agrobacterium* carrying the *pMLBART_35S::hpRNAi-EBS* and *pMLBART_35S::EBS* respectively were able to form root (Figure 15D). These regenerated plants were then transferred to a plastic container filled with sterile wet perlite (Figure 15E). This phase served as a hardening step for the regenerated plants

as they transit from the growth medium to sterile soil. The plants were kept for one week on perlite and transplanted into soil and finally kept in green house (Figure 15F). However, only 10 independent putative lines harbouring the knock-down construct and one from the over-expression construct were able to survive the soil (Table 2).

Table 2 Summary of *E. californica* stable genetic transformation and regeneration efficiency

Binary vector	Total number of explant used for transformation	Total number of explants that form shoot	Total number of explants that form root	Total number of plants regenerated on soil	Total number of plant positive for transgene integration
pMLBART_*35S::hpRNAi-EBS*	180	40	12	10	6
pMLBART_*35S:: EBS*	181	28	22	1	0

DNA extracted from leaf tissue of the regenerated plants and wild type plants was used for PCR based genotyping. Primers amplifying partial sequence from the *CaMV35S* gene were used for genotyping. The result showed that out of the 11 independent lines, 6 and 0 of them carry the *35S::hpRNAi-EBS* and*35S::EBS* transgene respectively. Strong amplified DNA fragment was observed for the transgenic line #2 and #6 while faint DNA fragment was detected in transgenic #3, #4, #8 and #17 (Figure 16A). This might possibly be due to copy number variation. Independent lines were chosen and quantitative RT-PCR was done to investigate the relative expression of *EBS* in wild type and transgenic lines using gynoecium. The result shows that *EBS* transcript was significantly down-regulated in the three transgenic lines analyzed in comparison to the wild type plant (Figure 16B). However, it was not possible to see any altered phenotype in the knock-down lines compared to the wild type plants. This implies that the level of down-regulation was not sufficient enough to reveal a phenotype or there might be another gene which act redundantly to *EBS* which makes is difficult to identify the function of EBS.

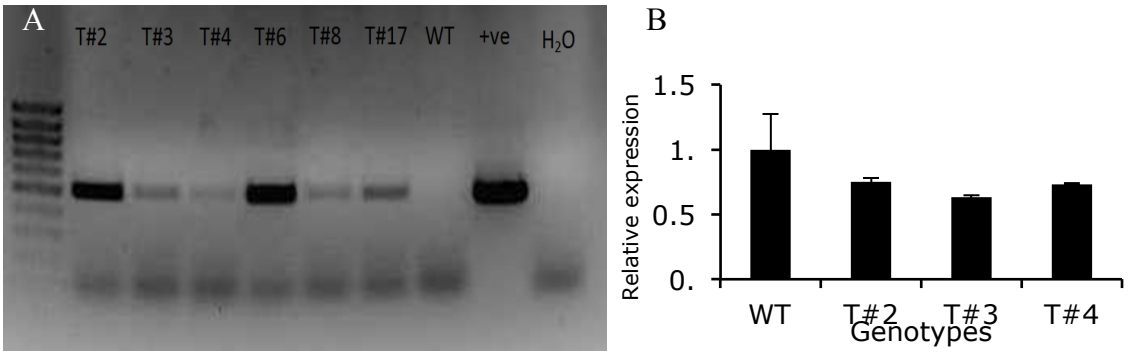

Figure 16: Characterization of regenerated lines. (A) PCR based genotyping of transgenic lines. (B) Relative expression of *EBS* transcripts in wild type and transgenic lines.

5. Discussion

5.1 *ABS* Loss-of-Function Affects Several Aspects of Plant Development

B_{sister} genes have recently become the focus of several research fields mainly due to their role in seed development (For review [155]). *ABS* function was the first to be identified during screening for altered seed pigmentation in *Arabidopsis*, where it is crucial for the expression of genes required for the synthesis of proanthocyanidin and for inner integument development [132].

The *Arabidopsis* seed coat is an essential part of the seed since it is required both for nutrient transport and facilitating seed germination [206]. *Arabidopsis* seed coat is the product of two types of ovule integuments: the outer integument of the ovules makes the testa while the inner integument forms the tegmen. While testa is comprised of cells which contains mucilage, the tegmen is made from cells that contains PA [207].

The seed testa not only provides protection for the developing embryo, but also regulates the plant fertility. Several ovule mutations have shown the presence of a direct link between integument development and female fertility [91]. As mentioned in section 1.3.2 of this dissertation, *bel1* mutants completely lack the ovule inner integument while the outer integument is replaced with a modified structure that fails to fully cover the nucellus tissue. Consequently the megagametophyte development is severely affected leading to a complete female sterility [105]. Similarly, *ant* mutants that fail to initiate both ovule integuments also have flaws in their female gametophyte formation [110,208]. Furthermore, *sin1* mutants that show an incomplete inner integument development leaving partially covered nucellus were also reported to have an altered embryo sac development [105]. Change in the identity of the inner integument is also reported in *ats* mutants which in fact do not affect the female gametophyte development but causes a reduction in mature seed number [209]. In contrast to these ovule mutations, most mutations that affect the ovule outer integument, such as *ino* and *goa-1*, were not reported to affect the plant fertility [136, 111].

