Sepallata genes and their role during floral organ formation

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The candidate confirms that the work submitted is her own and that appropriated credit has been given where reference has been made to the work of others.

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Abstract

Charles Darwin called the ability and success of angiosperms to colonise various parts of the earth even under unfavorable conditions an abominable mystery. This mystery is still not solved but one idea to explain the success of angiosperms is the development of the flower. *SEPALLATA* genes are common across angiosperms and play a major role in the development of all four floral organs and meristem determinacy. *SEP* genes occurred via Whole Genome Duplication (WGD) and are described as redundantly acting genes in the model organism *A. thaliana*. However, this study shows non-redundant functions affecting all floral organs for all four genes, especially under elevated growth conditions affecting the robustness and reproductive fitness of the plant, suggesting a diversification. *SEPALLATA4* in particularly has a specific role within this gene family based on its early expression pattern compared to the other *SEP*s and its effect on flowering time, floral meristem maintenance, floral organ identity and organ number. The identification of genome wide targets of SEP4 and expression analysis revealed a bi-functional role for this transcription factor during flower development. Comparison between SEP3 and SEP4 targets revealed a large number of common but also independent targets, indicating that flower development is regulated to a large degree redundantly but also has independent ways of regulation. This suggests that maintenance of multiple genes after a WGD event in angiosperms causes diversification in-between these genes and contributes to the robustness of the plant to environmental perturbations and has influenced their ability to radiate and occupy different ecological niches. This might be one explanation to explain the tremendous success of flowering plants.

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Abbreviations

Ac	*ACTIN2*
AG	*AGAMOUS*
AGL2	*AGAMOUS LIKE 2*
AGL3	*AGAMOUS LIKE 3*
AGL4	*AGAMOUS LIKE 4*
AGL9	*AGAMOUS LIKE 9*
AGL24	*AGAMOUS LIKE 24*
A. majus	*Antirrhinum majus*
AP1	*APETALA1*
AP2	*APETALA2*
AP3	*APETALA3*
ARR6	*A. thaliana* two-compound response regulator
A. thaliana	*Arabidopsis thaliana*
bp	base pairs
˚C	degree Celsius
ca	carpel

Abbreviations

CAL	*CAULIFLOWER*
cDNA	complementary DNA
ChIP	Chromatin Immuno precipitation
ChIP-SEQ	Chromatin Immuno Precipitation followed by deep Sequencing
Col	Columbia ecotype
CUC3	*CUP-SHAPED COTYLEDON 3*
DEF	*DEFICIENS*
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleic acid mix of A, T, G, C
E coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid,
et al.	et *alii* (and others)
EtBr	Ethidium Bromide
FAR	*FARINELLI*
FDR	False Discovery Rate

Abbreviations

Fig.	Figure
FLC	*FLOWERING LOCUS C*
FT	*FLOWERING LOCUS T*
FUL	*FRUITFULL*
gDNA	genomic DNA
Genta	Gentamycin
GFP	GREEN FLUORESCENT PROTEIN
GRN	Gene Regulatory Network
GLO	*GLOBOSA*
HSF	*HEAT SHOCK FACTOR*
LB	Luria-Bertani medium
LBD	*LATERAL ORGAN BOUNDARY*
LD	Long Day
LEUNIG	*LUG*
MS	Muashige and Skoog medium
PCR	Polymerase Chain Reaction

Abbreviations

PI	*PISTILLATA*
qPCR	quantitative Real Time Polymerase Chain Reaction
rep.	replicate
RNA	Ribonucleic acid
TAE	Tris-acetate- Ethylenediaminetetraacetic acid
TF	Transcription Factor
Tris-HCL	2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride
SAM	Shoot Apical Meristem
SEP 1-4	*SEPALLATA 1-4*
SHP	*SHATTERPROOF*
SOC1	*SUPPRESSOR OF CONS TA NS 1*
STK	*SEEDSTICK*
SVP	*SHORT VEGETATIVE PHASE*
WGD	Whole Genome Duplication
WT	Wild type

Chapter 1

1. Introduction

1.1 Flower development in A. thaliana is controlled by ABCE genes

The flower of the model organism *A. thaliana*, like many angiosperm flowers, consists of four organ types arranged in a series of concentric whorls. From outside to inside, four sepals are in whorl one and four petals are in whorl two. These sterile organs form the perianth. The third whorl contains six stamens that produce pollen, the male gametophyte. Two fused carpels arise in the fourth whorl and contain the ovules, which contain the female gametophyte. Later on, the fertilized carpels will give rise to the fruit. Flowers play a central role in the biology of seed plants as they produce the gametophytes and are responsible for pollination, fertilization and propagation by seed production (Kaufmann K. et al., 2005) . Flower development can be divided into four steps, the first is controlled by a large group of flowering time genes (Nilsson O. et al., 1998; Michaels S.D. and Amasino R.D., 1999) that regulate the switch from vegetative to reproductive growth in response to environmental (e. g. light, temperature, photoperiod) (Weigel D. and Meyerowitz E.M., 1993; Levy Y.Y. and Dean C., 1998) and endogenous (e.g. auxin, gibberellin) signals (Blázquez M.A. and Weigel D., 1999). These genetic pathways monitor the developmental stage as well as environmental conditions. In the second step, the convergence of those pathways results in the activation of floral integrators, a small subset of genes including *SUPRESSOR OF OVEREXPRESSION 1* (*SOC1*) (Moon J. et al., 2003; Lee J. and Lee I., 2010), *LEAFY* (*LFY*) (Blázquez M.A. and Weigel D., 2000) and *FLOWERING LOCUS T* (*FT)* (Kardailsky I et al., 1999; Samach A. et al., 2000). These genes in turn activate floral meristem identity genes, whose role it is to specify floral identity and, at the same time, to repress genes that maintain the vegetative phase. (Kaufmann K. et al., 2005). In the third step, floral meristem identity genes, like *APETALA 1* (*AP1*) (Gustafson-Brown C. et al., 1994) and *CAULIFLOWER* (*CAL*) (Kempin S.A. et al., 1995), control the transition from vegetative to inflorescence meristems identity. Inflorescence meristems produce floral meristems on their flanks, thereby activating the floral organ identity genes. In the final step, floral organ identity genes activate downstream genes that are necessary for the specification of the different cell

types and tissues that contribute to the development of the four floral whorls and organ types (Yanofsky M.F., 1995; Jack T., 2004). In the early 90s, studies on homeotic mutants of snapdragon (*Antirrhinum majus*) and *Arabidopsis. thaliana* (*A. thaliana*) led to the proposal of the original ABC model (Coen E.S. and Meyerowitz E.M., 1991; Meyerowitz E. M. et al., 1991). Schwarz-Sommer and colleagues proposed a slightly different model (Schwarz-Sommer Z. et al., 1990). The ABC model (Fig. 1a) proposes that three different classes of homeotic genes, named A, B and C (hereafter named A-function, B-function and C-function), have overlapping activities (Coen E.S. and Meyerowitz E.M., 1991). In the first whorl, the A-function specifies sepal identity, while the A- and B-function genes together control petal identity in the second whorl. The B- and C-function genes are required for stamen development in the third whorl, while the C-function alone specifies carpel identity in the inner whorl and terminates floral meristem activity. In addition, the A- and C-functions mutually repress each other's expression, enforcing the proper domains of activity. This explains why the C-function expands into the outer whorls in A-function mutants, causing the ectopic development of reproductive organs in the first two whorls. *APETALA1* (*AP1*) (Mandel M.A. and Yanofsky M.F., 1995) and *APETALA2* (*AP2)* (Okamuro J.K. et al., 1997) are the two A-function genes in *A. thaliana*. *AP2* is the only floral organ identity gene that does not belong to the MADS-Box transcription factor (TF) family, being a member of the AP2/EREBP (ethylene responsive element binding protein) transcription factor family (Okamuro J.K. et al., 1997). In *ap1* and *ap2* mutants, organs in the first two floral whorls fail to develop with the correct identity and develop instead as reproductive organs. Flowers of *ap2* mutants produce carpels in the first and stamens in the second whorls. In addition to organ identity defects, *ap1* mutants are affected in floral meristem identity too, resulting in a partial conversion of flowers to shoots and the development of secondary flowers. *PISTILLATA* (*PI)* (Goto K. and Meyerowitz E.M., 1994) and *APETALA3* (*AP3)* (Jack T. et al., 1992) are the B-function genes and *AGAMOUS* (*AG)* is the C-function gene (Yanofsky M.F. et al., 1990) in *A. thaliana*. Mutants of either of the B-function genes, *AP3* or *PI*, show a conversion of petals to sepals in the second whorl and stamens to carpels in the third whorl. In *ag* mutants, the third whorl develops as petals and the fourth whorl as sepals and this pattern can repeat itself many times, resulting in large, indeterminate flowers that consist only of perianth organs. At the beginning it was supposed, that the ABC genes are persistently expressed in the regions of the flower that show defects in the respective mutants. By the time it became clear that this is not true for all of them. The gene *AP1* has a dual function; firstly it specifies the identity of floral meristems and secondly determines sepal and petal development (Mandel M.A. et al., 1992). Thus, *AP1* RNA is expressed in young flower primordia when they initially arise on the flanks of inflorescence meristems and increases during the growth. Later on, during development, its expression is

restricted to the first and second whorl (Mandel M.A. et al., 1992). The expression of the A-function gene *AP2* is not limited to the first two whorls as predicted by the ABC model. Expression can be detected throughout all floral whorls and non-floral organs (Jofuku K.D. et al., 1994; Würschum T. et al., 2006). It has been proposed that *AP2* is expressed ubiquitously, but with transiently stronger accumulation in different organ primordia (Alvarez-Venegas R. et al., 2003) and it had been a question for a long time, how the broadly expressed *AP2* gene can repress *AG* expression in the perianth. The answer to this mystery was the discovery that *AP2* expression is regulated at the post-transcriptional level by microRNA (miRNA), miR172 (Rhoades M.W. et al., 2002; Aukerman M. and Sakai H., 2003; Chen X., 2004). The overproduction of miR172 disrupts the floral structure, which is similar to the phenotype observed in strong *ap2* mutants (Aukerman M. and Sakai H., 2003; Chen X., 2004) and overexpression of a miR172-resistant *AP2* gene under the control of its native promoter shows strong floral phenotypes, such as indeterminate flowers with numerous reproductive organs and enlarged floral meristems (Zhao L. et al., 2007). RNA of the B-function gene *PI* accumulates in the second, third and fourth whorl of developing flowers. This pattern of expression is unexpected, as it does not coincide with the domain of the *pi* mutant phenotype, which is in the second and third whorls. (Goto K. and Meyerowitz E.M., 1994). *AP3* RNA is detected in petals and stamens and in low levels in siliques (Jack T. et al., 1992). The gene *AG* is expressed in stamens and carpels (Yanofsky M.F. et al., 1990) as predicted by the ABC model. Although the ABC model nicely explained how floral organ development occurs, these three gene functions alone are not sufficient to produce flowers. Johann Wolfgang von Goethe proposed more than 200 years ago that flower organs are modified leaves (Goethe, 1790) and indeed he was right. Flowers of triple mutants lacking the ABC genes (*ap2-1 ap3-1 ag-1*, *ap2-1 pi-1 ag-1*, or *ap2-2 pi-1 ag*-1) show a conversion of all floral organs into leaf like structures (Bowman J.L. et al., 1991). However, attempts to convert vegetative leaves into each of the distinct floral organs by overexpressing ABC genes failed (Mizukami Y. and Ma H., 1992; Krizek B.A. and Meyerowitz E.M., 1996), suggesting that other flower-specific co-factors are needed to explain this conversion. The four *SEPALLATA* genes (*SEP1-4*), also called E-function genes are these missing co-factors. They received their name based on the finding that flowers of the s*ep1sep2sep3* triple mutant show a conversion of all floral organs into sepals and indeterminacy, whereas single and double mutants are indistinguishable from WT flowers (Pelaz S. et al., 2000). This phenotype reported in *sep1sep2sep3* triple mutants is similar to *ap3 ag* or *pi ag* double mutants (Bowman J.L. et al., 1991), but interestingly the expression of the three *SEP* genes is not affected in these double mutants. Expression of the B- and C-function genes is also not affected in the *sep1sep2sep3* triple mutant, suggesting that the *SEP* genes are not required for the initial activation of the B- and C-function genes (Pelaz S. et al., 2000).

Removing all four *SEP* genes in the *sep1sep2sep3sep4* quadruple mutant leads to indeterminate 'flowers' containing floral organs converted into leaf-like structures (Ditta G.S. et al., 2004), a phenotype similar to that seen in ABC triple mutants (Bowman J.L. et al., 1991). The observation that triple and quadruple mutants have dramatic phenotypes, whereas the single and double mutants are aphenotypic, led to the idea that *SEP* genes act redundantly. Furthermore these experiments showed that *SEP*s are needed for the development of all floral whorls and to impose floral determinacy (Pelaz S. et al., 2000; Ditta G.S. et al., 2004). The *SEP* genes have a similar temporal expression pattern during early and intermediate stages of flower development. *SEP1*, *SEP2* and *SEP4* are expressed in all four floral whorls whereas *SEP3* expression is restricted to the inner three whorls and cannot be detected in the first whorl (Ma H et al., 1991; Flanagan C.A. and Ma H., 1994; Huang H. et al., 1995; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Pelaz S. et al., 2000; Ditta G.S. et al., 2004). In addition *SEP4* is expressed in cauline leaves and is therefore not only restricted to the flower (Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G.S. et al., 2004). The finding that *SEP* genes are necessary for the development of all floral whorls led to an extension of the original ABC model. The *SEP* genes were included into this model as E-function genes and thus floral organ formation occurs by different combinations of these four gene functions (Fig. 1 b, 1 c). Sepal identity needs a combination of A- and E-function genes; petal identity is conferred by A-, B- and E-function genes. Stamens are made by a combination of B-, C- and E-function genes and finally C- and E-function genes confer the identity of carpels.

Fig. 1.1

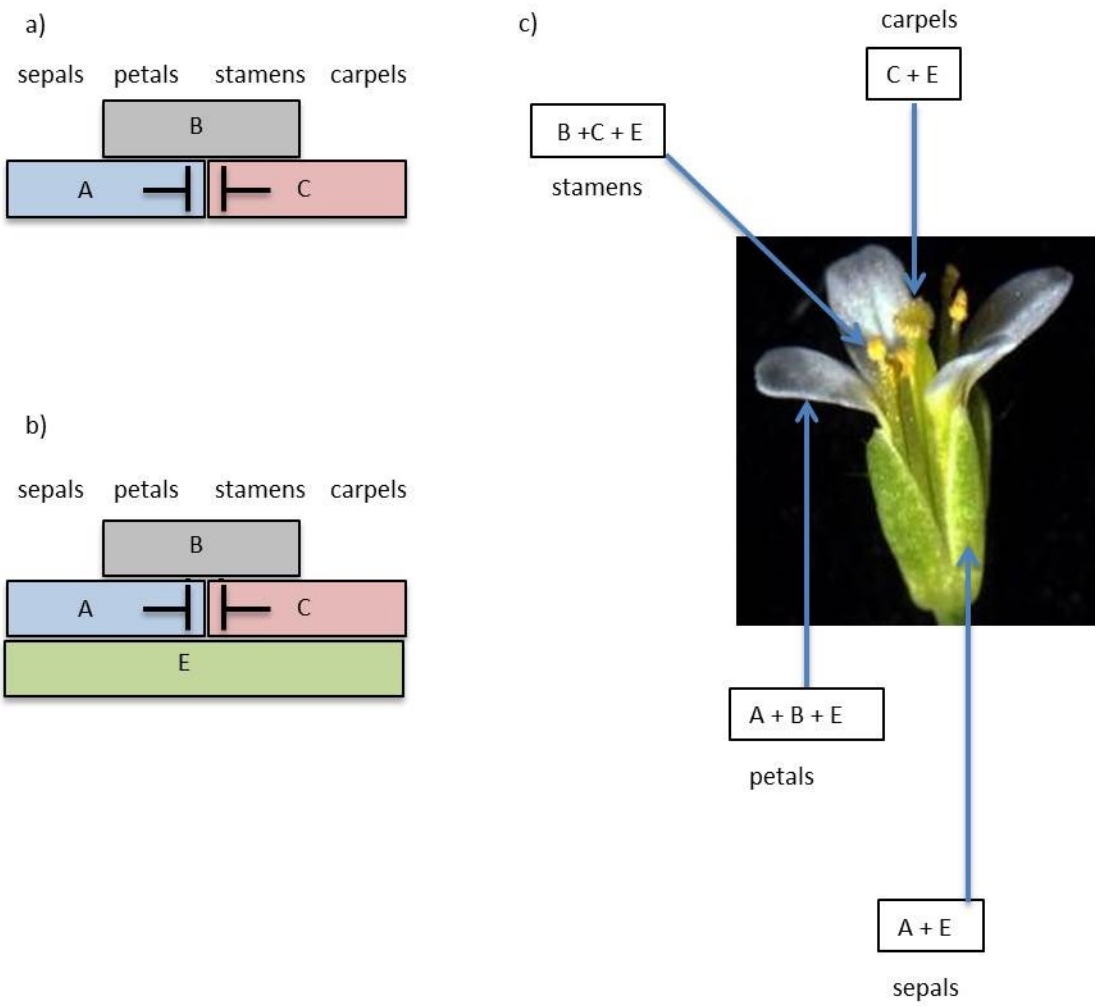


Figure 1.1: ABC and ABCE models for floral organ developmenta) graphical representation of the classical ABC model explaining how the four floral organs are regulated by a combination of ABC genes, note that A- and C-function genes are mutually repressive, A-function genes alone confer sepal identity, A- and B-function genes confer petal identity, B- and C-function genes are necessary for stamen identity and the C-function gene alone confers carpel identity, b) graphical representation of the extended ABCE model for the specification of floral organs, c) schematic representation of the ABCE model. Sepal identity is conferred by a combination of A- and E-function genes, while petal identity needs a combination of A-, B- and E-function genes. A combination of B-, C- and E-function genes makes stamens while carpels need a combination of C- and E-function genes

1.2 Problems with the original model led to a new (A)BC model

Although the original ABC model is widely accepted and provides a good framework to explain floral organ formation, the A-function genes raise questions. The original model was based on homeotic mutants in *A. thaliana* and *A. majus*, but the problem is that there is no true recessive A-function mutant identified for *A. majus* or any other species other than *Arabidopsis*. A mutant that showed the typical loss of A-function phenotype of carpel, stamen, stamen, carpel was discovered to be a dominant gain-of-function mutant of the C-function gene *PLENA* (*PLE)* in which *PLE* becomes ectopically expressed (Bradley D. et al., 1993). While B- and C-function genes are conserved across different plant species at a molecular level, no A-function analogous to that found in *A. thaliana* is encoded in species other than *A. thaliana* (Heijmans K. et al., 2012). Another deviation is the expression pattern of the A-function genes from the classical ABC model. According to that model, each homeotic gene should be expressed in two adjacent whorls to give floral organ identity. The A-function gene *AP2* has a broad expression pattern as it is expressed in all four whorls of the flower as well as in non-floral organs (Jofuku K.D. et al., 1994; Alvarez-Venegas R. et al., 2003; Würschum T. et al., 2006). Both *AP1* and *AP2* genes are involved in non-floral actions, *AP1* functions as a meristem identity gene and a floral organ identity gene reflecting its broad expression pattern. During the early stages of flower development, when floral meristems are determined, *AP1* is expressed throughout the floral primordium, while later when perianth organ identity is specified, expression becomes restricted to the first two whorls (Mandel M.A. et al., 1992; Gustafson-Brown C. et al., 1994). *AP2* has three roles during flower development, firstly to prevent *AG* expression in the perianth and confine reproductive organ identity to the inner whorls, secondly, confer perianth organ identity (Bowman J.L. et al., 1989; Smyth D.R. et al., 1990) and thirdly a role in ovule and seed coat development (Jofuku K.D. et al., 1994). Another deviation from the prediction of the original model is the control of the perianth organ identity. Strong *ap2* mutants can affect first and second whorl organs, producing carpeloid organs instead of sepals and stamenoid organs instead of petals, as predicted in the ABC model. However, *ap1* and *ap2* mutants can also show other phenotypes, including leaf or bract-like structures in the first and missing floral organs in the second whorl or the development of secondary flowers in the second whorl (Litt A., 2007). These fates cannot be explained by the ABC model and suggest a failure in the establishment of meristems. A-function genes are not absolutely necessary for petal development as evidenced by sepal and petal development in plants constitutively expressing *SEPALLATA3* in combination with *ap1* mutants (*ap1 35S:SEP3*) (Castillejo C. et al., 2005), or the double mutants *ag ap1* (Bowman J.L.

et al., 1993) and *agl24 ap1* (Yu H. et al., 2004). Taken together, there is little evidence for "true" A-function genes and their absolute requirement for the specification of petal identity. Thus Causier et al. (Causier B. et al., 2010) proposed a new (A) BC model (Fig. 1.2 a), where the (A)-function is necessary to enable B- and C-functions to exert their control to regulate floral organ identity. According to this model, the new (A)-function fulfils several roles, it establishes floral meristem identity, facilitates the production of sepals and activates and regulates B- and C-functions, resulting in the establishment of B- and C-function boundaries (Causier B. et al., 2010). The new (A)-function includes the E-function (Fig. 1.2 b, c) and has all requirements necessary to enable the B- and C-functions to exert their control over floral organ identity (Causier B. et al., 2010).