Even though *abs* is an inner integument mutation, previous studies do not report any problem in plant fertility in the single mutants. In this study, however, additional phenotypes associated with *ABS* loss-of-function (*abs* mutant) are reported. Among

them includes the reduction in mature seed number. Compared to the wild type plants, seed number in *abs* mutant is reduced by 33%. However, in the *goa-1* mutant (an outer integument mutant), no significant difference was found in seed number in comparison to the wild type. This strengthens the speculation that, the inner integument development is strongly linked with the plant fertility in comparison to the outer integument. This might be due to the presence of regulatory pathway that originates from the inner integument to the female gametophyte tissue. Yang and Sundaresan [114] stated that, altered gametophyte development phenotype observed in *sterile apetala (sap)* mutant and *spl* female gametophyte mutants, is strongly due to the absence of essential signals that originate from the integuments. Even though *SAP* is expressed strongly in both integuments and ovule primordia, *sap* mutants do not affect the integument development but instead failed to form the megagametophyte indicating targets of *SAP* gene in the integument are necessary during gametogenesis [210]. In order to investigate the fertility problem associated with the *abs* genotypes, attempts were made to analyze the transmission tract proper development by using alcian blue and neutral red staining method. It has been previously reported that proper development of the gynoecium transmitting tract tissue is important for the seed setting efficiency in *Arabidopsis* [211]. However, the result shows no difference between the wild type and *abs* mutant with regard to the transmission tract development. This indicates the fertility problem is possibly associated with the altered maternal tissue development.

Another new phenotype observed in the *abs* mutants is the reduced silique length. Similar phenotype was reported in canola B_{sister} genes, *BnTT16s*, knock-down genotypes. Down-regulation of *BnTT16s* causes a reduction in mature seed number along with reduction in fruit size [137]. As mentioned in section 1.3.4 of this dissertation, elongation of the silique length is an immediate response to the hormones released from fertilized ovules [40]. This direct relationship between fruit growth induction and fertilization was shown in other studies that identified genes which are required to inhibit the fruit development in the absence of fertilization [212]. Taking this into account, the presence of an incomplete fertilization in the *abs* mutants can be the main reason for the reduction in silique length, instead of ABS being involved in fruit development directly.

5.2 *ABS* is Crucial for Seed Germination

With or without the presence of abiotic stress, only few seeds from *abs* mutant germinate compared to the wild type seeds. However, seed from *goa-1* mutants, which is not a pigmentation mutation though, do not show any deviation from the wild type during germination. These differences probably might be associated with the high permeability of *abs* genotypes for surrounding substances, such as the sodium hypochlorite and ethanol solutions used for seed surface sterilizing. And when it is coupled with additional stresses, such as high salt concentration in growth medium, it could results in poor germination efficiency. This indicates that, though the deposition of PA by itself has a significant role in seed germination, alteration of seed testa structure will expose the seed to unfavorable environmental conditions that will hamper the proper seed germination.

5.3 *ABS* and *SHP1 SHP2* Loss-of-Function Affects Seed Morphology

Upon lack of convincing phenotype in *abs* genotype during earlier studies, it was suggested that protein complexes which are formed between B_{sister} proteins and floral homeotic proteins might reveal novel function of *ABS* [37]. A recent study reported that*abs stk* double mutants shows fertility problems and lack the inner ovule integument [133].

Investigation of *abs shp1-1 shp2-1* triple mutants showed the importance of these three proteins for proper seed development. The results show a loss of function of these three genes affecting the plant fertility significantly in comparison to the wild type and the parental lines. In addition to the fertility problem, seeds from the triple mutant lines have an altered structure. This suggests that the *SHP1* and *SHP2* genes act redundantly with *ABS* in maintaining both the plant fertility and seed grain filling.

In order to find out the molecular reasons behind the fertility problem and altered seed grain filling phenotype observed in the triple mutants, attempts were made to analyze the expression of selected genes which are involved in different aspects of ovule and seed development. An expression analysis for the candidate genes was carried out based on the assumption that both ecotypes have similar pattern of development, and expression of the candidate genes probably might not differ significantly. However the result shows a significant difference in the expression of several candidate genes. For

example, expression pattern of *SULFURTRANSFERASE1 (STR1), At1G53350,* and *At3G14460* differ significantly between the two wild types (Figure 17). Such variation of expression was also reported in previous studies where only 50-60% of analyzed genes have similar expression pattern in the ovule between the Col-0 and WS-4 ecotype [213].

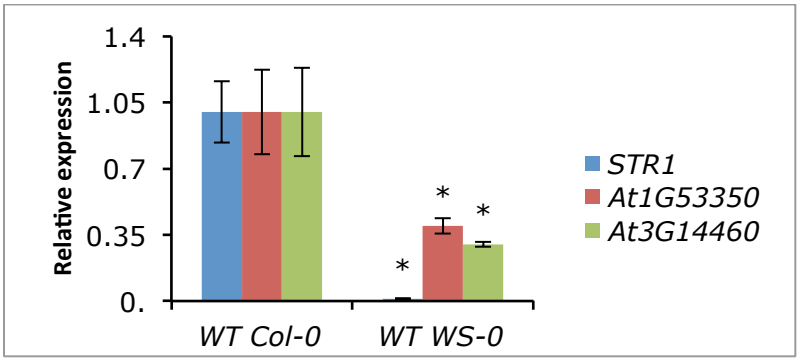

Figure 17: Pre-fertilzation expression of *At1G53350*, *At3G14460* and *STR1* in two different wild type plants. Significant difference in expression level is observed between the two ecotypes.Astrix above the bars indicated significance difference inferred by two-way T-test statistical analyses ($p<0.05$) and bars indicates standard deviation.

Though it is difficult to make conclusive comparative analyses between the wild type and the triple mutant lines for the candidate genes, a gene regulatory network was constructed by comparing the expression pattern of the candidate genes in the wild type and the parental lines. Since the expression of the candidate genes vary during pre and post-fertilization stage, the gene expression regulatory model was constructed separately for both developmental stages (Figure 18).

The expression data shows that *ABS* and *SHP1 SHP2* genes negatively regulate each other's gene expression at both developmental stages. Furthermore, there is a significant up-regulation of genes involved in PCD in *abs* mutants prior to fertilization. So far no information is avaialable that links up-regulation of PCD genes with fertility problem. However, up-regulation of PCD genes has been previously reported to be linked with ovule abortion in *Vitis vinifera*. Expression of *Vitis vinifera* metacaspases (*VvMC*) such as *VvMC1*, *VvMC3*, and *VvMC4* is significantly up-regulated during the developmental stage when endosperm abortion occurs in seedless variety of *Vitis vinifera* [214]. Moreover, both *ABS* and *SHP1 SHP2* genes negatively regulate the expression of *FUL* gene. Previous studies suggested that, the CArG box binding site in the promoter region of *FUL* is essential for the initiation of its transcription [215]. *ABS* and

SHP1 SHP2 proteins possibly might competitively bind to this CArG box binding site independently or as a complex and inhibit the binding of a protein complex required for its transcription initiation and regulate the expression of *FUL* gene.

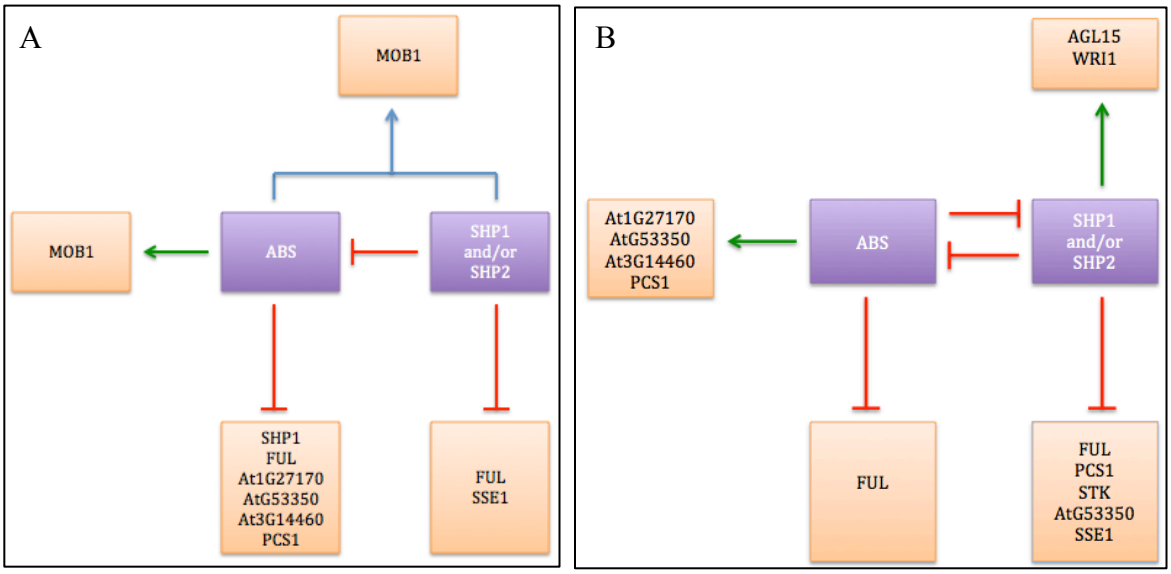

Figure 18: Regulation of candidate genes expression by *ABS* and *SHP* genes. (A) Pre-fertilization regulatory network. (B) Post-fertilization regulatory network. Red lines indicates negative regulatory pathway and Green lines indicates positive regulatory pathway.

Brambilla et al. [109] stated that high order protein complex formation is crucial not only for the regulation of genes at transcript level but also controls the availability of a particular protein found in the complex. They affirmed that the protein complex formed between SHP1-SHP2-STK is required to stabilize the high order protein complex formed between AG-SEP-BEL. The AG-SEP-BEL protein complex is essential since it causes scarcity of a freely available AG protein in the ovule tissues that otherwise lead to the phenotypes observed in *bel1* mutant [107]. In general, since it is difficult to associate the expression pattern of candidate genes with the *abs shp1-1 shp2-1* triple mutant phenotype, it might be plausible to speculate that the protein complex formed between ABS-SEP-SHP1-SHP2 is crucial not only to regulate the expression of target genes, but also might be involved in stabilizing protein complexes that are crucial for the ovule development. Taking all the results of this and previous works, *ABS* is shown to be crucial for the plant fertility and seed development (Figure 19).

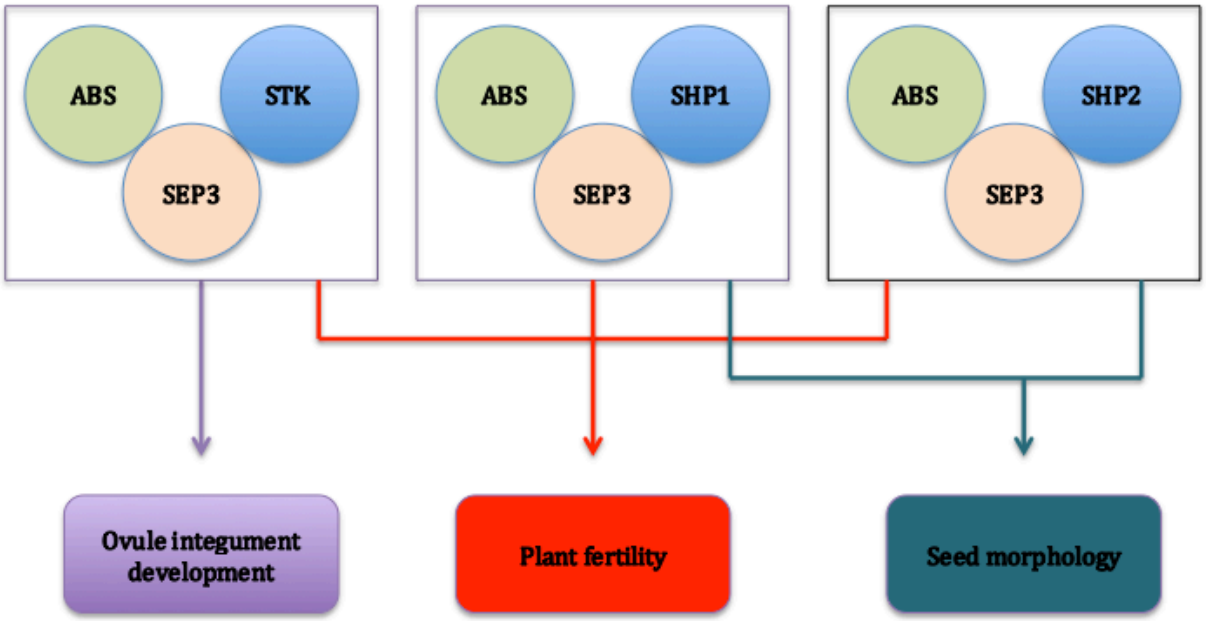

Figure 19: Functional relevance of protein complexes involving ABS protein [37,89,132,133].