Fig. 1.2

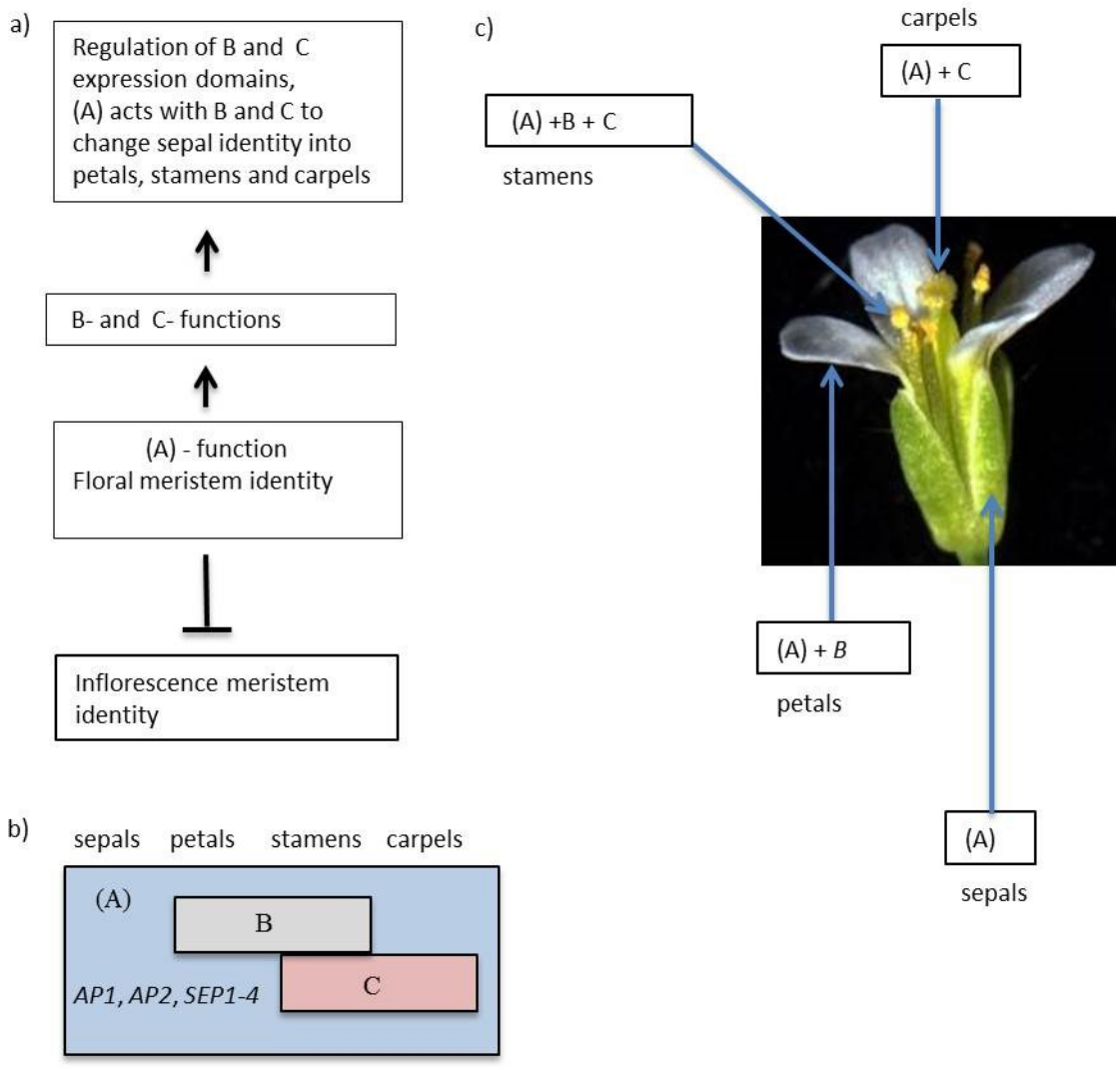


Fig. 1.2: The new (A)BC model of flower development

a) the new (A)-function represses inflorescence meristems and activates floral meristem identity to promote the floral ground state. At the time the floral ground state is established, the (A)- function induces expression of B- and C-function genes and restricts their function to specific domains The (A)-function acts with B- and C-functions to change the identity of the floral ground state, b) graphical representation of the (A)BC model where A-function includes the E-function to control floral organ identity, c) schematic representation of the (A)BC model explain floral organ development by a combination of the (A)BC genes

1.3 MADS-Box Genes

MADS-Box transcription factors can be found in all eukaryotes (Gramzow L. et al., 2010b). In *A. thaliana,* at least 107 MADS-Box genes have been identified (Parenicová L. et al., 2003) and this gene family belongs to one of the best studied transcription factors (TF) in *A. thaliana*. In contrast compared to *A. thaliana,* yeast, fruit flies and nematode genomes have a small number of MADS-Box genes, ranging from 1-4 (Theissen G. et al., 2000; Gramzow L. and Theissen G., 2010a). MADS-Box genes are involved in all stages of the life cycle of *A. thaliana*, for example the transition from the vegetative to reproductive phase, flower, fruit and seed development as well as root development. All ABCE floral organ identity genes except *AP2* belong to the MADS-Box TF family (Coen E.S. and Meyerowitz E.M., 1991; Kaufmann K. et al., 2005; Gramzow L. and Theissen G., 2010a). MADS-Box genes can therefore be characterised as the architects of flower development. The name MADS-Box was proposed by Schwarz-Sommer and colleagues (Schwarz-Sommer Z. et al., 1990; Yanofsky M.F. et al., 1990) and derives from the initials of the first four cloned genes of this kind. *Minichromosome Maintenance 1* (*MCM1*) factor in *Saccharomyces cerevisiae (*Passmore S. et al., 1988), *AGAMOUS* (*AG*) in *Arabidopsis* (Yanofsky M.F. et al., 1990)*, DEFICIENS* (*DEF*) in *Antirrhinum* (Sommer H. et al., 1990) and *Serum Response Factor* (*SRF*) in humans (Norman C. et al., 1988). Plants MADS-Box TFs can bind the DNA as homodimers, heterodimers or higher-order complexes. Dimers of MADS-domain transcription factors bind to the consensus sequence 5'-CC[A/T]$_6$GG-3' (or similar) called the CArG-box, to regulate transcription of target genes. All MADS- Box genes possess a highly conserved ~ 180 bp DNA sequence, the so-called MADS-Box (Fig. 1.3) that is required to encode the DNA-binding domain (Kaufmann K. et al., 2005). Furthermore, MADS-Box TFs can be divided into two types throughout the eukaryotes, Type I (Fig. 1.3 a) and Type II (Fig. 1.3 b). Both types differ in plants in a number of features. Type I MADS-Box genes are smaller and have usually one exon, whereas Type II genes contain five to eight exons (De Bodt S. et al., 2003). Three groups of type I genes are known, Mα, Mβ and Mγ, based on their phylogeny and the presence or absence of conserved carboxy-terminal regions (De Bodt S. et al., 2003; Parenicová L. et al., 2003). Type II MADS-Box genes are also called MICK-type genes, referring to the typical domain structure of the encoded proteins. Type II MADS-Box proteins reveal a structure composed of MADS (M), Intervening (I), Keratin-like (K), and a Carboxy-terminal (C) domain (Fig.1.3 b) (Becker A. and Theissen G., 2003). The characteristic DNA binding domain, the MADS-Box, is usually found in one exon, representing the amino-terminal domain of the protein (Parenicová L. et al., 2003), followed by the I, K and C domains that are less conserved (Parenicová L. et al., 2003). The I domain influences the specificity of DNA-binding dimer

formation and together with the MADS-domain, is often sufficient for the formation of DNA-binding dimers. The C domain is known to be important for the activation of transcription of target genes (Honma T. and Goto K., 2001). For Type II MADS-Box proteins, the K domain is, after the MADS domain, the best conserved and can be further divided in K1, K2 and K3. These subdomains are reported to form amphipathic α-helices that are predicted to form coiled coils and thereby mediate protein-protein interactions (Yang Y. et al., 2003). K1 is required for DNA-binding dimer formation. K1 and K2 together support the formation of DNA-binding dimers and K3 might contribute to multimerization (Kaufmann K. et al., 2005). However exceptions can be found as well. Recently it has been shown that the C domain is not required for multimerization and the K3 subdomain is essential, at least in the absence of the C domain (Melzer R. et al., 2009b). Type II MADS-Box genes are further subdivided into two groups, $MIKC^C$ and MIKC* (Fig. 1.3 b), distinguished by the number of exons that encode the I domain and structural differences in the K domain (Gramzow L. and Theissen G., 2010a) .Genes of the $MIKC^C$ group can be found in all land plants whereas MIKC* genes have been detected in moss only (Henschel K. et al., 2002). MIKC genes were duplicated during the whole-genome duplication that occurred early in angiosperm evolution, whereas many type I genes in Arabidopsis show signs of intrachromosomal duplication events happening gradually and more recent in evolution (Martinez-Castilla L.P., 2003). Compared to plant type II MADS-Box genes, plant type I MADS Box gene sequences have undergone a faster rate of birth and death during evolution. Therefore, early reports concluded that type I genes were probably of minor functional importance for plants in comparison to type II genes (De Bodt S. et al., 2003; Kofuji R. et al., 2003). Both types are involved in different developmental steps. Type I genes are more numerous (64 presumed functional and 37 nonfunctional type I genes and 43 presumed functional and 4 nonfunctional type II genes) (Nam J. et al., 2004). Type I genes are involved in plant reproduction (Masiero S. et al., 2011), whereas Type II genes are known to play a role in determination of meristem identity, reproductive development, organ identity, flowering time and root development (Ng M. and Yanofsky M.F. , 2001; Liu C. et al., 2009). Type II MADS-Box genes are distributed over all five chromosomes, whereas Type I genes are mainly found on chromosome I and V (Masiero S. et al., 2011).

Fig. 1.3

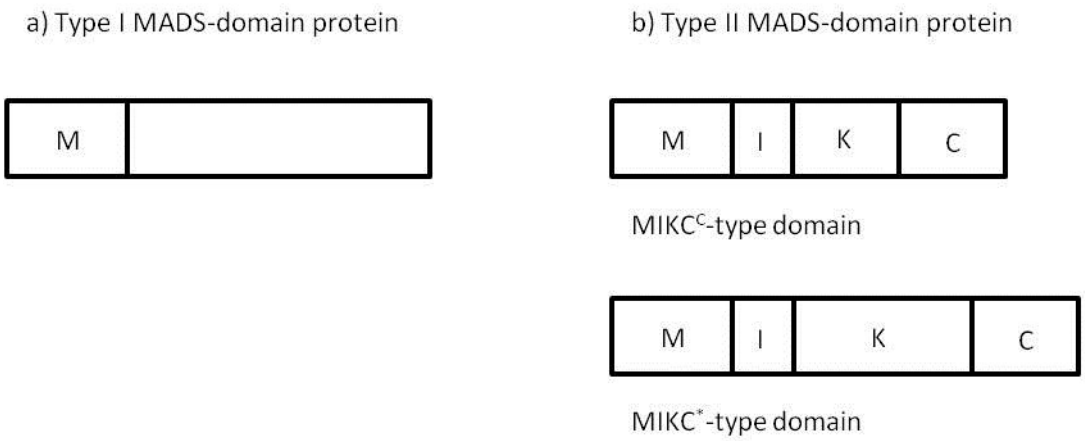


Fig. 1.3: Diagram of Type I and Type II MADS-domain proteins

a) Plant Type I MADS-Box transcription factors possess a conserved DNA binding domain, called MADS domain (M) and a long variable C-terminal domain, b) Plant type II MADS-Box transcription factors possess four domains, the DNA binding domain, MADS (M), the intervening (I), keratin like (K) and C-terminal (C) domain. Type II MADS-domain proteins can be further subdivided into MIKC* and MIKC^C. MIKC*-type proteins are longer than MIKC^C proteins due to their longer K domain.

1.4 The Floral quartet model

The ABCE model does not provide a molecular mechanism for the combinatorial interactions of floral homeotic genes during the specification of the floral organs. The introduction of the yeast two hybrid system, a method to study protein-protein interactions in the in the early 90s (Fields S. and Song O.K., 1989) shed more light on that question as it was shown by Egea-Cortines et al. that the *A. majus* orthologues of AP3, PI and AP1 (*DEF* and *GLO* are the B-function genes, *SQUA* is the *AP1* orthologue) form multimeric complexes in different assays (Egea-Cortines M. et al., 1999). The DNA-binding affinity of these complexes differs from those of individual dimers and thus Egea-Cortines et al. suggested a model in which the protein complex is a tetramer, composed of a DEF–GLO heterodimer and a SQUA–SQUA homodimer. Within this tetramer, the two dimers recognise different CArG-boxes (Egea-Cortines M. et al., 1999). Together with the finding that ABC genes alone are not sufficient to specify floral organs, but need the *SEPALLATA* genes (Pelaz S. et al., 2000), this led to the postulation of the "floral quartet model" (Fig. 1.4 a) (Theissen G. and Saedler H., 2001). This model tries to explain how different combinations of floral proteins (the products of ABCE genes) specify the identity of the floral organs. According to the "floral quartet model", two dimers of floral homeotic proteins bind to two nearby *cis*-regulatory DNA elements and interact with each other by looping the intervening DNA. Four different combinations of four different floral homeotic proteins determine the identity of the different floral organs. For each floral organ, a unique combination of different protein complexes is needed. For the sepals these are AP1-AP1-SEP-SEP, for the petals AP1–AP3–PI–SEP, for stamens AP3–PI–AG–SEP and for the carpels AG–AG–SEP–SEP. These complexes are assumed to bind to promoters of their target genes to activate or repress them as appropriate for the development of the floral organ. The transcriptional activation ability is maintained by the recruitment of AP1 or SEP (particularly SEP3) proteins, as these are the only proteins so far shown to add transcriptional activation to quartet complexes (Honma T. and Goto K., 2001). Specifity of target selection could be achieved by the four complexes having different binding affinities for CArG-boxes and by the characteristic distribution of these pairs of sequence elements in the promoter regions of the different target genes. The reported mutual antagonism between *AG* and *AP1* in the ABC model could be explained by a repression of *AG* by protein complexes that contain AP1 protein complexes. (Theissen G., 2001). Biochemical experiments have shown that the two B-function proteins PI and AP3 only weakly bind DNA, even under favourable conditions (when suitable CArG boxes are present in the right distance), whereas the same dimer together with SEP3 co-operatively binds DNA much quicker, underscoring the ability of SEP proteins (in

particular SEP3) to add transcriptional activation to these complexes (Melzer R. and Theissen G., 2009a; Melzer R. et al., 2009b). This co-operative binding involves DNA looping and largely depends on the K3 domain of the SEP3 protein, a domain suggested to be important for protein–protein interactions (Kaufmann K. et al., 2005). The strength of tetramer formation is strong in the SEP3-SEP3/AP3-PI heterotetramers, less strong in SEP3 homotetramers and weak in AP3-PI/AP3-PI heterotetramers (Melzer R. and Theissen G., 2009a). This finding is in agreement with the idea that SEP proteins are important components of protein complexes, interacting with other MADS domain proteins, depending on their temporal order of availability. For a long time the quartet model was based on *in vitro* experiments and only recently the first *in vivo* studies have shown that the MADS domain proteins AP1, AP3, PI, AG, SEP3 can interact in floral tissues as proposed in the quartet model as well as the reconstitution of these core complexes of floral MADS domain proteins (Smaczniak C. et al., 2012). In addition, other TF family members were identified as components of the MADS domain protein complexes, for example Homeobox TFs and nucleosome remodelling factors (Smaczniak C. et al., 2012). Thus, ABCE proteins act as part of larger complexes with other non-MADS-Box proteins to regulate floral organ identity. These findings show that the protein complexes are bigger than expected in the original “floral quartet model”. Another deviation from the “original” model is the role for the A-function. Based on the model, a combination of A, B- and E -function proteins (AP1–AP3–PI–SEP) form a quartet complex to regulate petal identity. However, biological experiments have shown that petal development is possible in plants constitutively expressing *SEP3* in combination with *ap1* mutants (Castillejo C. et al., 2005) or in *ap1 ag* (Bowman J.L. et al., 1993) or *ap1 agl24* double mutants (Yu H. et al., 2004), making the AP1 protein unnecessary for petal specification. Subsequently, the original ABCE model changed into the (A)BC model based on the finding that there is no true “A”-function. According to this, the new (A)- function includes the E-function to provide the context in which the B- and C-functions are active and regulate their expression (Causier B. et al., 2010). Therefore it would be suitable to change the floral quartet model in a similar way, where (A)-function proteins include the former A- and E-function proteins (Fig. 1.4 b).