5.4 Conserved B_{sister} Expression Pattern Observed *in E. californica*

A gene expression pattern analysis is a pre-requisite to identify the relevant plant organs where the gene acts. In this study, RNA *in situ* hybridization was carried out on *E. californica* floral buds from three different flower developmental stages: stage 6, 7, and 9. According to Becker et al. [169], the stage 6 floral bud is comprised of an ovule free gynoecium, stamens with locules and undeveloped petals. During flower development stage 7, ovule primordial formation starts along with the development of male sporogenous tissue formation and later during Stage 9 the megagametophyte development commences and after this stage, the flower opens and ready for fertilization.

Here the result shows that during stage 6 flower development, *EBS* mRNA is weakly detected in the developing stamens and gynoecium tissue. But these might potentially also be a background stain since qRT-PCR result shows very low *EBS* expression during this stage. No expression was detected in the sepal and petal tissue. However during stage 7, strong presence of *EBS* mRNA is detected in the entire floral bud including vascular tissue connecting the stamen. Nevertheless, the expression becomes restricted to developed ovule during the stage 9 floral development. Within the ovules, strong expression was detected in the inner integument and nucellus region. This *in situ*

hybridization result is also supported by the qRT-PCR data that shows higher expression of *EBS* in the gynoecium, fruit and seed but not in stamens and petals. Expression in other floral organs and leaf remains very low compared to the expression in the female reproductive organ. Taking the *in situ* hybridization result, It is possible that expression in the fruit tissue arises possibly from the expression present in the developing seeds within the fruit since *EBS* mRNA is absent in the ovary wall. *EBS* expression declines in orderly fashion of unfertilized gynoecium, fruit and mature seed. This indicates that *EBS* is probably required mainly during those developmental stage that is close to fertilization in the ovule development and maintained at low level after fertilization.

Several researchers have carried out RNA *in situ* hybridization to investigate the B_{sister} gene spatio-temporal expression pattern using different species. *Petunia hybrida* is one of the closest eudicot species for *E. californica* with its B_{sister} gene (*FBP24*) functionally characterized. Expression pattern of *FBP24* is quite similar to what was observed in *E. californica*, where the *FBP24* mRNA is localized in the developing ovule specifically in the nucellus and inner integument and its expression decrease after fertilization took place [139]. Such expression pattern is also highly conserved in both gymnosperms and monocot species too. According to Becker et al. [131], weak expression of *GGM13*, *G. gnemon* B_{sister} gene, was detected when young female cones are investigated prior to the onset of ovule formation. Once the ovules are formed, *GGM13* expression becomes exclusively present in the nucellus, middle envelope, and inner envelope. Analyses of *ZMM17*, *Zea mays* B_{sister} genes also shows the presence of weak expression in all organ primordia of the female spikelet prior to ovule formation and the expression becomes exclusively present in the ovules and silk at late stage development [131]. Furthermore, *Triticum aestivum* B_{sister} gene (*WBsis*) is reported to be exclusively expressed in the inner integument and nucellus region of the ovule [157]. In *Arabidopsis*, even though there is no spatio-temporal expression analyses of *ABS* across different flower developmental stages, Mizzotti et al. [133] reported that *ABS* transcript is present in the inner integument of mature ovules. Taking all the expression data together, the expression pattern of B_{sister} genes, except for *GOA*, is highly conserved across species

from different lineages and the inner integument and the nucellus region are the main ovule parts where B_{sister} genes are highly expressed and known to function.

5.5 *EBS* Protein-Protein Interacting Partners Differs From Other Eudicots

In order to specify the identity of floral organs in angiosperms, MADS-domain proteins are known to function by forming a higher order protein complexes with one another [36]. Therefore understanding the protein-protein interaction partners of a given protein helps to gain insights with regards to its function and functional conservation.

In this study, it is reported that EBS protein interacts with different floral organ identity proteins that were not previously reported to interact with other angiosperms B_{sister} proteins. EBS strongly interact with B-class and C-class proteins and able to form a homodimer. Previous studies reported the ability of other B_{sister} proteins to form such protein complex with other floral organ proteins [139, 216]. However most of the interacting partners known from other angiosperms vary from what is observed in *E. californica*. *ABS* and *FBP24* proteins have similar protein interaction partners. They form high order protein complex with E-class and D-class proteins [139, 216]. Even though the higher order protein complex formation (tetramer) is not tested using EBS, a two-hybrid assay in yeast shows that EBS do not interact with candidate from E-class and D-class floral organ identity proteins in yeast system. Nevertheless, BiFC experiment result shows that EBS is able to form a protein dimer with EcSEP3 (an E-class protein) but not with the other proteins.

The only B_{sister} protein that share similar protein-protein interaction partner with EBS is the gymnosperm B_{sister} gene GGM13. GGM13 is reported to form a strong protein interaction with GGM2 (B-class) and GGM3 (C-class) MADS-domain proteins [217]. Taking this into consideration, the protein interaction between EBS and B-&C-class genes might be a facultative heterodimerization that might have originated from the obligate heterodimerization present in gymnosperms.

5.6 *EBS* Down-regulation and Gain-of-Function Indicates Its Role in Plant Development

The weak phenotype associated with *ABS* loss-of-function and the absences of any phenotype in *FBP24* loss-of-function mutant genotypes were the justifications for this

study to characterize another B_{sister} gene using a model basal eudicot species [138, 213] Since there are no available *EBS* mutant lines, the VIGS method was used to transiently infect seedlings and knock-down *EBS* transcripts. Out of 52 seedlings infiltrated with an *Agrobacterium* harboring the *pTRV2_EBS* and *pTRV1* constructs, only two plants were found to have a strong reduction of *EBS* transcripts (>75%). Efficiency of the VIGS method in this study was poor possibly due to:

(1) Weak induction of the *Agrobacterium* virulence gene prior to infiltrating the seedling and/or,

(2) The VIGS system may not be efficient to knock-down genes which are expressed in fewer amounts with in specific cells and are expressed at late stage of plant development; such as ovule formation.

In spite of that, developing seed from the two plants with strong down-regulation showed altered seed morphology in comparison to the wild type plants. Wild type developing seeds (20 DAP) have a round/oval shape where as those from VIGS #8 and VIGS #23 have a wrinkled and shrunken structure.

Though they differ in their mechanism of regulating ovule development, similar shrunken seed phenotype was reported in majority of B_{sister} loss-of-function studies done so far [130,137,139]. Down-regulation of canola B_{sister} causes flattened or wrinkled seed morphology due to aborted embryo and defective embryo respectively [137]. The absence of PCD in the nucellus and nucellar projection region caused by an indirect effect of down-regulated rice B_{sister} gene transcript, *OsMADS29*, was reported to be the reason behind the shrunken seed phenotype [130]. Down-regulation of *Petunia hybrida* B_{sister} gene using an over-expression/co-suppression method also was proved to alter the identity of seed endothelial layer that also led to altered seed morphology [139]. Therefore, the phenotype observed in *EBS* knock-down lines is consistent to what is described in plants with loss-of-function of several B_{sister} genes. In general regulation of plant fertility and seed morphology can be regarded as the common function of B_{sister} genes investigated so far indicating these might be the ancestral and conserved function of B_{sister} genes (Figure 20).

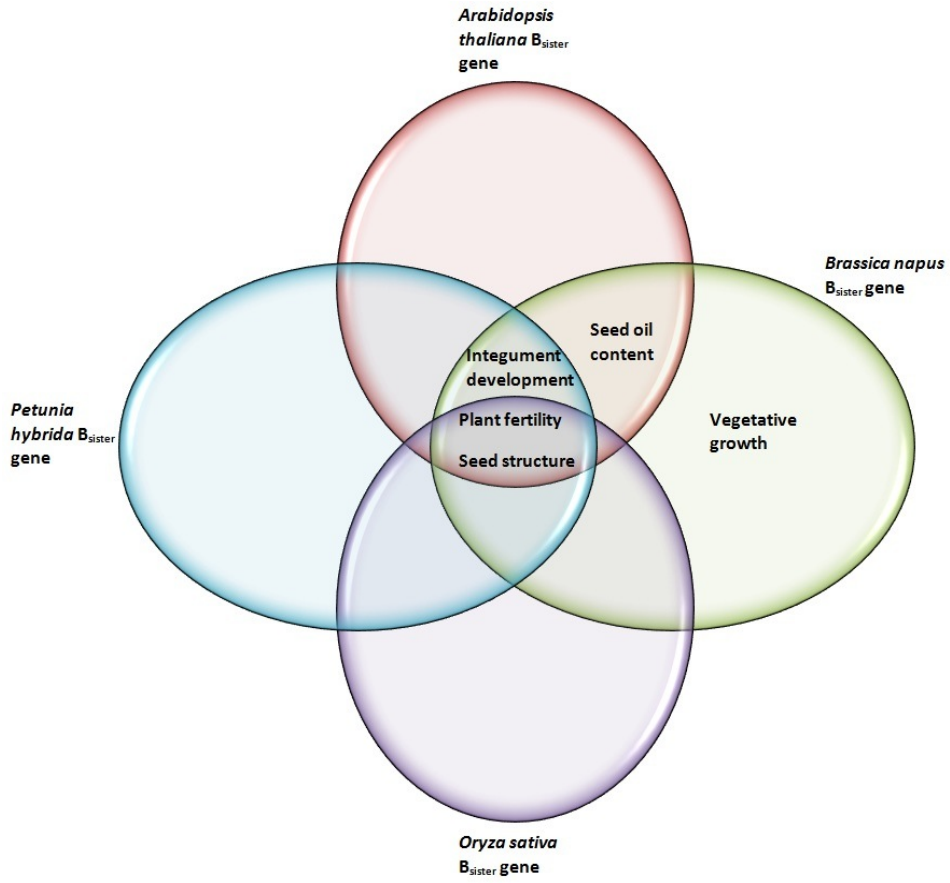

Figure 20: Functional relationship between B_{sister} genes characterized from four species. Down-regulation of B_{sister} gene is reported to affect seed structure and plant fertility in all species characterized [130,137,138,139].

In addition to *EBS* loss-of-function analyses, more information was obtained when heterologous *EBS* expression was carried out in *Arabidopsis*. Constitutive expression of *EBS* leads to a number of defects in the vegetative and reproductive organs development. Some of the independent lines showed more sever defect in their floral organ development. Even though there is no homeotic conversion of floral organs, absence of the petal, short stamens and reduction of the flower size were observed in transgenic lines. Such defects might have been a result of the interference caused by EBS on proteins required for proper development of the stamen and petal. As mentioned in section 5.4 of this dissertation, EBS is able to form a protein complex with B-class and C-class proteins. It might be plausible that EBS can also interact with similar class of proteins in *Arabidopsis*, implying it can competitively form a protein complex with proteins which otherwise are required for petal and stamen development. Similar floral defects were reported when *ABS* is constitutively expressed [216]. This indicates that the restriction of B_{sister} genes expression in the ovule is crucial to avoid

their interference in other floral organ identity formation and also avoid male sterility. Though *EBS* and *ABS* gain-of-function causes somewhat similar aberrant phenotypes, complementation assay of *EBS* in *abs* background was not able to restore the wild type pigmentation of mature seed indicating *EBS* do not activate or repress *ABS* target genes. Similar observation was reported when *FBP24* was expressed in *abs* background and fail to complement *ABS* despite both proteins interacts with similar class of floral homeotic genes [138].