Fig. 1.4

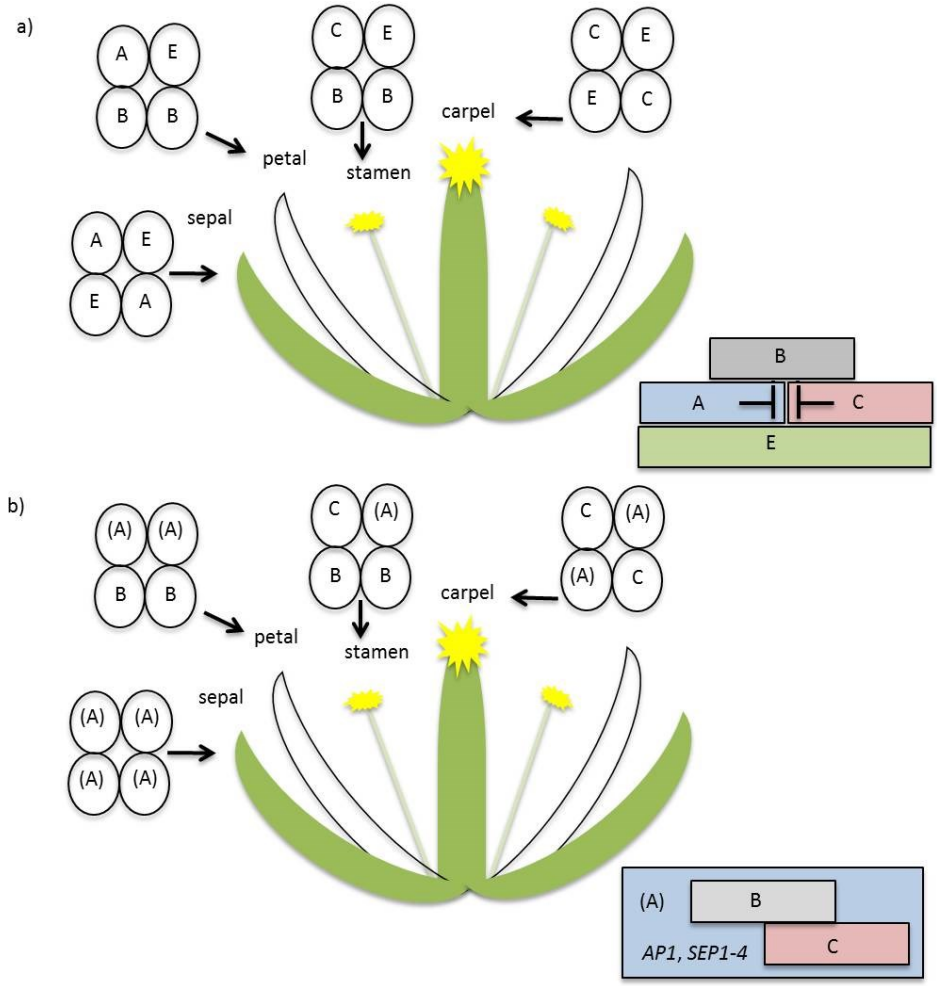


Fig. 1.4: The floral quartet model

a)ABCE MADS-Box protein complexes (shown as circles) are sufficient for the specification of each floral organ. A combination of A- and E-function proteins regulates sepal identity, petal identify is conferred by a complex of A-, B-, and E -function proteins, stamen are specified by B, C and E proteins and the carpels are made by a complex of C- and E-function proteins. Rectangles represent the A (*AP1,*), B (*AP3, PI*), C (*AG*) and E (*SEP1-4*) -function genes, b) floral quartet model based on the new (A)BC model, where the new (A)-function proteins include A- and E-function proteins, thus sepal identity is conferred by (A)-function proteins, petals identity by (A)- and B-function proteins, stamens are made by (A)-, B- and C-function protein and the carpel identity is conferred by (A)- and C-function proteins, Rectangles represent the (A) (*AP1,SEP1-4*), B (*AP3, PI*) and C (*AG*)-function genes.

1.5 *SEP* genes across angiosperms

SEP genes form a separate subfamily in the MADS-Box gene family (Becker A. and Theissen G., 2003), present in all major angiosperm lineages (Fig. 1.5). Multiple members of this gene family are found in angiosperm species, including basal angiosperms (the base or root of all angiosperms), eudicots and monocots, but *SEP*s are not present in gymnosperms (Becker A. and Theissen G., 2003; Malcomber S.T. and Kellogg E.A., 2005; Zahn L.M. et al., 2005). The co-occurence of many duplicated genes, including MADS-Box subfamilies near the origin of the angiosperms suggests that these duplicates arose via Whole Genome Duplication WGD (Zahn L.M. et al., 2005). *SEP* genes originated prior to the origin of extant angiosperms, as shown with the identification of two Amborella *SEP* genes (Zahn L.M. et al., 2005). Amborella represents a species that diverged early from other angiosperms and is placed at the base of the flowering plant lineage (Soltis P. and Soltis D., 2013). The branching-point for Amborella is situated 'between' gymnosperms and all other angiosperms, making this plant as an interesting candidate to study developmental processes that distinguish these two seed plant lineages (Soltis D.E et al., 2008). An early gene duplication event occurred before the origin of extant angiosperms which produced the *SEP3* (also called *AGL9* clade) and *LOFSEP* clade (also called *AGL2/3/4* clade) (Fig. 1.6) (Zahn L.M. et al., 2005). Duplications within the *LOFSEP* clade near or at the base of core eudicots produced the *SEP1/2*, *FBP 9/23* and *SEP4* clades. The *FBP9*/*23*- clade is not present in *A. thaliana*, thus most available functional information of this clade is derived from studies in Solanaceae (Malcomber S.T. and Kellogg E.A., 2005; Zahn L.M. et al., 2005; Liu D. et al., 2013). *SEP1* and *SEP2* were reportedly formed as a result of a recent genome duplication (Ermolaeva M.D. et al., 2003; Moore R.C. et al., 2005). *SEP1* and *SEP2* are closely related to *SEP4* (Becker A. and Theissen G., 2003; Parenicová L. et al., 2003; Vandenbussche M. et al., 2003). The *SEP* gene family shows a large diversity within angiosperms. Although *SEP* genes are generally similar in specifying meristem and floral organ identity, the number of these genes as well as their developmental and biochemical function varies across different plant species. Reported as being redundant in *A. thaliana*, *SEP*s in other plants have non-redundant roles as reported for the *Gerbera ASTERACEAE SEP3* gene (*GhGRCD1*) affecting stamenoide identity (Kotilainen M. et al., 2000), fruit maturation in tomato (*LeMADSRIN*) (Vrebalov J. et al., 2002) and plant architecture in petunia (petunia *FBP9/23* gene) (Immink R.G. et al., 2003) (Table 1.1). Some examples will be described to show the diversity of this gene family and their functions during plant development in different species.

Fig. 1.5

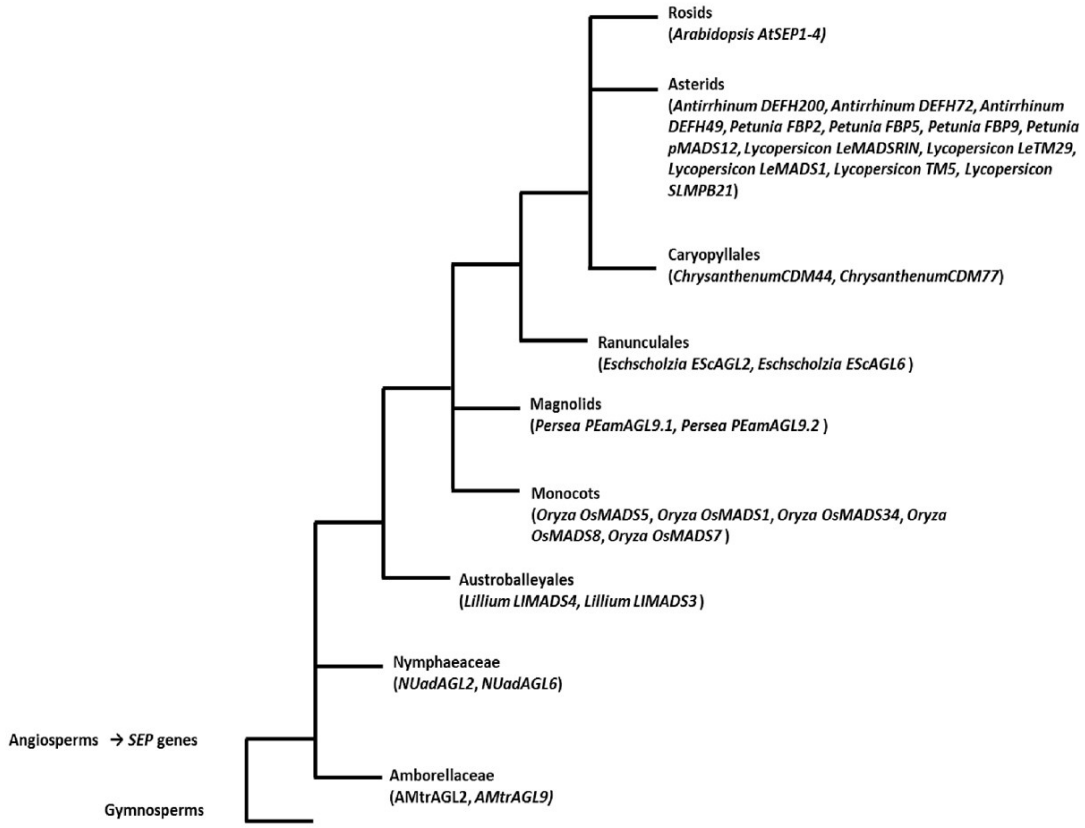


Fig. 1.5: **Simplified tree of *SEPALLATA* genes in Angiosperms**

Multiple *SEP* genes are present in all angiosperms but have not been detected in gymnosperms.

Tree modified after Zahn et. al (Zahn L. et. al, 2005)

Fig. 1.6:

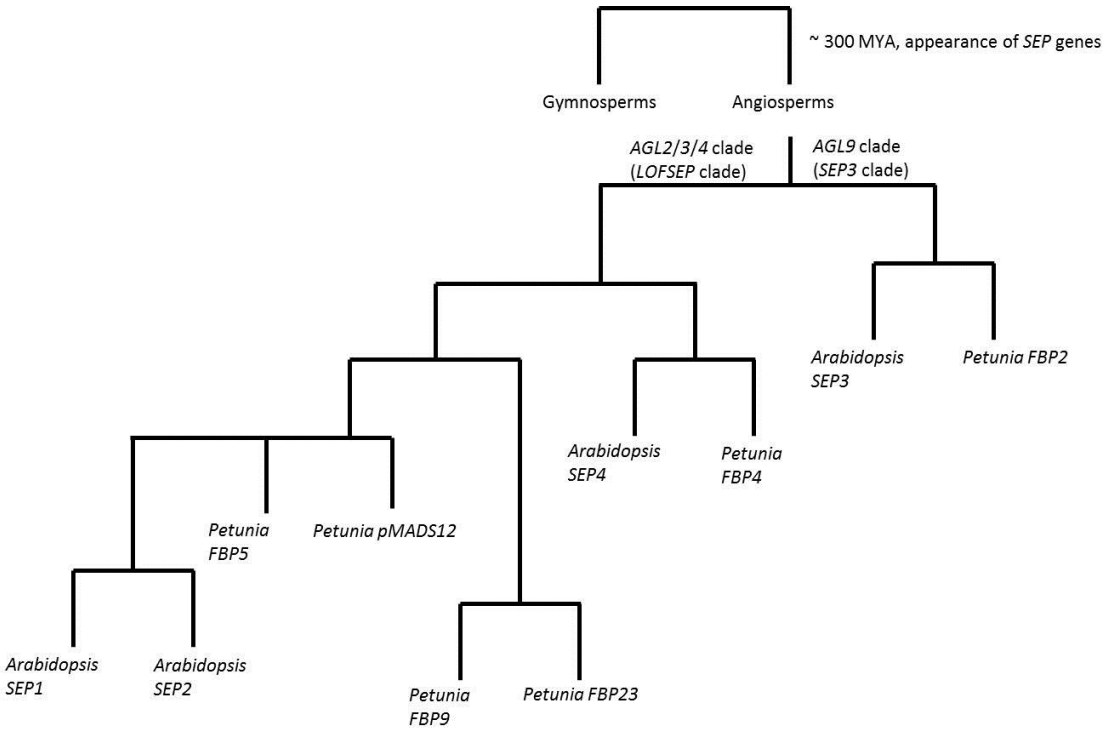


Fig. 1.6: **Gene duplication events in *Arabidopsis* and *Petunia SEPALLATA* genes**

SEP genes have experienced several gene duplication events; here shown for the two plant species *Arabidopsis thaliana* and *Petunia*, the first produced the *AGL2/3/4* (*LOFSEP* clade) and *AGL9* clade (*SEP3* clade). Within these clade, further duplication events occurred and produced the *SEP3* and *FBP2* clade and for the *LOFSEP* clade, the *SEP4*/*FBP4*, *FBP5* and *pMADS12*, *FBP9* and *FBP23* and *SEP1* and *SEP2* clade

MYA = Million Years Ago, tree modified from Ruelens et. al. (Ruelens P. et al., 2013)

1.51 *Arabidopsis thaliana*

The four *SEPALLATA* genes *SEP1-4* (previously known as *AGL2, AGL4, AGL9* and *AGL3*, also called E-class genes) are expressed before the onset of B and C class gene expression (Ma H. et al.; 1991, Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Pelaz S. et al., 2000; Ditta G.S. et al., 2004). *SEP1, SEP2, SEP4* are expressed in all floral whorls, while the expression of SEP3 starts slightly later and is restricted to the inner three whorls (Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Pelaz S. et al., 2000; Ditta G.S. et al., 2004). Later during development, *SEP1*, *SEP2*and *SEP4* remain expressed in all four whorls; *SEP3* is still expressed in the three inner whorls while *SEP4* expression is high in the central dome of whorl four and less strong in the first whorl. All *SEP*s are expressed in the developing ovules (Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G.S. et al., 2004). In addition, *SEP4* is expressed in vegetative tissue; the cauline leaves (Ma H et al., 1991; Huang H. et al., 1995, Ditta G.S. et al., 2004). Single *sep* mutants were described as indistinguishable from WT whereas flowers of triple and quadruple mutants display severe phenotypes (Pelaz S. et al., 2000; Ditta G.S. et al., 2004). These observations lead to the idea that the E-function acts redundantly in *A. thaliana*. Flowers of the *sep1sep2sep3* triple mutant are composed only of sepals and show indeterminacy in the fourth whorl (Pelaz S. et al., 2000), whereas floral organ identity is completely abolished in the *sep1sep2sep3sep4* quadruple mutant, revealing indeterminate floral structures composed only of leaf like organs (Ditta G.S. et al., 2004). The quadruple mutant resembles the phenotype of triple mutants lacking all three components of the ABC-function (Meyerowitz E.M. et al., 1991), suggesting that the ABC floral organ identity genes need *SEP* genes to specify all floral whorls. Although described as a redundantly acting gene in *A. thaliana*, more recently a phenotype has been described for *sep3* mutants, with a partial conversion of petals into sepals or occasionally a phenotype with axillary flowers that develop at the base of the sepals (Pelaz S. et al., 2001b). In contrast, over-expression of *SEP3* causes early flowering (Kaufmann K. et al., 2009). Yeast two hybrid assays and in vitro studies revealed that SEP3 has the ability to mediate the interaction between AP1 and AP3/PI or PI/AP3 and AG (Fan H.Y. et al., 1997; Honma T. and Goto K., 2001) in addition to its role in activating AP3 and AG (Castillejo C. et al., 2005). Simultaneous ectopic expression of *AP1*, *AP3, PI* and *SEP3* leads to a conversion of vegetative leaves into petaloid structures (Honma T. and Goto K., 2001; Pelaz S. et al., 2001a) and ectopic expression of AP3, AG, PI and SEP3 leads to the conversion of leaves into stamenoid structures (Honma T. and Goto K., 2001). These data suggest that floral homeotic MADS domain and SEP proteins work together to confer the identity of petals and stamens. In addition to the development of all four floral whorls, SEPs are involved in ovule development as shown in yeast-two and three- hybrid assays (Favaro R. et al., 2003; Pinyopich A. et al., 2003). SEP proteins are necessary to form

transcription factor complexes that control ovule development. *SEEDSTICK* (*STK*), *AG*, *SHATTERPROOF 1* (*SHP1*), *SHATTERPROOF 2* (*SHP2*) and *SEP3* are all expressed during ovule development (Favaro R. et al., 2003). Yeast two hybrid experiments showed that STK cannot interact with AG, SHP1 or SHP2, since none of these proteins could form hetero or homodimers. However, yeast three hybrid experiments revealed that ternary complexes were formed using all possible combinations of SHP1, SHP2, STK, and AG as long as SEP3 was added as a mediator (Favaro R. et al., 2003).

1.52 *Petunia*

The *SEP* like genes in petunia are *FBP2* (Angenent G.C. et al., 1994), *FBP4, FBP5, FBP9, FBP23* (Immink R.G. et al., 2003) and *pMADS12* (Ferrario S. et al., 2003), but only phenotypes for *FBP2* and *FBP5* and *FBP9* have been reported so far. Unlike the above-described *A. thaliana SEP*s, mutations in single *SEP* genes in petunia reveal changes in floral organ development. *FBP2*, the petunia *SEP3* orthologue is expressed in the inner three whorls of the flower like *SEP3* (Ferrario S. et al., 2003). Cosuppression of the *FBP2* gene causes alterations in whorls 2, 3, and 4 with a smaller, green corolla (petals), stamens transformed into petaloid structures, incompletely fused carpels that lack ovules and inflorescences with floral buds that develop in the axils of the fourth whorl. Additionally, the tips of the second and third whorl organs develop trichomes on both sides, normally seen on sepals and leaves (Angenent G. et al., 1994). This phenotype is similar to the *sep1sep2sep3* triple mutant in *A. thaliana* (Pelaz S. et al., 2000). *35S::FBP2* can complement the s*ep1sep2sep3* mutant phenotype in *A. thaliana* (Ferrario S. et al., 2003). Vandenbussche et al. (Vandenbussche M. et al., 2003) used insertion lines and reported similar phenotypes for *fbp2* mutants, with petals having an overall diffuse green hue and secondary inflorescences in the third whorl, a conversion of petal epidermal cells into sepal-like cells and trichomes on both sides of the petals that are usually on sepals and leaves. Flowers of *fbp5* mutants are affected in female fertility, whereas the *fbp2fbp5* double mutants show an increased conversion of petals into sepals and sepal like structures on the top of the anthers that are covered with trichomes. The number of carpels is increased (three instead of two) and remains unfused. Inside, the development of leaf-like organs is observed at positions normally occupied by ovules and thus it can be inferred that both *FBP2* and *FBP5* are involved in ovule development (Vandenbussche M. et al., 2003) . *fbp9-1* mutants display aberrations in plant architecture during the reproductive phase (Vandenbussche M. et al., 2003).

Biochemical experiments, such as the use of yeast two hybrid assays, revealed similarities and differences in protein-protein interactions. For example, it has been shown that FBP2, FBP5 and FBP9 interact in a similar way with the petunia C-class proteins FBP6 and pMADS3

(Ferrario S. et al., 2003). However selectivity for interactions have been shown for individual SEP proteins for example, FBP9 does not interact with FBP7 and pMADS12 interacts with the C- class protein FBP6 but not with pMADS3, whereas pMADS12 interacts with the D-class protein FBP7 but not with FBP11 (Ferrario S. et al., 2003; Immink R.G. et al., 2003).

As these examples show, *SEPALLATA* genes in petunia have non-redundant functions, as seen in phenotypes for *fbp2* and *fbp5* single mutants as well as different protein-protein interaction partners, shown by yeast two- and three-hybrid experiments.

1.53 *Antirrhinum majus*

DEFH200, *DEFH72* and *DEFH49* belong to the *SEP* clade in Antirrhinum. The first two genes are expressed in all floral organs, having the highest expression levels in petals and carpels whereas *DEFH49* is mainly expressed in carpels (Davies B. et al., 1996). Yeast two-hybrid analysis has shown that the loss of the MADS-Box and the 5'-UTR region does not affect the ability to form heterodimers but deletion of the K-Box results in a loss of heterodimerization. The C-terminal domain of the MADS-Box has a function in stabilisation of the formed heterodimers (Davies B. et al., 1996). It is worth noting, that the genome of Antirrhinum is not yet sequenced and there are presumably more *SEP* like genes in this model organism than the three described above.