5.7 *E. californica* is Amenable to Genetic Transformation

Stable genetic transformation protocol is one of the essential techniques required to efficiently use a given model species for several research purposes. *E. californica* is one of the frequently used plant species for different researches [34,83,218,219]. A couple of research work groups had published a transformation protocol for *E. californica* using different explants and *Agrobacterium* strains even though is difficult to reproduce the methods in our laboratory [175,176,220]. However, in this collaboration work, it was possible to regenerate transgenic *E. californica* plants by establishing a new protocol that uses developing seeds as explants. In this protocol, developing seed (22 DAP) are recommended to be a crucial stage to initiate a better somatic embryogenesis (Aravinda Yellina, Personal communication). Furthermore, this protocol provides the optimum concentration of BASTA that is sufficient for selecting transgenic *E. californica* calli. The stable integration of the transgene was verified by a PCR procedure targeting CaMV35S promoter and a qRT-PCR based expression analyses of *EBS*. Though the *EBS* transcript is significantly lower than the wild type plants, there was no observable alteration in the transgenic plants development compared to the wild type. In general this method is proven to be efficient and requires a short period of time, 4 month, to obtain a transgenic plant.

6. Conclusion and Outlook

The significance of *ABS* in *Arabidopsis* plant development has been questionable since the discovery of its weak phenotype. However, in recent works and in this study, several aspects of the phenotypes associated with *ABS* loss-of-function were reported. The revelation of these phenotypes indicates the importance of *ABS* in the seed development and makes a vital statement as to why the gene is still conserved for more than 350 million years. So far, investigation of *ABS* unfolded several developmental processes including:

1) Ovule inner integument development [132,133]

2) Plant fertility

3) Seed morphology

4) Seed germination, and

5) Silique development.

Based on these results, it can be concluded that, *ABS* is crucial for both plant fertility and germination of viable seeds. The poor fertility and sillique development result also shows the presence of direct relationship between the inner integument development and female fertility plus a direct relationships between fertilization and silique development. Furthermore majority of these key functions of *ABS* were identified by analyzing the *ABS* along with its protein interacting partners. The presence of severe and novel phenotypes in *abs shp1-1 shp2-1* triple mutants and regulation of different genes expression patterns show that *ABS*, *SHP1,* & *SHP2* act independently or together to direct proper expression of genes essential for ovule and seed development. In addition to the phenotypical descriptions and gene expression data, complete understanding of how *ABS* and *SHP1 SHP2* genes are acting in redundant manner in ovule/seed development requires further investigation using histological, molecular and genetic methods. Further analyses of the downstream target genes of *ABS*, *SHP1* and *SHP2* can give more insight regarding the ovule organogenesis. In addition to that, thorough investigation for presence of shared regulatory network among the

Arabidopsis TRANSPARENT TESTA genes helps to understand more of *Arabidopsis* ovule development. This is mainly because, similar phenotypes were found among them in regulating ovule integument development and seed pigmentation [221,222]. Especially, similar phenotype was reported in both *abs* and *tt1* mutant plants indicating these genes might act in similar regulatory pathways [132,223].

In this study, a closer look was carried out on *Eschscholzia californica* B_{sister} gene. The results show that, just like the majority B_{sister} genes described before, *EBS* is expressed strongly in specific tissues of the ovule, the nucellus and inner integument. Except for *GOA*, all B_{sister} genes investigated so far were found to be exclusively expressed in either of the two ovule tissues mentioned above [129,131,134,138,139]. Based on these data, it is conclusive that the B_{sister} genes have a specific role in ovule/seed development and this function has been conserved for more than 400 million years. Though selected floral homeotic proteins were used for the analyses of EBS protein-protein interaction partners, the protein interacting partners of EBS are different from what is known in other eudicot. Nevertheless, *E. californica* and *G. gnemon* B_{sister} proteins interact with similar proteins that indicate the protein interaction observed in *E. californica* might be a result of facultative heterodimerization, which might be more functionally relevant to the gymnosperms. In order to identify the list of potential and functionally relevant EBS protein interacting partners, a yeast based cDNA library screening and BiFC should be carried out and further investigation of the ability of EBS to form high order protein complexes similar to what is shown in *Arabidopsis* and *Petunia* can help to understand the evolution of B_{sister} proteins.

In this study, it was possible to show the down-regulation of *EBS* transcript using the VIGS methods. Even though the majority of the treated plants do not show any altered phenotype in comparison to the wild type, it was possible to see a sever amorphisim in the developing seed of two lines. This phenotype is consistent with what is reported in other B_{sister} mutants/knock-down genotypes [133,135,141,142]. Based on these observations, it is conclusive that B_{sister} genes are crucial in maintaining the seed morphology. Further investigation is required to understand the mechanism behind how *EBS* is involved in seed development using histological, molecular and genetic methods. Nevertheless, since *E. californica* is an obligate outcross species, homogenous wild type population is

a pre-requisite to avoid any ambiguity during morphological characterization of experimental plants. In addition, molecular interaction data and overlapping expression pattern with other MADS box genes should be taken into consideration to further characterize *EBS* and to find out if there are functions similar to what is observed in other species. Besides to knock-down experiments, heterologous expression of *EBS* in *Arabidopsis* thaliana was shown to cause altered plant development. Similar observation was documented in ectopic expression of other B_{sister} genes [132,139]. This suggests that, restricting the expression of the B_{sister} genes to specific tissues in the ovules is crucial to avoid the meddling caused by ectopic expression of B_{sister} genes. Additional characterization of shared regulator elements that are present in the promoter and intragenic regions of B_{sister} genes can give insight the regulatory pathways present in B_{sister} genes expression. In conclusion, this study also confirms the exclusive expression pattern of B_{sister} gene within the ovule tissue of a basal eudicot species and showed the importance of restricted pattern of expression for the sake of proper development of other floral organs.