1.54 *Gerbera hybridia*

Flowers of the plant Gerbera (Gerbera hybridia) display a different architecture to *A. thaliana* and petunia. Gerbera plants have an indeterminate inflorescence, although they produce a fixed number of flowers. The inflorescence meristem is consumed and replaced by floral meristems leading to a terminal symmetrical flower in the centre of the inflorescences (Harris E. M. , 1995; Teeri T.H. et al., 2006). The inflorescence is dense (known as the capitulum) and bears individual flowers playing different roles. Marginal (ray) flowers have an extended fused corolla (fused petals) and are female only. Stamen development starts in this flower type normally, but progresses slowly and stamens abort before the flower opens, thus these structures are called sterile staminoides. Central flowers (disc flowers) contain male and female organs. The main role of central flowers is to produce pollen in the inflorescence (capitulum). The third flower type is called "trans" and similar to the ray type (only female organs) but has a smaller corolla (Teeri T.H. et al., 2006).

Gerbera has two *SEP* like genes, *Gerbera Regulator of Capitulum Development 1* (*GhGRCD1*) and *Gerbera Regulator of Capitulum Development 2* (*GhGRCD2*). *GRCD1*, a *SEP3* homologue is involved in stamen identity. Transgenic plants in which *GhGRCD1* expression is down-regulated by introducing a nearly full-length *GhGRCD1* cDNA (20 nucleotides missing at the 5‘ end) in the antisense orientation under the control of the cauliflower mosaic virus 35S promoter, reveal homeotic changes of whorl 3 organs. Female ray flowers normally bear sterile staminodes, whereas transgenic *GhGRCD1* lines develop petals at that position (Kotilainen M. et al., 2000). Down-regulation of *GRCD2* leads to a loss of carpel identity, where the innermost floral organs reveal a mixture of carpel, petal and sepal cells. The inflorescence is affected as seen with bracts, floral primordia and altered flowers growing at the position where ovules are normally formed. Floral meristem identity is affected in these mutants, as they reveal a phenotype with meristems reverting back to inflorescence identity. (Uimari A. et al., 2004). The inflorescence in down-regulated *GhGRCD2* transgenic plants is affected, as plants produce more flowers than WT and the inflorescence meristems remain undifferentiated until flower production ceases due to senescence (Uimari A. et al., 2004). As described above for Antirrhinum, the genome of Gerbera is not yet sequenced and there are possibly more than two *SEP*-like genes. Nevertheless the two known genes have non-redundant functions, since genetic experiments show *GhGRCD1* and *GhGRCD2* affecting different floral organs.

1.55 *Lycopersicon esculentum*

Five *SEP* genes have been identified in tomato to date, *TM5*, *TM29*, *RIN*, *LeMADS1* and *SLMBP21*, and all appear to have unique functions. The tomato *MADS Box gene 5* (*TM5*), the homolog of the *A. thaliana SEP3* gene, is expressed in the inner three whorls (Pnueli L. et al., 1994), whereas *TM29* shows similar expression profiles to *SEP1* and *SEP2,* with expression in all four floral whorls. In addition, *TM29* is expressed in inflorescences and vegetative meristems. (Ampomah-Dwamena C. et al., 2002). Down-regulation of *TM5* causes a conversion of floral organs into sepaloid structures in the three inner whorls and a loss of floral determinacy in the center of the flower (Pnueli L. et al., 1994). Transgenic plants that either co-suppress *TM29* or antisense *TM29* lines show a partial conversion of petals and stamens into sepaloid structures and ovaries that later develop into parthenocarpic fruits with secondary flowers emerging from the fruits in down-regulated plants. The *TM29* phenotype affects the inner three whorls similar to the one observed in antisense *TM5* transgenic flowers (Pnueli L. et al., 1994) and both phenotypes resemble the *sep1sep2sep3* triple mutant phenotype (Pelaz S. et al., 2000). In contrast *LeMADS-RIN* (*ripening inhibitor*), the *A. thaliana SEPALLATA4* homolog exclusively accumulates in the fruit during ripening, but is not expressed

in other tissues like leaf, stem and flowers (Vrebalov J. et al., 2002). *LeMADS-RIN* regulates ripening as shown in antisense lines that fail to ripen (Vrebalov J. et al., 2002). The gene *SLMBP21,* belongs to the FBP9/23 subclade, that is not present in *A. thaliana*. Recently, experiments have shown that downregulation of *SLMBP21,* using antisense constructs and RNA interference (RNAi) abolishes the development of the abscission zone (AZs) in the pedicel (Liu D. et al., 2013). Abscission is a process, where plants shed their organs and this occurs at a specific region, named the abscission zone (AZ) (Nocker S.V., 2009). Downregulation of *LeMADS1* caused only subtle phenotypes with flowers having elongated sepals (Liu D. et al., 2013).

As these examples show, angiosperms have multiple *SEP* genes and show a diversity regarding their developmental and biochemical functions in different experiments. Genetic experiments have shown individual functions for single *SEP* genes affecting plant development. Biochemical studies applying yeast n-hybrid screens revealed identical and non-identical interaction partners in every species.

One cannot help but ask whether *Arabidopsis thaliana* is the only angiosperm that has redundant E-class genes?

Table 1.1

Species	Genes	Phenotype	reference
A. thaliana	*SEP 1-4*	Single and double mutants are aphenotypic, *sep1sep2sep3* triple mutants reveal a conversion of floral organs into sepals, *sep1sep2sep3sep4* quadruple mutants develop flowers composed of leaves	Pelaz S. et al.,2000, Ditta G.S. et al., 2004
Gerbera hybridia	*GRCD1* , *GRCD2*	Down-regulation of *GRCD1* causes flowers with petal-like sterile staminode. Down-regulation of *GRCD2* causes indeterminate flowers, petal-like carpels and larger inflorescences with more flowers.	Kotilainen M. et al., 2000, Uimari A. et al., 2004
Lycopersicon esculentum	*TM5*, *TM29*, *RIN, LeMADS1, SLMBP21*	*TM5* antisense transgenic tomato plants show changes in the three inner whorls with green petals rather than yellow, green and separated stamens and variable carpel defects. Down regulation of *TM29* affects the inner three whorls with a partial conversion of petals and stamens into sepaloid structures and parthenocarpic fruits developing from ovaries. *rin* mutants fail to ripen. Down-regulation of L*eMADS1* causes longer sepals. *slmbp21* mutants fail to produce an abscission zone .	Pnueli L. et al., 1994, Ampomah-Dwamena C. et al., 2002, Vrebalov J. et al., 2002, Liu D. et. al., 2013
Petunia	*FBP2, FBP4, FBP5,FBP9, FBP23, pMADS12*	Flowers of *fbp2* insertion mutants develop secondary inflorescences in the third whorl, mild conversion of petals to sepals. *fbp5* insertion mutants have a reduced fertility. *fbp2fbp5* double mutants develop secondary inflorescences in the third whorl, enhanced conversion of petals towards sepals, *fbp9* mutants are affected in their plant architecture during the reproductive phase .	Vandenbussche M. et al.,2003, Ferrario S. et. al. 2003

Table 1.1: *SEPALLATA* genes in different plant species

Only those genes from different plants have been included for which there is evidence in their biological function described from phenotypic analysis

1.6 Genetic Redundancy

Redundant genes are common phenomena in many organisms (Brookfield J., 1992; Thomas J.H., 1993) . How do we define redundancy and how do redundant genes evolve? The lack of distinguishable mutant phenotypes in knockout experiments has been mainly attributed to genetic redundancy (Brookfield J., 1992). Redundancy is usually defined as existing when single mutants show little or no phenotypic defects, but double and higher order mutant combinations show a significant phenotype (Chen H.W. et al., 2010). Genetic redundancy is usually observed between homologous genes that evolved via gene duplication events (Pickett F.B. and Meeks-Wagner D.R., 1995). Plants contain many large gene families and it is known that genes can become duplicated via different mechanisms, varying from single genes to whole genomes (Airoldi C.A . and Davies B., 2012). The duplication that generated MADS-Box genes that are mainly associated with flower development can be attributed to Whole Genome Duplication (WGD) events (Zahn L.M. et. al, 2005, Airoldi C. A . and Davies B., 2012). It is estimated that the last WGD event in *A. thaliana* occurred between 24 and 48 MYA ago (Blanc G. et al., 2003; Bowers J.E. et al., 2003) and around 30 % of genes have surviving duplicated copies (Moore R.C. et al., 2005). A disproportionate number of duplicated transcriptional regulators has been maintained in the genome of *A. thaliana* (Blanc G. and Wolfe K. H., 2004). Gene duplication creates two functionally identical copies, the ancestral or original one and the duplicated gene, which should act fully redundantly immediately after the duplication event. It is generally assumed that one of the redundant copies is initially free of all selective restraint (Ohno S., 1970). What happens to duplicate genes? The most likely outcome of such an event is that one duplicate gene acquires deleterious mutations, and classic evolutionary theory predicts that it is subsequently lost or becomes a pseudogene (Ohno S., 1970; Wagner A., 1998; Tautz D., 2000). This process is also called nonfunctionalization (Ohno S., 1970). A second possibility is a process termed neofunctionalization, which means that the duplicate gene acquires advantageous mutations that become subject to selection leading to a new function. The third possibility, known as subfunctionalization and means both genes accumulate mutations that lead to the sub-division of the functions of the ancestral/original gene. Another possibility is that duplicated genes can undergo a combination of sub- and neofunctionalization. The duplicated gene experiences subfunctionalization, followed by neofunctionalization (Kramer E.M. et al., 2004; He X. and Zhang J., 2005). Neo- and subfunctionalization mainly occur through *cis*-acting mutations in regulatory sequences, rather than mutations in the coding sequence (Blanc G. and Wolfe K. H., 2004; Langham R.J. et al., 2004).

Nevertheless, we are still left with the question why so many MADS-Box genes are retained in the plant genome? It appears that some genes are preferentially retained due to encoding proteins that are highly connected in networks, such as TFs or signal transduction components (Blanc G. and Wolfe K. H., 2004). This overrepresentation of specific genes can also be seen in other organisms such as *Xenopus laevis* or yeast (Hughes M.K. and Hughes A.L., 1993; Papp B. et al., 2003). Several theories have addressed the question how to explain the retention of duplicated genes after WGD. According to the Gene Balance Hypothesis (Birchler J.A. et al. 2001, Birchler J.A et al., 2005, Veita R.A., et al., 2008, Edger P.P. and Pires J.C., 2009), highly connected proteins are more dosage sensitive and duplicated genes encoding them need to be retained to maintain the stoichiometry of the complex components. Assuming that the right balance or stoichiometry is needed to maintain function, an abundance or lack of one subunit, as would happen if not all genes are maintained after a WGD event would disturb the whole network or complex, leading to decreased fitness (Birchler J.A. et al. 2001, Birchler J.A et al., 2005). Increased lethality has been shown in artificial overexpression experiments in yeast for genes that encode protein subunits (Papp B. et al., 2003). However, it is worth mentioning that this theory applies well in yeast, to explain gene retention after WGD. On the other hand, plants have a much smaller effective population size and a greater tolerance of mutations that are initially slightly deleterious (Guo H. et al., 2013). This might cause differences in the fates of genes and mutations between yeast and plants (Lynch M. et al., 1995). The Subfunctionalization Theory predicts that after a WGD both copies specialize in complementary functions and thus split the functions of the ancestral genes (Lynch M. and Force A., 2000) The Gain of Function Hypothesis argues that gene duplication followed by innovation in one daughter gene is the primary source of new genes (Lewis E.B., 1951; Ohno S., 1970). Innovation can be the development of novel functions in one of the genes (also known as neofunctionalization), whereas the other duplicate maintains the ancestral function. The ABC MADS domain proteins provide good examples of different fates adopted after WGD (loss, neo- and sub-functionalization). We will take the C-function gene *AGAMOUS* (*AG*) as an example of the different fates a gene can adopt after WGD. In *Arabidopsis, ag* mutants lack reproductive organs and have petals and indeterminate mutant flowers in the third and fourth whorls (Yanofsky M.F. et al., 1990), mutations in *PLENA* (*PLE*), the C-function gene in *A. majus* result in a similar phenotype (Bradley D. et al., 1993). A WGD event around 120 million years ago gave rise to one pair of duplicated C-function genes in the last common ancestor of *A. thaliana* and *A. majus*, that we call ancestral *AGAMOUS/FARINELLI* (*AG/FAR*) and ancestral *PLENA/SHATTERPROOF* (*PLE/SHP*) respectively (Fig. 1.7). In *A. majus*, one of these duplicated genes evolved into *PLE*, the gene responsible for male and female organ development (Bradley D. et al., 1993) and the other into *FAR*, a gene involved in stamen development (Davies B. et

al., 1999). In *A. thaliana*, *AG*, the orthologue of *FAR* specifies both, male and female organs (Yanofsky M.F. et al., 1990), whereas *SHP1* and *SHP2* are the orthologues of *PLE in A. thaliana*. Both genes redundantly affect fruit development as evidenced by *shp1shp2* double mutants, which fail to dehisce. *SHP1* and *SHP2* have an expression pattern restricted to the fourth whorl (Liljegren S.J. et al., 2000). However, overexpression experiments revealed that the two *SHP* genes can regulate carpel and stamen development (Pinyopich A. et al., 2003) leading to the idea that a modification in the expression pattern of the two genes resulted in a neo-functionalization event. The two *SHP* genes arose from a recent WGD in the Brassicas and this duplication event also produced two copies of *AG*, one of which was subsequently lost (Causier B. et al., 2005; Airoldi C.A . and Davies B., 2012). On the contrary, ectopic expression of *FAR* does not confer the ability to specify male and female organ identity, as has been reported for the *SHP* genes. Ectopic expression experiments of *FAR* have shown that the gene can regulate male organ identity only (Causier B. et al., 2005; Airoldi C. A . and Davies B., 2012). This example of the C-function genes in the two plant species *A. majus* and *A. thaliana* shows all three fates, genes can adapt after a WGD event: gene loss, because one *AG* duplicate was lost in *A. thaliana*, neofunctionalization of the two *SHP* genes and subfunctionalization of *PLE* and *FAR.*

Although these genes provide examples for the Gain of Function and Subfunctionalization Hypothesis, neither make predictions concerning whether certain gene classes are significantly over retained after WGD, but instead propose that any gene might be retained following duplication. Therefore Langham (Langham R.J. et al., 2004) suggested that both functionalization processes occur largely after genes are retained in duplicate copies due to the gene-dosage constraints.

Nevertheless, the question remains why functional divergence, as seen in neo- and subfunctionalization, does not disturb the balance of the complex or network and cause a dosage imbalance (which can be seen as an intragenomic conflict)? According to Birchler (Birchler J.A. and Veitia R.A., 2007a; Birchler J.A. et al., 2007b), the duplicated TF needs to escape from the balanced stoichiometry to evolve a novel regulatory complex. One theory predicts, that a lesion in a *cis*-dominant regulatory region of a target locus may allow a shift in the balance of the complex. This could modify the gene expression, for example only in single cells or at certain developmental stages (spatiotemporal and not globally). This change in expression could progressively occur to other target genes and if this scenario happens to multiple target genes, a new balanced state of the complex is either tolerated or deleted to resolve the resulting intragenomic conflict. If the newly balanced state is tolerated, a spatio-temporarily regulated gene regulatory relationship might give rise to novel morphologies (Birchler J.A. et al., 2007b, Edger P.P. and Pires J.C., 2009). This seems to be a reasonable

explanation, as experiments have shown that one third of the human TFs are specific to a single tissue (Lynch V.J. and Wagner G.P., 2008). One advantage of tissue specific expression is the limitation of pleiotropic effects in case of a disadvantageous mutation for the organism.

All these theories explain why certain gene classes are retained after WGD, but more important is to address the question, what advantage does an organism gain from that retention? It is known from plant and animal species that divergence of TF function co-occurs or correlates with new morphological structures and WGD. Some studies suggested, that plants with doubled genomes (as after a WGD) had better chances to cope with changing environmental conditions and colonize different ecological habitats and there is evidence that gene duplication fuels long-term diversification and evolutionary success through novel gene functions (Otto S.P. and Whitton J.; Taylor J.S. and Raes J., 2004). A WGD event in the timeframe of 60-70 mya might have provided advantageous to flowering and seed plants and allowed them to adapt better to changing environmental conditions and to survive in different ecological niches (Fawcett J.A. et al., 2009) . At that time multiple environmental catastrophic events happened (referred as KT) and caused the extinction of many terrestrial plants (Vajda V. et al., 2000). Angiosperms occupied many terrestrial niches, whereas gymnosperms disappeared from parts of the landscape. The speed of angiosperm diversification from their first appearance to today's dominance is impressive and also referred as Darwin's "abominable mystery" (Friedman W.E., 2009). Plants with duplicated genomes had an increased fitness, which helped them to survive under unfavourable conditions and to occupy many parts of the landscape. As described, plants and other organisms provide examples of what can happen to genes after a WGD and considering the extended version of the gene balance theory, there is no contradiction in the diversification. If duplicated genes fuel long-term diversification (Otto S.P. and Whitton J.; Taylor J.S. and Raes J., 2004) and enhance the fitness of an organism, how can redundancy be explained?

One explanation might be a lack of precision in the phenotypic analysis regarding the screened individuals. Maybe the sample size was not large enough to identify a phenotype. A second explanation could be that the advantage for the WT lies in an environmental condition that is tested under laboratory conditions (Brookfield J., 1992). It should be considered, that most experiments are performed under ideal laboratory conditions with controlled temperatures, light/dark cycle and nutrient supply. This leads to the idea, that multiple genes might be maintained in the genome and deploy their function in stressful situations. Stress for plants could be variable environmental conditions, such as drought, increased or decreased temperature or lack of nutrients. Some of these genes could only have an effect under "emergent" conditions, therefore multiple genes that have acquired new functions could enhance the robustness and reproductive fitness of the plant to guarantee propagation.

Another idea might be that genes have a minor effect in laboratory conditions and time but a major effect over the evolutionary period (Tautz D., 2000). And finally, one should consider that most phenotypic characterizations are carried out by human eyes, relying on judgement of the degree to which a mutant varies from the WT. Kieffer et. al (Kieffer M. et al., 2011) provides an example of how the use of advanced methods of phenotypic analysis, in that case computer aided morphological analysis reveals a phenotype for “redundantly” acting genes.

Fig. 1.7

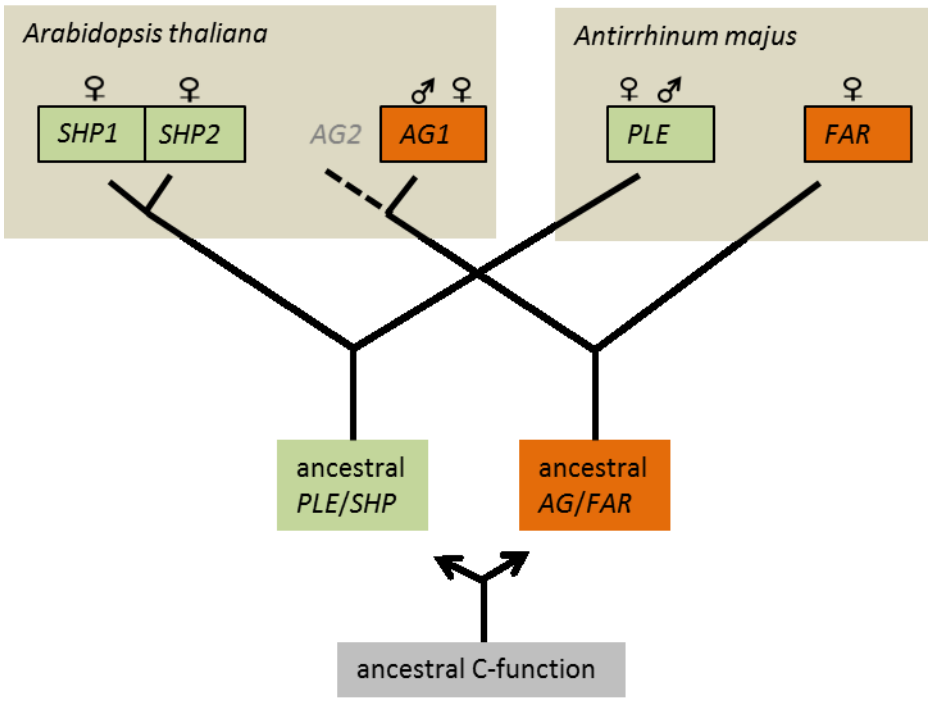


Fig. 1.7: Evolution of the C-function genes *in Antirrhinum* and *Arabidopsis*

A WGD event in the last common ancestor of monocots and dicots generated duplicated copies of a single C-function gene. The two copies are here called ancestral *PLE/SHP* and ancestral *AG/FAR.* Another WGD event in the Brassicaceae generated the two *SHP* genes and two *AG* genes. The *SHP* duplicates were retained, but one copy of *AG* was lost. In *A. thaliana*, *AG* controls development of female and male organs, whereas *SHP* genes redundantly control seed pod shatter. In *A. majus, PLE* specifies male and female reproductive organ development and *FAR* has a subtle role in stamen development. This clade provides examples for neofunctionalization with *AG* and *SHP* genes in *A. thaliana* and subfunctionalization for the two genes *PLE* and *FAR* in *A. majus*.

1.7 Scope of the thesis

Genetic redundancy has been reported frequently in different organisms and in plants many of these redundant genes evolved via genome duplication events. However, according to Ohno, duplicated genes should diverge in function over time with long term retention of truly redundant genes unlikely (Ohno S., 1970). Thus, the reported redundancy for *SEP* genes in *A. thaliana* is a contradiction, because the advantage to maintain multiple genes that do not differ in their function is questionable. In this study the four *SEP* genes from *A. thaliana* were chosen to assess the degree of redundancy. *SEP* genes are necessary co-factors for flower development thanks to their ability to form multimeric protein complexes with the other ABC floral organ identity genes. The four *A. thaliana SEP* genes are reported to act redundantly. Based on the idea that multiple genes can positively affect the reproductive fitness or robustness of the plants, two experimental approaches will be used to shed more light into this topic.

Firstly, the redundancy is assessed by performing experiments of single and multiple *SEP* mutant combinations, under both standard laboratory conditions and under conditions that mimic a change in the environment, in this case an increase in temperature. Based on the idea that multiple *SEP* genes positively affect the robustness and reproductive fitness, single or mutant combinations could be affected under these changing temperature conditions causing a disadvantage for the plant and its propagation.

Secondly, I will dissect the reported genetic redundancy at a molecular level. As previously shown SEP proteins have both common and distinct interaction partners (de Folter S. et al., 2005; Immink R.G. et al., 2009), suggesting individual as well as overlapping roles on a protein level. Little information is available about target genes of individual SEPs, not to mention their genome-wide binding profiles. Therefore I aim to identify genome-wide targets, particularly for SEP4 to compare how targets differ between SEPs. This will give more information about the biological roles of individual and multiple *SEP*s with regards to the development and fitness of the plant. These experiments will also shed more light into evolution of this gene family.

Chapter 2

2. Material and Methods

2.1 General methods

2.11 Media and agar plates

Luria Broth Medium (LB)

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

make up to 1 L with distilled water.

Sterilize by autoclaving from 121-124 °C for 20 minutes

Agar plates

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Agar	4 g

make up to 1 L with distilled water.

Sterilize by autoclaving from 121-124 °C for 20 minutes

2.12 Chemicals and antibiotics

All chemicals and antibiotics of analytical quality have been purchased from Sigma Aldrich (The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT), Roth (Schoemperlenstr. 3-5, 76185 Karlsruhe, Germany), Merck (Merck Chemicals Ltd., Boulevard Industrial Park, Padge Road, Beeston, Nottingham NG9 2JR), Duchefa (DUCHEFA BIOCHEMIE B.V., P.O. Box 809, 2003 RV Haarlem, Netherlands), Severn Biotch Ltd (Unit 2, Park Lane, Kidderminster, Worcestershire, DY11 6TJ).

2.13 Enzymes and kits

Restriction enzymes were purchased from Fermentas (HELENA BIOSCIENCES LTD. Colima Avenue, Sunderland Enterprise Park, Sunderland, SR5 3XB). Modifying enzymes came from Invitrogen (3 Fountain Drive, Inchinnan Business Park Paisley PA4 9RF, UK), New England Biolabs (New England Biolabs, 75-77 Knowl Piece, Wilbury Way, Hitchin Herts SG4 0TY), Biorad (Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX) and Promega (Delta House / Enterprise Road, Chilworth Science Park, Southampton, Hampshire SO16 7NS). Oligos/Primers were generated by Integrated DNA technologies and Sigma. Plasmid purification and DNA extraction kits were supplied from QIAGEN (Lloyd Street North , Manchester M15 6SH). A Green Fluorescent Protein (GFP) Antibody was supplied from Milteny Biotech (Almac House, Church Lane Bisley, GU24 9DR Surrey).

2.14 Cloning Vectors and Constructs

Following vectors were used in this work

pDONOR 207 vector (Invitrogen) was used as a donor vector in Gateway based cloning

pMDC107gb (prepared by Dr. K. Kaufmann) as a binary target vector containing a GFP cassette and Hygromycin resistance.

*pAlligator*2 and *pAlligator*3 (cnrs.gif.fr) as a binary gateway target vector containing a *CaMV* 35S promoter cassette and Spectinomycin resistance

2.2 Plant material and methods

2.21 Plant Material

Arabidopsis thaliana plants used were derived from the Col-0 accession. The transgenic lines *sepallata1-1*, *sepallata2-1*, *sepallata3-2* and *sepallata4-1* were obtained from Dr. Chiara Airoldi (Center for Plant Sciences, Leeds, UK) and Dr. Kerstin Kaufmann (Plant Research International, Wageningen, NL) and previously published (Pelaz S. et al., 2000; Ditta G.S. et al., 2004). The *sep1-2* (SALK_011077C) and *sep4-2* (SALK_06229) alleles were obtained from the SALK T-DNA insertion collection (http://signal.salk.edu). The *sep2-2* (GABI-Kat246B03) allele was obtained from the GABI-Kat T-DNA insertion collection (ww.gabi-kat.de), *cuc3-105* seeds were given by Dr. Mitsuhiro Aida, (NAIST, Ikoma, Japan) and previously published (Hibara K. et al., 2006).
Double and triple mutants described in this work were generated using following alleles: *sepallata1-1*, *sepallata2-1*, *sepallata3-2* and *sepallata4-1* and cuc3-*105.*
PCR, resistance to Hygromycin or Spectinomycin or the presence of GFP was used to determine the genotypes.

2.22 Plant growth conditions

Arabidopsis thaliana seeds were germinated on soil or half MS2 medium containing 0.5 % sucrose and 0. 8 % agarose. Before planting seeds out on soil, they were put on wet filter paper (Whatman™, GE Healthcare UK Limited, Buckinghamshire UK) and stored for 3 days at 4 ° C. Plants were grown at 22 °C or at 27 °C under standard greenhouse conditions in a Sanyo MLR-352 H growth chamber with additive humidity under a typical long day cycle (16hr light:8 hr dark, 65 % RH) respectively.

2.23 Crossing of plants

At a stage when the flowers were closed and the pollen of the anthers was not ripe the anthers of the acceptor flower were removed completely using very fine forceps. All remaining older and younger flowers were also removed. The stigma of the carpels was pollinated with pollen from the donor plant.

2.24 Plant transformation

Plants were transformed according to the "floral dip" method (Clough S.J. and Bent A.F., 1998). Two days prior transformation 10 ml of pre-culture containing Agrobacterium carrying the appropriate plasmid were incubated in LB medium with the appropriate antibiotics at 28 °C /200 rpm overnight.

This pre-culture was used to inoculate the final 500 ml culture. The next day, bacteria were pelleted for 15 min at 6000 rpm and suspended in 250 ml 5 % Sucrose solution. Before dipping the plants, Silwet L-77 was added to the suspension at a final concentration of 0.05 %. Plants were dipped in this solution for about 30 seconds and then placed horizontally in a tray, sealed with a lid and transferred to the growth chamber for 24 hours to maintain humidity.

2.25 Selection of transformants

Seeds of transgenic plants carrying the appropriate transgene with the Hygromycin resistance gene were selected on MS Agar plates containing 50 µg/ml Hygromycin.

2.26 Seed sterilization

Seeds were incubated for 5 minutes in sterilized water containing 1 % SDS and centrifuged down at full speed. Supernatant was removed and seeds were sterilized by incubating in a 30 % bleach solution for 10 minutes. After removing the bleach, seeds were washed four times with sterile water to remove residual bleach and finally suspended in 0.1 % agarose. The seed/Agarose mixture was spread out on MS Agar plates (1.1 g/l MS, 1 % sucrose, 0.8 % Agar at pH6) containing the appropriate antibiotics. The plates were sealed with micropore tape and incubated for 7-14 days in a chamber at 16 °C and 16/8 hours day /night.

2.3 Bacterial methods

2.31 Bacterial strains

Standard cloning was performed using the strain DH5α. *Agrobacterium* t*umefaciens,* strain GV3101 was used for plant transformations according to the floral dip method (Clough S.J. and Bent A.F., 1998)

2.32 Plasmid DNA preparation from bacteria

Plasmid preparation was performed using a QIAprep® spin mini prep kit according the manufacturer's recommendations.

2.33 Restriction enzyme digestion

Reactions were set up in a volume of 10 µl with a maximum of 10 units of appropriate restriction enzyme (Fermentas, fast digest) and the appropriate buffer (Fermentas, fast digest green buffer). Digestions were incubated for 30 min up to 2 hours at 37 °C.
Digest reactions were run on a 1 % agarose gel and size of bands was determined.

2.34 Bacterial growth conditions

The bacteria stain *E. coli* DH5α was grown overnight at 37 °C either in LB media (shaking at 200 rpm) or on Agar plates containing the appropriate antibiotics.

2.35 Generation of the reporter constructs

For the generation of the reporter constructs the Gateway System (Invitrogen) was used. A 2-3 kb 5' upstream region of genomic *SEP1*, *SEP2* and *SEP4* DNA was amplified via PCR. PCR products were subsequently purified as described in section 4.12 and recombined into the Donor vector pDONR207. After successful sequencing the entry clone was recombined into the Destination vector pMDC107gb.

2.36 Transformation of bacteria

25 µl of chemically competent *E. coli* α select cells (Bioline) were defrosted on ice for 5 minutes and 2-5 µl of a standard miniprep was added and to the cells and gently mixed before incubating them 30 min on ice. Afterwards cells were heat-shocked at 42 °C for 30 sec. in a water bath and immediately transferred on ice for 2 minutes. 800 µl of LB medium was added to the cells before incubating them at 37 °C, shaking at 200 rpm for 2 hours. Subsequently 100 µl of cells were plated on Agar plates containing the appropriate antibiotics and incubated overnight at 37 °C. Single colonies were picked and grown overnight in LB medium with the appropriate antibiotics at 37 °C, shaking at 200 rpm. Plasmids were purified using a plasmid purification kit (Qiagen) according to the manufacturer's instructions. The presence of the

vector of interest was confirmed by restriction enzyme digestion. Samples were loaded on a 1 % Agarose gel with a final concentration of Ethidium Bromide of 0.5 µg/ml and visualized using a UV transilluminator.

2.37 Introduction of plasmid into Agrobacterium

10ml overnight culture (ON) containing *Agrobacterium tumefaciens* GV 3101 were grown in LB at 28 °C containing the appropriate antibiotics (Gentamycin 25 µg/ml and Rifampycin 34 µg/ml). The next day, 100 µl of ON culture was inoculated into 10 ml LB containing the appropriate antibiotics and grown for six hours at 28 °C until they reached moderately cloudy stage. For each transformation 2 ml of the culture was pelleted and suspended in 1 ml ice cold 10 mM Tris (pH 7.5), pelleted again and resuspended in 200 µl of ice cold Elution Buffer (QIAGEN). 1-2 µg of Plasmid DNA was added to the cells and the mixture was incubated for five minutes in liquid nitrogen. Cells were immediately transferred for five minutes to a 37 °C waterbath. 1 ml of fresh LB was added to the cells and incubated for 2-3 hours at 28 °C to recover. 200-500 µl of the cells were plated onto selection plates containing the appropriate antibiotics and incubated for three days at 28 °C.

2.4 Molecular methods

2.41 Primer design

All primers were designed with Oligonucleotide Properties Calculator
(http://www.basic .northwestern.edu/biotools.oligocalc.html), or
mfe primer (http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0).
For the cloning steps with the Gateway System, primer pairs were designed including the attB sequence.

2.42 Rapid genomic DNA extraction from plant tissue (Quick and dirty protocol)

For PCR based genotyping, plant genomic DNA was isolated according to the following protocol. A small amount of plant material (e.g. a young rosette leave) was put in a 1.5 ml eppendorf tube with 400 µl extraction buffer (250 mM TRIS HCL, pH 7.7, 25 mM EDTA, 250

mM NaCl, 1 % SDS), ground with an eppendorf pestle and centrifuged at 14 000 rpm for ten minutes. The supernatant was transferred to a new eppendorf tube containing 0.9 volumes of isopropanol, inverted 5 times and centrifuged for 30 minutes at 14 000 rpm. The supernatant was removed and the pellet washed in 1 ml 70 % ethanol. After centrifugation at 14 000 rpm for four minutes the ethanol was removed, the pellet air-dried and suspended in TE buffer (10 mM TRIS-HCL, pH 7.6, 1 mM EDTA, pH 8).
The prepared genomic DNA was stored at – 20° C.

2.43 Genomic DNA extraction from plant tissue II

Genomic DNA was extracted by using the Bioline Isolate Plant DNA mini kit (Fermentas) according to the manufacturer's instructions.

2.44 Polymerase Chain Reaction (PCR)

PCR reactions contained dNTPs (0.2 mM final concentration), 5' and 3' primers (0.2 mM final concentrationeach), 10-100 ng template, DNA polymerase and the appropriate buffer containing $MgCl_2$ (20mM) according to the manufacturer's instructions. For genotyping of plants, Dream Taq DNA Polymerase (Thermo Scientific) was used. For cloning the proofreading high fidelity Phusion® Polymerase (Finnzymes) was used. PCRs were performed in a volume of 50 μl as shown in Table 2.1 and Cycling programs were run according to the manufacturer's recommendations as shown in Table 2.2.

Table 2.1

10 x Dream Taq Buffer	5 µl
dNTP Mix	1 µl (0.2 mM final concentration)
Forward primer	1 µl (0.2 mM final concentration)
Reverse primer	1 µl (0.2 mM final concentration)
Template DNA	10-100 ng
Dream Taq DNA Polymerase	1.25 U
Water, nuclease free	up to 50 µl

Table 2.1 Mastermix for a PCR per 50 µl reaction using Dream Taq DNA Polymerase (Thermo Scientific)

Table 2.2

Step	Temperature	time
1)initial denaturation	95 °C	3 min.
2) denaturation	95 °C	30 sec.
3)primer annealing	X	30 sec.
4) extension	72 °C	1 min. / 1 kb
5) final extension	72 °C	10 min.

Table 2.2: Cycling program using Dream Taq DNA Polymerase (Thermo Scientific).

Step 2- 4) are repeated 34 times, x= primer specific annealing temperature that was calculated using primer mfe (http://biocompute.bmi.ac ac.cn/CZlab/MFEprimer-2.0/).

2.45 DNA agarose gel electrophoresis

Agarose gels were made using 1 % Agarose w/v (Melford Laboratories, Ltd) in 1x Tris-acetate-EDTA buffer (TAE buffer) (Severn Biotech Ltd.). The solution was boiled in a microwave and Ethidium bromide added to a final concentration of 0.5 µg/ml. Loading dye (6x DNA loading dye, Fermentas) was added to each DNA sample prior to loading on a gel and samples were run alongside a 1 kb plus molecular ladder (Invitrogen). Gels were typically run for 30 minutes at 100 mV and visualized using a UV transilluminator.

2.46 DNA extraction from Agarose gels

Electrophoretically separated DNA fragments were cut from gels with razorblades and the DNA extracted and purified using the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.47 RNA extraction from plants

Total RNA was isolated from inflorescences stage 1-12 (Smyth D.R. et al., 1990) using the RNeasy® Plant Mini Kit (QIAGEN). Samples were *DNASE* I treated to avoid DNA contamination and eluted in 40 µl RNAse free water. The RNA concentration was measured using the Nanodrop ND 1000 instrument or the Agilent 2100 Bioanalyzer. For measurement with the Agilent 2100 Bioanalyzer, samples were prepared using the RNA 6000 LabChIP® kit following the instructions of the manufacturer.

2.48 cDNA synthesis and Quantitative Real time PCR (qPCR)

1 µg of total RNA was used to synthesize cDNA using oligo(dT) and the SuperScript II Reverse Transcriptase ®Kit (Invitrogen). Absence of genomic DNA contamination was tested by PCR using primers of a reference gene, *Elongation Factor1*α designed to amplify an intron sequence before performing Quantitative real time PCR (qPCR) on an Opticon continuous fluorescence detection system (C1000™Thermal Cycler, Biorad) using the IQ™SYBR® Green Supermix (Biorad). Reactions were performed in a total volume of 25 µl as shown in Table 2.3 and the cycling programme was used according to the manufacturers instructions (Table 2.4).Gene expression was calculated relative to *Actin2* using the standard curve method.

Table 2.3

IQ™ SYBR® GREEN supermix (2 x)	5 µl
Forward primer	0.5 µl (0.2 mM final concentration)
Reverse primer	0.5 µl (0.2 mM final concentration)
Template DNA	1 µl (1: 50 dilution)
Water, nuclease free	10. 5 µl

Table 2.3: Reaction set up for Quantitative Real time PCR per 25 µl reaction.

Table 2.4

Polymerase activation and DNA denaturation at 95 °C	Denaturation at 95 °C	Annealing/Extension + Plate read at an optimized temperature (55 °C-60 °C)	Cycles	Melt Curve Analysis
3 min.	10-15 sec.	30-60 sec.	39	55 °C – 95 °C, 0.5 °C increment 2-5 sec. / step, (or use instrument default setting)

Table 2.4: Thermal cycling protocol using C1000™Thermal Cycler, Biorad.

2.5 Microarray methods

2.51 Microarray

Plants were grown in a Sanyo MLR-352 H with humidity under a typical long day cycle (16 hours light and 8 hours dark) The temperature was maintained at 21-23 °C and the humidity was maintained at 65 % RH. Inflorescences from stage 1-12 were taken with forceps from 3-4 weeks old plants. RNA was extracted and purified using the Qiagen RNeasy® Plant Mini Kit according to the manufacturer's instructions. The optional on-column DNase digestion was done according to the manufactures recommendations and finally RNA was eluted in 40 µl Elution Buffer (Qiagen RNeasy® Plant Mini Kit). RNA quality was checked on the Agilent 2100 Bioanalyzer and a total of 1 µg in 10 µl of a total volume was sent to Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham UK for hybridisation to ATH1 arrays.