Attempts were also made to knock-down *EBS* transcript using stable genetic transformation method. Optimized transformation protocol was developed in collaboration by using developing seeds (22 DAP) as an explants. Integration of the transgene was verified by PCR and qRT-PCR techniques indicating that somatic embryogenesis is possible in *E. californica* and provides an additional technique that help significantly for genetics experiments. In conclusion, this transformation protocol is efficient and requires much less time to regenerate a transgenic plant and it can be used as an alternative method to other transient methods.

7. References

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8. Appendix

8.1 Relative Expression of *EBS* in wild type and VIGS treated plants

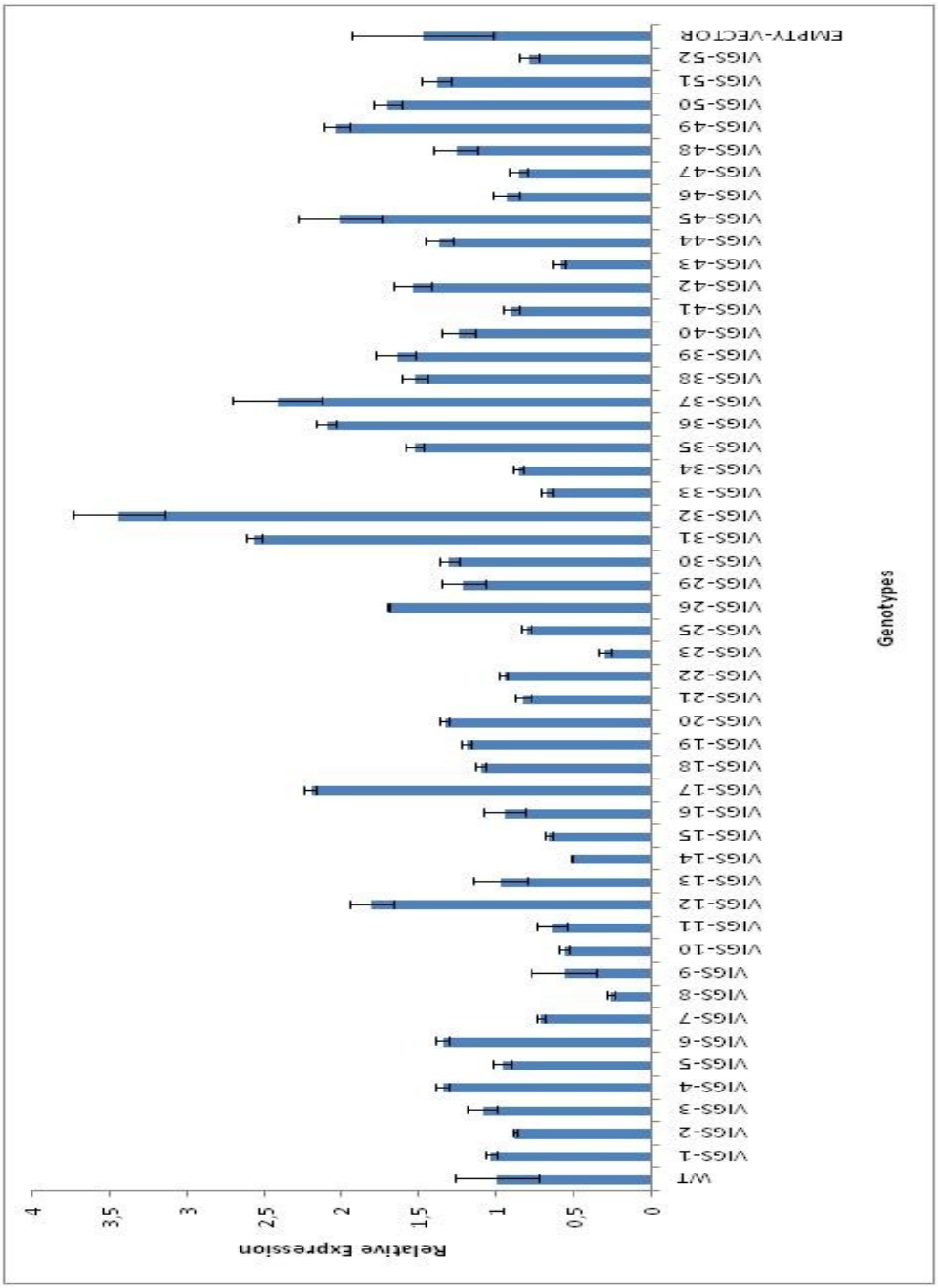

Figure 21. Relative expression analysis of wild-type and treated plants of VIGS experiment

8.2 List of Primers

Name	Sequence (5'-3')	Purpose
abs-1_Fw	GGATTTTCATTTGGCCCAGAAGTT	Genotyping
abs-1_Rev	GTCGCAATCCGTTGGTATGC	Genotyping
abs-1_TDNA	CAACCCTCAACTGGAAACGGGCCGGA	Genotyping
shp1-1_Fw	GATGCACTCGAAATCAGCCAATTTTAGAC	Genotyping
shp1-1_Rev	GTGACGGAAGGAGGGTTGACG	Genotyping
shp1-1_TDNA	GTCTACTGATGAGTTGTCACTAGG	Genotyping
shp2-1_Fw	GAGGATAGAGAACACTACGAATCGTC	Genotyping
shp2-1_Rev	CAGGTCAAGTCAATAGATTCCCTAC	Genotyping
goa1_Fw	TGAATCGGAACTTGAAAATGG	Genotyping
goa1_Rev	AGGATCAGTGTTCAGATTGCG	Genotyping
LB1.3	ATTTTGCCGATTTCGGAAC	Genotyping
agl16-1_Fw	CCATGCATTTTCGGTTTTATG	Genotyping
agl16-1_Rev	CTCTTCTTTTGCCTTTTGCAG	Genotyping
CaMV35S_Fw	CCCAGCAGTGATCCAGCCCC	Genotyping
CaMV35S_Rev	TCGCCAGTCTTCACGGCGAG	Genotyping
RTq-AtABS_Fw	CTCTGTGACGCTCACATCGG	RTq-PCR
RTq-AtABS_Rev	AATGAGTTGAGGCATCCTGTTCTG	RTq-PCR
RTq-AtSHP1-Fw	AAG AAT GAG CTG TTA GTG GCA GAG	RTq-PCR
RTq-AtSHP1-Rev	CTTCGGCTATCTTTGCTCGCAG	RTq-PCR
RTq-AtSHP2-Fw	GCCAACAACAGTGTGAGAGGA	RTq-PCR