2.52 Microarray hybridisation

Microarray Hybridisation was performed at NASC, UK. Approximately 1 µg of total RNA from each sample was used to produce cDNA using the GeneChip® One-cycle cDNA synthesis kit (Affymetrix), as per manufacturer's instructions. Double stranded cDNA products were purified using the GeneChip® Sample Cleanup Module (Affymetrix). The synthesised cDNAs were in-vitro transcribed by T7 RNA polymerase using biotinylated nucleotides to generate biotinylated complementary RNAs (cRNAs) using the GeneChip® HT IVT labeling kit (Affymetrix), as per manufacturer's instructions. The cRNAs were purified using the GeneChip® Sample Cleanup Module (Affymetrix). The cRNAs were then randomly fragmented at 94 °C for 35 minutes in a buffer containing 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate to generate molecules of approximately 35 to 200 bp. Affymetrix *A. thaliana* ATH1-121501 GeneChip® arrays were hybridised with 15 µg of fragmented labelled cRNA for 16 h at 45 °C as described in the Affymetrix Technical Analysis Manual using the GeneChip® hybridization control kit and GeneChip® hybridisation, wash and stain kit (Affymetrix). GeneChip® arrays were stained with streptavidin-phycoerythrin solution and scanned with an Affymetrix 3000 7G GeneArray scanner. Following scanning, non-scaled RNA signal intensity (CEL) files were generated using GeneChip® operating software (GCOS; Affymetrix) (Craigon D.J. et al., 2004). All data are MIAME compliant and the raw data will be deposited in ArrayExpress at the beginning of 2014.

2.53 Microarray analysis

The non-scaled RNA CEL files were loaded into GeneSpring 12.1 analysis software (GeneSpring 12.1; Agilent Technologies, USA) using the Robust Multichip Average (RMA) pre-normalisation algorithm (Irizarry R.A. et al., 2003). Separate experiments were created for each mutant (sep3-2, *sep4-1*) and WT using the normalised CEL files for the mutant and corresponding wild-type RNAs. Further normalisations were performed for each experiment using a three step process: (i) probe-sets with a signal value <0.01 were set to 0.01, (ii) per chip normalisation to the 50th percentile, (iii) each gene signal was normalised to the median of that gene. Putative genes with differential hybridisation intensities between a single mutant and corresponding wild-type were identified using a two-step process: (i) genes that were 1.5-fold up- or down-regulated were selected, and (ii) a Welch's unpaired t-test was performed ($p<0.05$). Genes that were differentially expressed in more than 1 mutant were identified by comparing gene lists of differentially expressed genes in a single mutant using the Venn diagram function of GeneSpring (Agilent, version 12.1).

The agriGO analysis tool kit was used to search for ontologies that were over-represented amongst the commonly up- or down-regulated genes (Du Z. et al., 2010).

2.6 Chromatin immunoprecipitation (ChIP) methods

2.61 Chromatin immunoprecipitation

Up to 0.8 g of plant tissue (inflorescences stage 1-12) were harvested into a 50 ml tube on ice. The tissue was immediately fixed for 30 minutes under vacuum infiltration (VP100, VWR) in MC buffer containing 1 % Formaldehyde (10 mM sodium phosphate, pH 7, 50 mM NaCl, 0.1 M sucrose, 1 % Formaldehyde) and stopped by adding 2.5 ml 1.25 M Glycine. Vacuum was applied for another 2 minutes. Tissue was washed three times with MC buffer, gently dried with tissue paper, transferred to a 50 ml tube and snap frozen in liquid Nitrogen. Afterwards the tissue was thoroughly ground with a mortar in liquid Nitrogen for about 30 minutes and transferred to a new 50 ml tube with 20 ml M1 buffer (10 mM sodium phosphate, pH 7.0, 0.1 M NaCl, 1 M 2-methyl-2.4-pentanediol, 10 mM β-mercaptoethanol, Complete Protease Inhibitor Cocktail). The resulting slurry was filtered through a mesh (55 μm) and collected in a 50 ml tube on ice. The mesh was washed with 5 ml M1 buffer to collect all nuclei. The filtrate was centrifuged at 1000 g for 20 minutes at 4 °C. The nuclear pellet was washed five times with 5 ml M2 buffer (10 mM sodium phosphate, pH 7.0, 0.1 M NaCl, 1 M 2-methyl-2.4-pentanediol, 10 mM β-mercaptoethanol, Complete Protease Inhibitor Cocktail, 10 mM $MgCl_2$, 0.5 % Triton-X-100) and centrifuged at 4 °C for 10 minutes at 1000 g. Finally the nuclei pellet was washed with 5 ml M3 buffer (10 mM sodium phosphate, pH 7.0, 0.1 M NaCl, 10 mM β-mercaptoethanol, Complete Protease Inhibitor Cocktail) and centrifuged as in the previous step. The nuclei pellet was suspended in 1 ml Sonic buffer (10 mM sodium phosphate, pH 7.0, 0.1 M NaCl, 0.5 % Sarkozyl, 10 mM EDTA, Complete Protease Inhibitor Cocktail) and transferred to a 2 ml safe lock tube. Sonication of the chromatin was performed with a probe sonicator (MSE Soniprep 150) with 15 cycles for 15 seconds and 45 seconds cooling in between. Tubes were left on ice for three minutes and inverted several times. The supernatant was centrifuged 3 times for 10 minutes at 16000 rpm at 4 °C to remove all debris. 120 μl were kept as “input DNA” control and 20 μl were used to check the degree of sonication by loading on a 1.5 % Agarose gel after reverse cross-linking and purification. 1 ml IP buffer and 50 μl paramagnetic beads (μMACS Anti-GFP starting kit, Miltenyi Biotec) were added to the lysate and incubated at 4 °C for one hour on a rotating device. A μ Column (MACS® Separation Columns, Miltenyi Biotec) was placed in a magnetic field (μMACS™ Separator, Miltenyi Biotec) and equilibrated by adding 200 μl IP buffer (50 mM Heppes, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 10 μM $ZnSo_4$, 1 % Triton-X-100, 0.05 % SDS). Lysate was applied to the columns and

passed through. Beads were washed in the following order: 2 x with 400 µl IP buffer, 1 x with 200 µl IP buffer, 2 x 200 µl High salt wash buffer (500 mM NaCl, 0.1 % SDS, 1 % Triton-X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8), 2 x 200 µl LiCl wash buffer (10 mM Tris-HCl pH8, 1 mM EDTA, 1 % NP-40, 1 % sodium deoxychelate , 0.25 M LiCl) and 2 x 200 µl Tris-EDTA (TE) buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). Finally 20 µl Elution buffer (1 % SDS, 50 mM Tris, pH 8, 10mM EDTA, 50 mM DTT) was heated up to 95 °C and added to the columns and incubated for five minutes. 50 µl of hot Elution buffer was added for three times and eluted in a new 2 ml safe lock tube. 100 µl TE and 11.25 µl Proteinase K (20 mg/ml) were added to the samples and incubated ON at 37 °C. At the same time 150 µl of TE buffer and 11.25 µl of Proteinase K were added to the input samples and incubated over night at 37 °C. On the following day 11,25 µl Proteinase K (20 mg/ml) was added to the ChIP and input samples and incubated at 65 °C for six hours to reverse crosslink (and isolate the DNA). DNA was precipitated by adding 2.5 vol EtOH (100 %, pA), 1/10 vol NaAc (3 M, ph 5.4) and 3 µl Glycogen (Ambion, 5 mg/ml) overnight at -20 °C and pelleted by centrifugation for 30 minutes at 4 °C. DNA was suspended in 100 µl Millipore Ultra pure water and purified with a QIAquick® PCR Purification Kit (QIAGEN). DNA was eluted in 34 µl Elution buffer. Subsequently ChIP DNA was analyzed by qPCR (ChIP-qPCR) to check for enrichment. ChIP DNA was used in a 1:5 dilution input DNA was used in a 1:1000 dilution. IQ™SYBR® Green Supermix (Biorad) was used for qPCR reactions in a total volume of 25 µl (Table 2.5 and Table 2.6). Gene expression was calculated relative to the two reference genes *two-component response regulator ARR6* (*ARR6)* and *heat stress transcription factor A-1a* (*HSF1*) (Kaufmann K. et al., 2009) using the ΔΔCt method (Livak K.J. and Schmittgen D.T., 2001). The two reference genes should be expressed at relatively constant levels in all cells.

Table 2.5

IQ™ SYBR® GREEN supermix (2 x)	12.5 µl
Forward primer	1 µl (0.2 mM final concentration)
Reverse primer	1 µl (0.2 mM final concentration)
Template, ChIP DNA	5 µl (1: 5 dilution)
Water, nuclease free	3. 5 µl

Table 2.5: Reaction set up for ChIP-qPCR per 25 µl reaction

Table 2.6

Polymerase activation and DNA denaturation at 95 °C	Denaturation at 95 °C	Annealing/Extension at 60 °C	Cycles	Melt Curve Analysis
3 min.	15 sec.	60 sec.	39	55 °C – 95 °C, 0.5 °C increment 2-5 sec. / step, (or use instrument default setting)

Table 2.6: Quantitative real time PCR protocol on an Opticon continuous fluorescence detection system (C1000™Thermal Cycler, Biorad).

Gene expression was calculated relative to *ARR6* and *HSF* using the ΔΔCt method (Livak K.J. and Schmittgen D.T., 2001).

2.62 Preparation of Illumina multiplex libraries

Before ChIP samples were sent for sequencing they were converted into libraries using the NEXTflex™ChIP-Seq Kit, (Illumina Compatible, Catalog#:5143-02). This protocol was used in a modified version.

The first step was an End Repair step where 7 µl NEXTflex ™ChIP End Repair Bufffer Mix, 3 µl NEXTflex ™ChIP End Repair Enzyme Mix and ChIP DNA in a total concentration of 10 ng were pipetted together. The reaction was filled up to a total volume of 50 µl with Nuclease free Water. Samples were incubated for 30 min at 22 °C in a thermo cycler block.

Second step was a Bead Clean Up step where 90 µl of AMPure XP Beads were added to the mix and incubated at RT for 5 min. Afterwards the 96 well PCR Plate was placed on a magnetic stand at RT for 5 min. Supernatant was removed and beads were washed twice with 200 µl of freshly prepared 80 % EtOH with 30 sec incubation in-between each step. The plate was removed from the magnetic stand and beads were dried for 3 minutes on RT before suspending them in 17 µl Resuspension buffer. Beads were incubated for 2 minutes on RT before placing them back to the magnetic stand and incubated for 5 min. 16 µl of clear sample were transferred to a new well.

The third step was a 3 ‘Adenylation where 16 µl End-Repaired DNA (from the previous step) and 4.5 µl NEXTflex™ChIP Adenylation Mix were mixed together (in a total volume of 20.5 µl) and incubate on a thermo cycler at 37 °C for 30 min.

In the fourth step, the adapters were ligated. Therefore 20.5 µl of 3’ Adenylated DNA (from the previous step), 27.5 µl NEXTflex ™ChIP Ligation Mix and 2.0 µl NEXTflex ™ChIP Adapter (diluted 1:10) were mixed together in a total volume of 50 µl and incubated for 15 min at 22 °C on a Thermo cycler.

Step five was a clean up step. 55 µl AMPure XP Beads were added to the sample and incubated for 5 min at RT. Afterwards the 96 well was placed back in a magnetic stand and incubated for 5 min. Supernatant was removed and beads were washed twice with 200 µl 80 % freshly prepared EtOH with 30 sec pause in-between. The 96 well was removed from the magnetic stand and beads were dried at RT for 3 min before adding 52 µl Resuspension followed by a 2 min RT incubation. The PCR plate was placed back to the magnetic field and 50 µl of the clear supernatant were transferred to a new well. 55 µL of AMPure XP Beads were added to the sample and incubated for 5 min at RT. Afterwards samples were washed twice with 80 % EtOH and beads were dried at RT. Dried beads were resuspend in 38 µL Resuspension buffer, incubated for 2 min at RT, transferred to the magnetic stand and after 5 min incubation time, 36 µL of clear lysate was transferred to new well.

Step five was a PCR Amplification step performed according to the manufacturers instruction (see Table 2.7 and Table2.8), followed by an Agarose Gel Size Selection. Therefore, a 2 %

Agaroe gel w/v (Melford Laboratories, Ltd) in 1x TAE buffer (Severn Biotech Ltd) and Ethidium bromide added to a final concentration of 0.5 µg/ml was prepared. The gel was poured in a tray and the entire PCR sample was loaded into one lane of the gel. If more than one sample was processed, several gels were prepared to avoid cross contamination. 6 µl of MW Ladder Ready-to-Load 100 bp was loaded into one lane of the gel, skipping at lest two lanes between it and the sample. The gel was run at 100-120 V for around 60 min. in 1 x TAE buffer and visualized on a UV transilluminator . A clean razor was used to cut out a slice of gel from each sample lane corresponding to the 250-300 bp marker and purified using QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Samples were concentrated using a mini-elute kit (Qiagen) and the enrichment was checked for each library again as described in section 2.61 (Table 2.5 and Table 2.6) using the same positive and negative control genes as for the ChIP protocol. If enrichment was sufficient, libraries were sent for sequencing.

Table 2.7

Ligation Product (from step G1)	36 µl
Nextflex™ ChIP PCR Mastermix	12 µl
Nextflex™ ChIP Primer	2 µl (0.25 mM final conc.)

Table 2.7: PCR reaction set up for multiplex-library preparation per 50 µl reaction using NEXTflex™ChIP-Seq Kit.

Table 2.8

Temperature	Time
98 °C	2 min.
98 °C	30 sec.
65 °C	30 sec.
72 °C	1 min. / 1 kb
72 °C	4 min.

Table 2.8: PCR cycling program for multiplex library preparation

The NEXTflex™ChIP-Seq Kit was used, steps 2-4 are repeated between 15-19 times

2.63 ChIP-SEQ analysis

ChIP SEQ peak calling was performed by Pedro Madrigal (Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland) and Jose M Muiño **(**Applied Bioinformatics, Plant Research International, Wageningen, The Netherlands) using CSAR.

2.7 Microscopy

2.71 Light microscopy

All pictures were taken with a MZ16 stereomicroscopes from Leica Microsystems. Pictures were afterwards modified using Adope Photoshop CS24 version 11.

2.72 Confocal Microscopy

GFP expression in plant tissue was studied in the Angenent lab in Wageningen, NL. Confocal Scanning Laser Microscopical (CSLM) imaging was performed with a Zeiss LSM 510 inverted confocal microscope using a 40× C-Apochromat (NA 1,2 W Korr) lens. The tissue was embedded in the wells of a Silicone with 0.3 % agar. GFP was excited with the 488 line of an argon ion laser. The emission of GFP was filtered with a 505–530 nm bandpassfilter, while the red autofluorescence of the plant tissue was filtered with a 650 nm long-pass filter. 3D projections of the obtained confocal z-stacks were made with the Zeiss LSM Image Browser Version 4 as previously described in Urbanus et al (Urbanus S.L. et al., 2009)

2.73 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to produce detailed images of surface structures from young inflorescences (stage 1-8) from plants. Samples were prepared according to Reynolds. E. S. (Reynolds E. S., 1963). Pictures were taken using a FEI QUANTA 200 F Field Emission Gun Environmental Scanning Electron Microscope (FEG-ESM) in the FBS Electron Microscopy (EM) Unit (School of Molecular and Cellular Biology, University of Leeds) with the help of Martin Fuller

2.8 Bioinformatics and computer resources

Table 9 summarizes the online resources that were used in this work to generate oligonucleotides, order T-DNA insertion lines and analyse microarray and ChIP-Seq experiments.

Table 2.9

Analysis	Tool	Web Address	Reference
Gene structure	TAIR	www.arabidopsis.org	
Insertion lines	T-DNA express, GABI-KAT	http://signal.salk.edu/cgi-bin/tdnaexpress, www.gabikat.de	Alonso J. M. et al., 2003, Kleinboelting N. et al. 2012
Primer design	MFEprimer-2.0	http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0	Wubin Q. et al., 2012
Microarray analysis	GeneSpring software version 12.1		Agilent Technolongies USA
Gene ontologies	agriGO	http://bioinfo.cau.edu.cn/agriGO/	Zhou D. et al., 2010
ChIP-SEQ analysis	Pricat, IGB version 7	http://www.ab.wur.nl/pricat/, http://bioviz.org/igb/overview.html	Muino J. et al., 2011, Nicol J. W. et al., 2009

Table 2.9: Used bioinformatics and computer resources

Chapter 3

3. Phenotypic characterization of *sep* single mutants and mutant combinations

3.1 Do *SEP* genes affect the robustness of flower development?

Plants are sessile organisms and thus require strategies to withstand environmental fluctuations and stress. They show a remarkable resilience to changing conditions, and at the same time, they can respond to changes by developing new phenotypes. Robustness can be defined as 'the ability of organisms to withstand genetic and environmental changes during development and the capacity to maintain specific functions' (Whitacre J.M., 2012). Considering the ecological dominance of angiosperms, one question in biology is what made them so successful in occupying different ecological niches? Genome duplications are associated with the opportunity to contribute to adaption and one theory is that such events were advantageous for the survival and success of the angiosperms (Fawcett J.A. et al., 2009). As described in chapter 1, MADS-Box genes became amplified in the genome via WGD and there is evidence that this extensive gene duplication and subsequent modification in various lineages resulted in diversified or novel protein functions (Irish V. F. and Litt A., 2005). New gene functions can evolve in response to a change in external conditions, but the origin of these underlying mutations must not necessarily occur at the same point as the change (Crow K.D. and Wagner G.P., 2006). For example a change in the environment could lead to novel protein functions, and the plant can benefit at a future time from that novel gene function. One possible explanation for the retention of multiple gene copies is to enhance the plant's robustness to fluctuating environmental conditions. *SEP* genes proliferated via WGD events and are reported to act redundantly in the model plant *A. thaliana* (Pelaz S. et al., 2000; Ditta G.S. et al., 2004). However, there is mounting evidence that these genes are not fully redundant, as shown with different protein-protein interaction partners for different SEP proteins (de Folter S. et al., 2005; Airoldi C.A. et al., 2010) or different expression patterns within the plant (Ma H et al., 1991, Rounsley S.D. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Pelaz S. et al., 2000; Ditta G.S. et al., 2004). This is further supported by the discovery of a subtle phenotype for *sep3,* with mutants displaying a partial transformation of petals towards sepals (Pelaz S. et al., 2001b).

If we assume that these genes enhance the robustness of the plant and their ability to propagate, then the loss of one or more *SEP* genes under non-standard experimental conditions should affect the fitness of the plant. In this case, such plants should have disadvantages with regards to their ability to propagate. Such changing environmental conditions could be for example an increase in the temperature. This is a situation that can be easily simulated under laboratory conditions.

In this chapter, the hypothesis that *SEP* genes affect robustness will be tested by growing WT, *sep* single and mutant combinations at two different temperature conditions; 22 °C (ideal growth conditions) and 27 °C (to simulate a change in the environment and expose the plants to elevated stress levels). In the case of full redundancy, single and double mutants should be similar to WT plants and unaffected in their development. If *SEP*s do not act fully redundantly and the plant maintained multiple copies of them to buffer its response to changing environmental conditions, a difference compared to the WT plants should be visible in single or double mutants when challenged by such conditions. This chapter describes the analysis of all four *SEP* single mutants and their double and triple mutant combinations under standard and non-standard temperature conditions.

3.2 Organ specific functions for single *SEPALLATA* genes

The four *SEP* genes have been described to act redundantly and it has been reported that single mutants have no, or only subtle, phenotypes (Pelaz S. et al., 2000; Ditta G.S. et al., 2004). The only *sep* single mutant reported to have a phenotype is *sep3* (*sep3-1* and *sep3-2*). *sep3* mutants are described as having petals that show a partial conversion towards sepals (Pelaz S. et al., 2001b) and inward folded petal tips for the *sep3-2* allele (Airoldi C.A. et al., 2010, Airoldi C. A. personal communication). However, in this study, all experiments were carried out with the *sep3-2* allele. To test the redundancy of the *sep* single mutants and their ability to specify normal floral development at elevated temperatures, the four published mutant alleles of the *SEP* genes (Pelaz S. et al., 2000; Ditta G.S. et al., 2004) were grown at 22 °C and 27 °C alongside a new *SEP1* allele, *sep1-2*. (SALK_011077, carries an insertion in exon 7). Furthermore, a second *SEP2* allele, *sep2-2* (GABIKAT 246B03, carries an insertion in the UTR after the stop codon) and a second *SEP4* allele, *sep4-2* (SALK_006229, carries an insertion in the first intron) were also grown alongside WT plants. *SEP* gene expression was analyzed in the new independent mutant alleles *sep1-2*, *sep2-2* and *sep4-2* by quantitative Real-Time PCR (qPCR), which confirmed a significant reduction in the RNA expression levels only of the affected genes (qPCR results shown in Supplementary data S1a *SEP1* expression in SALK_011077, S1b: *SEP2* expression in GABI-KAT246B03, S1c: *SEP4* expression in SALK_006229). The published alleles *sep1-1*, *sep2-1*, *sep3-2* and *sep4-1* were previously confirmed as RNA nulls (Pelaz S. et al., 2000; Ditta G.S. et al., 2004). All analysed mutants were confirmed by genotyping as being homozygous for the mutation.

sep1-1 and *sep2-1* have been previously described as aphenotypic (Pelaz S. et al., 2000) and this was confirmed in our analysis at 22 °C (Fig. 3.1 b, d). The same is true for the two new alleles *sep1-2* and *sep2-2* (Fig. 3.1 c, e). However, I observed phenotypes for mutants affecting either *SEP1* or *SEP2* (*sep1-1, sep1-2, sep2-1*) at 27 °C. Mutants affecting the *SEP1* gene (*sep1-1* and *sep1-2*) showed shorter carpels at 27 °C than the WT (Table 3.1, Fig. 3.1 k, l), while no other floral organs were affected. *sep2-1* flowers showed an increased frequency of bent carpels (Table 3.2, Fig. 3.1 m, n), 36 % in the *sep2-1* mutant vs. 12 % in the WT flowers and carpels of the mutant were occasionally split. The second *SEP2* allele (*sep2-2*, GABI-KAT 246B03) was indistinguishable from WT flowers at both growth conditions (Fig. 3.1 e, q). One explanation for the aphenotypic allele could be the position of the insertion. *sep2-1* carries an insertion in the coding region (in intron 6), whereas the second allele, *sep2-2* carries an insertion outside of the coding region (170 bp after the stopcodon). However, to be certain

that the phenotype observed for the *sep2-1* allele is attributable to loss of the *SEP2* gene, further experiments are necessary, for example a complementation analysis or a phenotypic analysis with other alleles. Unfortunately no other insertion lines for this gene was available at that time, so all further experiments will be carried out with the *sep2-1* allele and the *sep2-2* allele will not be included in further analysis. In addition to the bent carpel phenotype observed in *sep2-1* mutants, we noticed that petals were occasionally narrower compared to those of WT at 27 °C (Fig. 3.1 o, p), whereas the carpel length was not affected. No phenotype was observed in the *sep2-2* mutants affecting the size of the petals. Flowers of the *sep3-1* and *sep3-2* mutant were already described as having a phenotype affecting petals, with a partial transformation of petals into sepals (Pelaz S. et al., 2001b) and inward folded petal tips for the *sep3-2* allele (Airoldi C.A. et al., 2010, Airoldi C. A. personal communication). This phenotype affecting the petal blade was confirmed at both temperatures. At 22 °C, 72.5 % of *sep3-2* mutant flowers had petals that were folded at the tips, whereas WT flowers showed this phenotype very rarely (5 %), (Table 3.3, Fig. 3.1 f). If the petal blade was affected in the mutant, all four petals showed this phenotype. The same can be said for WT petals, if they revealed folded tips, usually all four petals were affected. At elevated temperatures, this phenotype was observed again with 50 % of *sep3-2* mutants having folded petal tips compared to 16 % of WT flowers (Table 3.3, Fig. 3.1 r). The partial conversion of petals into sepals was more difficult to observe. *sep3-2* single mutants revealed this phenotype occasionally but very mildly, independent of whether plants were grown at 22 °C or 27 °C. At 22 °C, 7.5 % of *sep3-2* mutants showed a partial conversion of petals into sepals, while this phenotype was not observed in WT flowers (Table 3.3, Fig. 3.1 f, shown with a yellow arrow). At elevated temperatures, 6 % of WT flowers and 7 % of *sep3-2* mutant flowers showed the partial conversion of petals into sepals (Table 3.3). The inward folding of the petal tips is therefore a more reliable phenotype in *sep3-2* mutants in this study, although only one mutant allele is available for this gene. The two *SEP4* mutant alleles (*sep4-1* and *sep4-2*) showed an increase in the number of perianth organs at both temperature conditions compared to WT (Fig. 3.1 g, h, i, s, t). At 22 °C, 28 % of sep*4-1* and 34 % of *sep4-2* mutant flowers displayed extra sepals and 29 % of sep*4-1* and 34 % of sep*4-2* flowers had additional petals (usually five instead of four sepals and petals) (Table 3.4), while WT flowers developed these additional organs, at a very low frequency of 1.6 % (Table 3.4). At elevated temperatures 31 % of sep*4-1* and 27 % of *sep4-2* mutant flowers produced extra sepals and 29 % had extra petals (Table 3.4), whereas only 3 % of WT flowers showed this phenotype (Table 3.4). Extra organs in the first and second whorls has never been previously described for the characterized *sep4-1* allele (Ditta G.S. et al., 2004). The second *SEP4* allele, *sep4-2*, has been first analysed in this work and shows the same phenotype as the *sep4-1* allele.

In summary, this analysis shows that two independent *sep1* mutants produce shorter carpels and *sep2-1* mutants have bent carpels, although the carpels of the second *sep2* allele (*sep2-2*) are unaffected necessitating further experiments. The newly discovered *sep1* and *sep2* phenotypes are only visible at elevated temperature conditions. In addition to the bent carpel phenotype for *sep2-1* mutants, a phenotype affecting the width of the petals was observed, as these mutants also show narrower petals at 27 °C. The previously reported phenotype for *sep3-2* mutants affecting the petal blade with folded tips was confirmed. The conversion of petals into sepals was observed at 22 °C, whereas at 27 °C this phenotype was not observed compared to WT flowers. In addition a new phenotype was discovered in two independent *sep4* alleles, increasing the number of perianth organs at both 22 and 27 °C. All phenotypes for *sep* single mutants are summarized in Table 3.5.

Table 3.1

	Average length of carpels in cm (± SEM)	Individual siliques analyzed / independent plants
WT at 27 °C	1.06 (± 0.031)	48/4
sep1-1 at 27 °C	0.85 (± 0.014) *	66/5
sep1-2 at 27 °C	0.87 (± 0.014) *	90/6

Table 3.1: Average carpel length of *sep1-1*, *sep1-2* and WT siliques grown at 27°C

Standard Error of the Mean (SEM) in brackets, *= $p < 0.01$

Table 3.2

	bent carpels per analyzed flowers	in %	individual flowers analyzed / individual plants
WT at 27 °C	5/40	12.5	40/5
sep2-1 at 27 °C	40/110	36	110/10
sep2-2 at 27 °C	6/50	12	50/7

Table 3.2: Carpel morphology of *sep2-1* and *sep2-2* compared to WT flowers at 27 °C.

Table 3.3

	Flowers showing folded tips at the edges of all four petals	% of analyzed flowers	number of flowers with at least one sepaloid petal	% of analyzed flowers	individual flowers analyzed / individual plants
WT at 22 °C	1/20	5	0/20	0	20/4
sep3-2 at 22 °C	58/80	72.5	6/80	7.5	80/10
WT at 27 °C	10/60	16	4/60	6	60/4
sep3-2 at 22 °C	36/71	50	5/71	7	71/9

Table 3.3: Analysis of *sep3-2* and WT mutants at 22 and 27 °C

The percentage of flowers affected in the petal blade with folded tips of all four petals and flowers that showed a transformation of sepals into petals.

Table 3.4

	4 sepals in %	5 sepals in %	6 sepals in %	4 petals in %	5 petals in %	6 petals in %	Individual flowers analyzed /individual plants
WT at 22 °C	100	0	0	98.4	1.6	0	60/4
sep4-1 at 22 °C	69.5	28.5	2	68.5	29.5	2	105/9
sep4-2 at 22 °C	62.9	34.1	2.9	62.3	34.7	2.9	85/10
WT at 27 °C	98.4	1.6	0	96.7	3.3	0	60/4
sep4-1 at 27 °C	68.9	31.1	0	70.1	29.9	0	119/12
sep4-2 at 27 °C	72.3	27.7	0	71	29	0	83/8

Table 3.4: Phenotypic analysis for sepal and petal number in *sep4-1*, *sep4-2* and WT flowers

Experiments were carried out at 22 and 27 °C, %= flowers having a defined number of sepals and petals.

Table 3.5

Mutant	sepals	Petals	stamens	carpels
sep1-1	-	-	-	short at 27 °C
sep1-2	-	-	-	short at 27 °C
sep2-1	-	narrow at 27 °C	-	bent at 27 °C
sep3-2	-	petal blade affected at both temperatures, mild partial conversion of sepals into petals at low frequency at 22 °C	-	-
sep4-1	extra organs at both temperatures	extra organs at both temperatures	-	-
sep4-2	extra organs at both temperatures	extra organs at both temperatures	-	-

Table 3.5: Summary of phenotypes from *sep* single mutants .

Legend: - = unaffected

Fig. 3.1

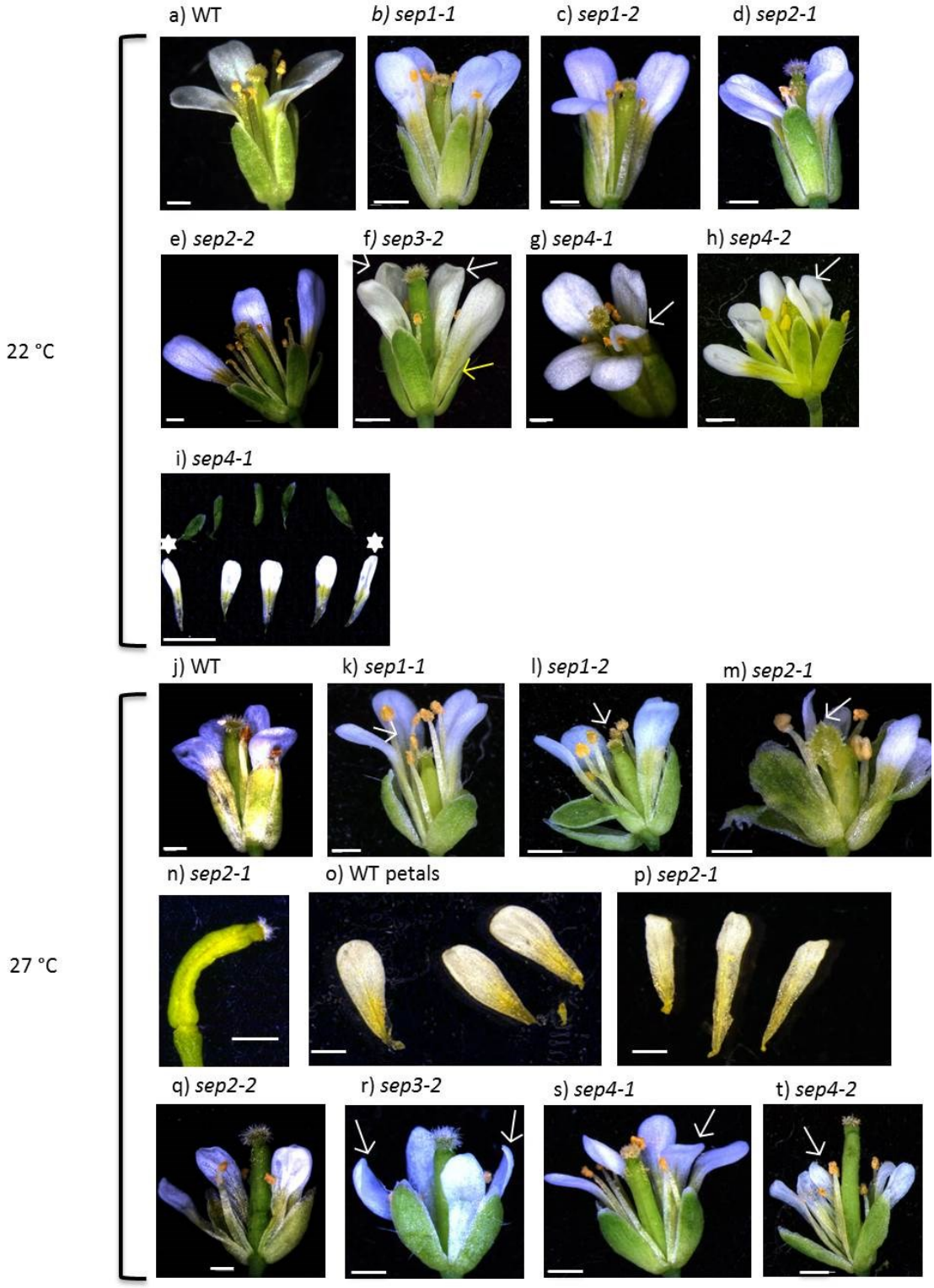


Fig. 3.1: *SEP* single mutants grown at 22 and 27 °C.

a) WT flower grown at 22 °C, b) *sep1-1*, c) *sep1-2*, one petal was removed from the flower, d) *sep2-1*, e) *sep2-2*, one petal was removed from this flower,

Legend to be continued

Fig. 3.1 continued…

f) *sep3-2* showing folded petal tips (marked with two white arrows) and one sepaloid petal (marked with a yellow arrow), g) *sep4-1* having an extra petal (marked with a white arrow), h) *sep4-2* having an extra petal (marked with a white arrow), i) dissection of *sep4-1* flower showing five sepals and petals, note that two petals are narrower than the others (marked with two asterisks), j) WT flower grown at 27 °C, k) *sep1-1* flower having a shorter carpel (marked with a white arrow), l) *sep1-2* flower having a shorter carpel (marked with a white arrow), m) *sep2-1* flower with a slightly bent carpel (marked with a white arrow), n) carpel of a *sep2-1* flower, o) dissection of three WT petals, p) dissection of three *sep2-1* petals, q) *sep2-2* flower, r) *sep3-2* flower with slightly folded petal tips (marked with two white arrows),
s) *sep4-1* flower having an extra petal (marked with a white arrow), t) *sep4-2* flower with an extra petal (marked with a white arrow). Bar in n = 0.5 mm, all other pictures, Bars = 1 mm

3.3 Double mutants reveal both, overlapping and distinct phenotypes

The single mutant analysis described in the previous section (3.2) showed that mutants for all four *SEP* genes cause morphological changes, although the phenotypes for *sep1* and *sep2* mutants are only obvious at elevated temperatures. The phenotype of each mutant is distinct from the others, suggesting that each SEP protein has both overlapping and individual functions. To study the interactions between the separate *SEP* mutants, the following double mutants were generated in this work: *sep1-1sep2-1* hereafter named *sep1sep2*, *sep1-1sep3-2* hereafter named *sep1sep3*, , *sep2-1sep3-2* hereafter named *sep2sep3, sep2-1sep4-1,* hereafter named *sep2sep4* and *sep3-2sep4-1,* hereafter named *sep3sep4.* The *sep1-1sep4-1* (hereafter named *sep1sep4*) double mutant was previously generated by Dr. Chiara Airoldi and used in this work. All double mutants were confirmed by PCR before analysed. The double mutants were analysed at both high and standard temperature conditions. These double mutant combinations revealed a wide range of phenotypes, especially at elevated temperatures, some of which could not have been predicted from the single mutant phenotypes.

Flowers of the *sep1sep2* double mutant

At 22 °C *sep1sep2* double mutants had a mild phenotype, showing a slight reduction in the number of sepals (Table 3.6), a slightly shorter carpel and narrower sepals (Fig. 3.2 b, c). The majority of analysed flowers had four sepals (94 %, Table 3.6) and the petal number was unaffected. A small proportion of analysed plants developed three instead of four sepals in early arising flowers and often one sepal was affected in its morphology. Figure 3.2 b shows a flower having one narrower sepal and Fig. 3.2 c shows an early arising flower that has one sepal that is not fully developed and much shorter than the others. Petal number was not affected in this double mutant. At 27 °C, these mutants showed an increased frequency of sepal loss, whereas the number of petals remained unaffected. Around 80 % of dissected flowers had two instead of four sepals, but all tested WT flowers had four petals (Table 3.6, Fig. 3.2 j, k). As well as a reduction in the number of sepals, branched trichomes frequently appeared on the *sep1sep2* sepals with a frequency of 40 %, whilst branched trichomes are seen on less than 1 % of WT sepals (Table 3.6).

Flowers of the *sep1sep3* double mutant

At standard growth conditions, the *sep1sep3* double mutant had a phenotype with folded petal tips at approximately the same frequency as the *sep3-2* single mutant (Table 3.7

compared to Table 3.3) and, in addition, 52 % of these flowers developed shorter stamens (Table 3.7, Fig. 3.2 d, marked with a yellow arrow). In contrast, at 27 °C, plants were severely affected as 95 % of analyzed flowers showed a conversion of petals into sepals (Table 3.7, Fig. 3.2 l). The majority of the plants (85 %, Table 3.7) had shorter stamens and these stamens did not produce pollen. As a consequence did these plants not produce seeds.

Flowers of the *sep1sep4* double mutant

At 22 °C the *sep1sep4* double mutants frequently developed extra perianth organs, 54 % had five sepals and 55 % had five petals and very rarely six petals were observed (Table 3.6, Fig. 3.2 e). A more striking phenotype was observed at 27 °C, where these flowers developed secondary flowers in the axils of the first whorl organs (Fig. 3.2 m, n). 61 % of the flowers developed secondary flowers along with a reduction in the number of sepals and petals, usually one sepal and one or two petals fewer than WT (Table 3.6). Secondary flowers have been reported in other mutants, for example in strong *ap1* alleles, and are interpreted as a reversion of a floral meristem to an inflorescence meristem (Mandel M.A. et al., 1992). In addition to secondary flowers, single flowers occasionally revealed sepal petal hybrids in the first whorl as seen in Fig. 3.2 n (marked with a yellow arrow). At elevated temperatures flowers rarely developed extra perianth organs, 7 % developed an additional sepal and petal whereas branched trichomes were found frequently on sepals (Table 3.6, 80 %). If flowers developed extra perianth organs, they never developed secondary flowers. This phenotype could not have been predicted based on the two single mutants, since *sep1* has a short carpel phenotype (Table 3.1, Fig. 3.1 k, l) and *sep4* has extra sepals and petals at 27 °C (Table 3.4, Fig. 3.1 s, t).