Name	Sequence (5'-3')	Purpose
RTq-AtSHP2-Rev	AGTATTAGCTTCGGTGATGGTCGG	RTq-PCR
RTq-AtFUL_Fw	GTCTGGTTTGCTCAAGAAAGCTC	RTq-PCR
RTq-AtFUL_Fw	CTCTCCATGCAAGAGTCGGT	RTq-PCR
RTq-3G14460-Fw	GAGCATTTCCTAAGTGGTTGGGT	RTq-PCR
RTq-3G14460-Rev	GCAGCTACTGAGAGTGACGGA	RTq-PCR
RTq-1G53350-Fw	CTTACCTCGTTCAATGCCTGCT	RTq-PCR
RTq-1G53350-Rev	GTCACACTACCGTGTTTCGTTGAG	RTq-PCR
RTq-1G27170-Fw	GTTTGCGACAAGGACTCGAAGG	RTq-PCR
RTq-1G27170-Rev	TCCCACTTTCTCTGGTGTATTGCT	RTq-PCR
RTq-AtMOB1-Fw	CACCCTAGGAAGTGGGAACTTGAG	RTq-PCR
RTq-AtMOB1-Rev	AATCCACAGTGTTTACAGCAAGCC	RTq-PCR
RTq-AtWRI1-Fw	CACCACAACGGAAGATGGGA	RTq-PCR
RTq-AtWRI1-Rev	GCTTCCTCCTGCGTATTATAGGT	RTq-PCR
RTq-AtPCS1_Fw	GCCGCTATTGTACCGAGTCC	RTq-PCR
RTq-AtPCS1_Rev	TCACGTAAGCCTCCATTCCCA	RTq-PCR
RTq-AGL11-Fw	CCAACAGGAATCTGATGGGAGAC	RTq-PCR
RTq-AGL11-Rev	GCAACTCATGCTTCTTGGACCT	RTq-PCR
RTq-AtSSE1_Fw	GGGCTATATCGCTTTCTGTGGA	RTq-PCR
RTq-AtSSE1_Rev	TTTCGTCTCCTCAGCTCATCCT	RTq-PCR
RTq-AtSTR1_Fw	CTTTCTCAGTAGGTCACTCCACT	RTq-PCR
RTq-AtSTR1_Rev	TTTGTCTCAACTCCAGTAGAAGCC	RTq-PCR
RTq-AtPECT1_Fw	CACTGATATCGTTGGTCGTATGCT	RTq-PCR

Name	Sequence (5'-3')	Purpose
RTq-AtPECT1_Rev	CCCATGACTAAATTGCCTTTGCAG	RTq-PCR
RTq-AGL15_Fw	TCAGAGAGCGAAAGGAACGATTG	RTq-PCR
RTq-AGL15_Rev	TCTTGAACCTGTCTACGCAAGGT	RTq-PCR
RTq-At-Actin_Fw	AGTGGTCGTACAACCGGTATTGT	RTq-PCR
RTq-At-Actin_Re	GATGGCATGGAGGAAGAGAGAAAC	RTq-PCR
EF1_Fwd	TGAGCACGCTCTTCTTGCTTTCA	RTq-PCR
EF1_Rev	GGTGGTGGCATCCATCTTGTTAC	RTq-PCR
qRT-EBS-Fw	CCGCCACCCATCACCAAGTACC	RTq-PCR
qRT-EBS-Rev	TGAAGGTTAGGCTGAGTGGGCTGA	RTq-PCR
RTq-EcActinfw	AAG AGC TCG AAA CTG CCA AG	RTq-PCR
RTq-EcActinrev	CAT CGG GAA GCT CGT AAT TT	RTq-PCR
RTq-EcGAPDHfw	GCT TCC TTC AAC ATC ATT CC	RTq-PCR
RTq-EcGAPDHrev	AGT TGC CTT CTT CTC AAG TC	RTq-PCR
EBS_insitu_F	GCAACAACAATTGGACAACCTACGC	In situhybridization
EBS_insitu_R_T7	CTTAATACGACTCACTATAGGGGATT AAAGCTGCAAGGAATTG	In situhybridization
EBS_insitu_F_T7	CTTAATACGACTCACTATAGGGTG AGCTCATGCAACAACAAT	In situhybridization
EBS_insitu_R	GCTGCAAGGAATTGCCAGGAAAA	In situhybridization
EcAGinsitu_F	GCA ACC AAA CTG CGT CAA CAA ATC G	In situhybridization
EcAGinsitu R_T7	CTTAATACGACTCACTATAGGGTG TCTGCTCCTGGTGGGAGTAATG	In situhybridization
T7	TAATACGACTCACTATAGGG	Sequencing

Name	**Sequence (5'-3')**	**Purpose**
M13_Fw	GTAAAACGACGGCCAGT	Sequencing
pJET_Fw	CGACTCACTATAGGGAGAGCGGC	Sequencing
Y2H-BD-Rev	TTTTCGTTTTAAAACCTAAGAGTC	Sequencing Y2H vector
Y2H-AD-Rev	AGATGGTGCACGATGCACAG	Sequencing Y2H vector
pNBV_GENE-YC-Rev	GAGCTGTACAAGGCCGGT	Sequencing BiFC vector
pNBV_GENE-YN-Rev	TATCATGGCCGGTGGAG	Sequencing BiFC vector

Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all materials that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus-Liebig University of Giessen in carrying out the investigations described in the dissertation.