Flowers of the *sep2sep4* double mutant

Analysis of the *sep2sep4* double mutant revealed that flowers more often had extra sepals and petals at both temperature conditions compared to WT, but no other floral organs were affected (Table 3.6, Fig. 3.2 f, p). This phenotype was similar at 27 °C with 25 % of flowers that had five instead of four sepals and 36 % of flowers developed five instead of four sepals (Table 3.6, Fig. 3.2 p).

Flowers of the *sep2sep3* double mutant

Flowers of the *sep2sep3* double mutants revealed mild phenotypes with petals that had folded tips at both temperature conditions. At 22 °C, 48 % had petals with folded tips and this phenotype was slightly increased at elevated growth conditions with 62 % of all analysed flowers showing that phenotype (Table 3.7, Fig. 3.2 g, o). Stamens were shorter at both

temperatures (32 % of stamens at 22 °C, Fig. 3.2 g, blue arrow and 20 % of stamens at 27 °C). Carpel morphology was affected in *sep2sep3* double mutants at 27 °C, as 30 % showed carpels that were bent to variable degrees (from slightly bent to strongly bent) (Table 3.7), whereas this phenotype was seen very rarely at 22 °C (Table 3.7).

Flowers of the *sep3sep4* double mutant

The *sep3sep4* double mutant was reported to have no phenotype compared to WT plants (Ditta G.S. et al., 2004),. However phenotypic analysis in this study revealed a phenotype. As previously described, *sep4* mutants revealed extra sepals and petals in both single mutants (*sep4-1* and *sep4-2*) and double mutants in combination with *sep1* or *sep2* at 22 °C (*sep1sep4* and *sep2sep4*), (Table 3.4, 3.5, 3.6). Interestingly the *sep3sep4* flowers did not show an increase in the observed number of perianth organs, only 7 % of flowers of *sep3sep4* double mutants grown at 22 °C developed extra perianth organs and 3 % showed a reduction in the number of sepals and petals (3 instead of four) (Table 3.6). The same was observed when grown under elevated temperature conditions, less than 5 % showed an extra sepal or petal whereas 4.6 % showed a reduction in the number sepals and 2.3 % developed fewer petals. Petal blade was affected at a high frequency at both temperatures, as evidenced with folded petal tips, 60 % at 22 °C and 70 % at 27 °C (Table 3.7, Fig. 3.2 h, q). In addition, changes in petal identity were observed at both temperature conditions, as *sep3sep4* double mutants showed sepaloid petals. 36 % of *sep3sep4* flowers developed sepaloid petals at 22 °C and, at 27 °C, 30 % revealed that phenotype which was rarelyobserved in WT flowers (Table 3.8, Fig. 3.2 h, q, shown with a yellowarrow at 27 °C). As seen for all double mutants containing a *sep3* mutant allele, stamens were shorter at both temperature conditions (47 % at 22 °C and 55 % at 27 °C, Table 3. 7, Fig. 3.2 h, shown with a yellow arrow).

In summary, the double mutant analysis revealed overlapping phenotypes that were already characterized in single mutants described in the previous section. The petal blade was affected in *sep3-2* single mutants at both temperature conditions and this was also observed in all double mutant combinations carrying a *sep3-2* allele at both temperature conditions at approximately the same frequency (Table 3.3, *sep3-2* single mutant, Table 3.7, double mutants carrying a mutant *sep3* allele).

A similar result can be seen with respect to the increased perianth organ number observed in mutants carrying a mutant *sep4* allele. The two *sep4* single mutants (*sep4-1*, *sep4-2*) and the *sep2sep4* double mutant revealed extra perianth organs at approximately the same frequency (Table 3.4, *sep4* single mutants, Table 3.6, *sep2sep4* double mutant) at both temperature

conditions. The *sep1sep4* double mutant shows a slightly higher frequency of extra perianth organs than the *sep4* single and the *sep2sep4* double mutants, with 54 % extra sepals and 54 % extra petals, (Table 3.4, *sep4* single mutant, Table 3.6, *sep1sep4* double mutant) at 22 °C. Interestingly, the *sep1sep4* double mutant grown at 27 °C revealed a new phenotype. Mutants developed secondary flowers in the axils of first whorl organs at a high frequency of 61 % (Table 3.6, Fig. 3.2 m, n); this phenotype is associated with a reversion of floral meristems to inflorescence meristems. An exception is seen in the case of the *sep3sep4* double mutant, which showed no increase in perianth organs (Table 3.4, *sep4* single mutant, Table 3.6, *sep3sep4* double mutant). Mutations in the *SEP3* gene do not affect organ number, whereas *sep4* mutants show an increased perianth organ number. Both mutants combined reveal flowers with roughly the normal organ number but show deviations over and under the normal number. The accuracy of setting the organ number is affected and leads to the idea that both genes are involved in a mechanism involved in setting the correct organ number.

The two *sep* single mutants *sep1-1* and *sep2-1* were affected in carpel length and carpel morphology. Carpel length was affected in *sep1* single mutants at 27 °C and in the *sep1sep2* double mutant at 22 °C. Carpel morphology, with a bent carpel phenotype, was seen *sep2-1* single mutants at 27 °C (Table 3.2) and in *sep2sep3* double mutants at 22 °C (Table 3.7). Remarkably, at 27 °C distinct and more severe phenotypes were observed that were not seen in *sep* single mutants. All double mutants carrying a mutant *sep3* allele (*sep1sep3*, *sep2sep3*, *sep3sep4*) showed shorter stamens (Table 3.7). As well as affecting stamen length, *sep1sep3* double mutants show a change in organ identity as evidenced by the development of sepals in the second whorl instead of petals (Table 3.7, Fig. 3.2 l). The number of floral organs was reduced in the *sep1sep2* double mutant at 27 °C, as these flowers developed two instead of four sepals at a high frequency, whereas the number of other floral organs was not affected (Table 3.6, Fig. 3.2 j, k). All phenotypes of *sep* single and double mutants are summarized in Table 3.9.

Table 3.6

	6 se %	5 se %	4 se %	3 se %	2 se %	6 pe %	5 pe %	4 pe %	3 pe %	2 pe %	sec. flowers	sepals with at least one branched trichome	Individual flowers analysed/ individual plants
WT	-	-	100	-	-	-	1.5	98.5	-	-	-	>5	55/8
WT	-	-	100	-	-	-	1.8	98.2	-	-	-	>5	60/4
sep1sep2	-	-	94	3	3	-	-	100	-	-	-	>5	42/6
sep1sep2	-	-	20	-	80	-	-	100	-	-	-	40	55/9
sep1sep4	-	54	45	-	-	2	53	43	-	-	-	>5	81/13
sep1sep4	-	7	48	40	5	-	7	46	35	12	61	80	80/12
sep2sep4		33.7	66	-	-	-	28.7	71	-	-	-	>5	80/12
sep2sep4	-	24.9	65	-	-	-	36.9	63	-	-	-	>5	30/3
sep3sep4	-	7	90	3	-	-	7	89.5	3.5	-	-	10	63/10
sep3sep4	-	4.4	90	4.6	-	-	4.6	94	2.3	-	-	20	55/8

Table 3.6: Morphological analysis of *sep1sep2*, *sep1sep4*, *sep2sep4*, *sep3sep4* and WT flowers at 22° C and 27 °C

The number of extra perianth organs was measured (% - flowers having a defined number of sepals (se), petals (pe)), the frequency of the development of secondary flowers was measured (% = measurement how often a secondary flowers was observed in all counted flowers) and branched trichomes on sepals (% = measurement of all individual flowers how often sepals revealed at least one branched trichome), white rows = experiments carried out at 22° C, red rows = experiments carried out at 27° C

Table 3.7

	petal blade (folded tips), %	short stamens, %	conversion of petals to sepals, %	bent carpels, %	individual flowers anlalyzed/ individual plants
WT at 22 °C	4	5	-	1	40/4
WT at 27 °C	5	5	-	10	50/4
sep1sep3 at 22 °C	71	52	-	-	73/7
sep1sep3 at 27 °C	no effect, conversion of petals to sepals	85	95	1	43/6
sep2sep3 at 22 °C	48	32	-	5	45/7
sep2sep3 at 27 °C	62	20	-	30	37/4
sep3sep4 at 22 °C	60	47	-	-	63/10
sep3sep4 at 27 °C	70	55	-	-	43/8

Table 3.7: Morphological analysis of WT, *sep1sep3*, *sep2sep3* and *sep3sep4* double mutant flowers at 22 °C and 27 °C.

Effects on petal, stamen and carpel morphology were analysed and compared to WT flowers at two temperature conditions (22 **°C** and 27 °C). Red rows = experiments carried out at 27 °C, white rows = experiments carried out at 22 °C, %= frequency how often the phenotype was observed in analysed flowers

Table 3.8

	sepaloid petals, %	individual flowers anlalyzed/ individual plants
WT at 22 °C	3	30/4
WT at 27 °C	3	25/3
sep3sep4 at 22 °C	36	63/10
sep3sep4 at 27 °C	30	43/8

Table 3.8: Phenotypic analysis of *sep3sep4* mutant and WT flowers at 22 °C and 27 °C

%= frequency how often sepaloid petals were observed in analysed flowers

Table 3.9

	1st whorl	2nd whorl	3rd whorl	4th whorl	sec. flowers	branched trichomes
sep1	normal	normal	normal	normal	no	no
sep1	normal	normal	normal	shorter	no	no
sep2	normal	normal	normal	normal	no	no
sep2	normal	narrower	normal	bent	no	no
sep3	normal	folded tips	normal	normal	no	no
sep3	normal	folded tips	normal	normal	no	no
sep4	↑	↑	normal	normal	no	no
sep4	↑	↑	normal	normal	no	no
sep1sep2	↓	normal	normal	slightly shorter	no	no
sep1sep2	↓	normal	normal	normal	no	↑
sep1sep3	normal	folded tips	short	normal	no	no
sep1sep3	normal	conversion into 1st whorl	short	normal	no	no
sep1sep4	↑	↑	normal	normal	no	no
sep1sep4	↓	↓	normal	normal	yes	↑
sep2sep3	normal	folded tips	short	bent	no	no
sep2sep3	normal	folded tips	short	bent	no	no
sep2sep4	↑	↑	normal	normal	no	no
sep2sep4	↑	↑	normal	normal	no	no
sep3sep4	variable, ↑ or ↓	folded tips, sepaloid, number of petals variable, ↑ or ↓	short	normal	no	↑
sep3sep4	variable, ↑ or ↓	folded tips, seploid, number of petals variable, ↑ or ↓	short	normal	no	↑

Table 3.9: Summary of all analyzed single and double mutants compared to WT flowers

The table shows the organs that are affected in each single mutant and double mutant combination. Red rows = experiments carried out at 27 °C, white rows = experiments carried out at 22 °C, ↑= increase in organ number, ↓= decrease in organ number,

Fig. 3.2

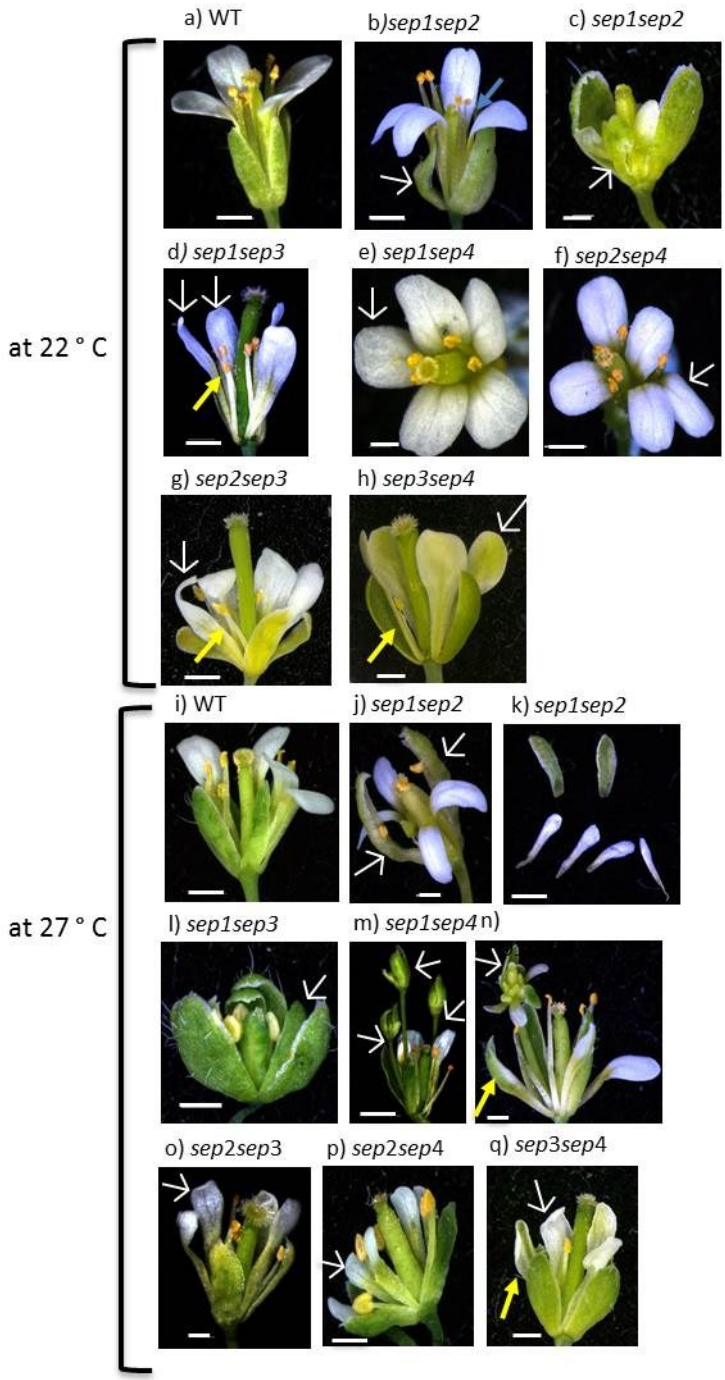


Fig. 3.2: *SEP* double mutants grown at 22 and 27 °C

a) WT flower grown at 22 °C, b) *sep1sep2* double mutant flower with a narrow sepal (white arrow) and slightly shorter carpel (yellow arrow), c) an early arising *sep1sep2* flower with one sepal shorter and not fully developed (white arrow), Legend to be continued

Fig. 3.2 continued…

d) flower of *sep1sep3* double mutant with petals that have folded tips (white arrows) and shorter stamens (yellow arrow), sepals were removed from this flower e) flower of *sep1sep4* double mutant showing a fifth petal (white arrow), f) flower of *sep2sep4* double mutant having a fifth petal (white arrow), f) flower of a *sep2sep3* double mutant with petals that have folded tips (white arrow) and shorter stamens (yellowarrow), h) flower of a *sep3sep4* double mutant having sepaloid petals (white arrow) and short stamens (yellow arrow). The same mutants grown at 27 °C shown, i) WT flower, j) *sep1sep2* double mutant flower with two sepals (white arrows) and four petals, k) dissection of another *sep1sep2* flower shows two sepals and four petals, l) flower of *sep1sep3* double mutant shows a conversion of petals into sepals (white arrow), m) flower of a *sep1sep4* double mutants has three secondary flowers (white arrows), n) flower of a *sep1sep4* double mutant has one secondary flower (white arrow) and a sepal-petal hybrid (yello arrow), o) flower of a *sep2sep3* double mutant with petals that have folded tips (white arrow), p) flower of *sep2sep4* double mutant having a fifth petal (white arrow), q) flower of *sep3sep4* double mutant showing petals that have folded tips (white arrow) and a sepaloid petal (yellow arrow). Bars= 1mm for a, b, c, d, g, h, I, n, Bars= 0.5 mm for e, f,j, l,o, p Bar= 0.3 mm for m, Bar= 1.5 mm for k

3.4 Triple mutants are severely affected in floral organ development and reproductive fitness

Flowers of the *sep1sep2sep3* triple mutants were previously described and show a conversion of floral organs into sepals and a loss of floral meristem determinacy together with secondary flowers that occasionally develop in the axils of first whorl organs (Pelaz S. et al., 2000). The published *sep1sep2sep3* phenotype was also observed in this study, when growing the triple mutant at 22 °C and 27 °C (Fig. 3.3 b and Fig. 3.4 b). All analyzed flowers showed a conversion of floral organs into sepals (Table 3.10, 3.14).

The *sep1sep2sep4* triple mutant was reported to have no significant perturbation of flower development (Ditta G.S. et al., 2004). Based on the finding that single mutants showed morphological phenotypes at different growth conditions and that the double mutants showed overlapping as well as distinct phenotypes, all four triple mutants (*sep1sep2sep3*, *sep1sep2sep4*, *sep1sep3sep4*, *sep2sep3sep4*) were reanalyzed. The *sep1sep2sep3*$^{+/-}$ and *sep1sep2sep4* triple mutants were obtained from Dr. Chiara Airoldi. The *sep1sep3sep4* and *sep2sep3sep4* triple mutants were generated in this work. In order to confirm the genotype, the four triple mutants were genotyped by PCR. All triple mutants were severely affected and showed a variation in the strength of their phenotype.

Flowers of the *sep1sep2sep4* triple mutant

sep1sep2sep4 flowers grown at 22 °C develop secondary flowers at a high frequency of 64 %. The development of secondary flowers can be interpreted as a reversion of floral meristems into inflorescence meristems or an incomplete conversion of inflorescence to floral meristems. Additionally, a deviation from the standard number of four sepals and petals in both directions (up and down) was observed (Table 3.10, Fig. 3.3 c, d, e). Stamen number and development was not affected and this triple mutant was able to produce seeds.

Flowers of the *sep1sep2sep4* triple mutant grown at 27 °C were severely affected, showing a dramatic reduction in the number of sepals and petals. Flowers often produced only one or two sepals (Fig. 3.4 e) and in 21 % of flowers, a total absence of perianth organs was observed (Table 3.10, Fig. 3.4 d). 16 % of all analyzed flowers developed secondary flowers (Table 3.10) and stamen number showed a deviation from the 6 observed in WT flowers (Table 3.11). Also it is worth noticing that these stamens produce less or no pollen. Often, stamens were green (Fig. 3.4 d) and short and very rarely bifurcated (less than 2 %, data not shown). These mutants rarely produced seeds.

In summary: at 22 °C *sep1sep2sep4* mutants revealed changes in meristem identity with a reversion of floral meristems into inflorescence meristems evidenced with secondary flowers, together with a change in the perianth organ number. Phenotypes were much stronger in early arising flowers than in older flowers. At 27 °C all organs were massively affected in *sep1sep2sep4* triple mutants, starting from a change in the number of floral organs (fewer sepals, petals and stamens), lack of floral organs (no sepals or petals) and the development of secondary flowers. Plants were not able to produce seeds at elevated temperatures while at standard growth conditions viable seeds were produced.

Flowers of the *sep1sep3sep4* triple mutant

Flowers of the *sep1sep3sep4* triple mutants showed a wide range of phenotypes. At 22 °C mild phenotypes revealed flowers developing secondary flowers (Fig. 3.3 f). Organ number was affected as well, showing a deviation from the standard of four sepals and petals observed in WT flowers in both directions (increased and decreased number of sepals and petals) (Table 3.10). The number of stamens also showed a deviation from the six stamens of WT flowers in both directions (5.5 % had an increased number and 32,7 % had a decreased number of stamens) (Table 3.11). Around 10 % of flowers showed strong phenotypes with carpeloid sepals (Fig. 3.3 h). If flowers had carpeloid sepals, they usually developed two instead of four sepals. Another phenotype that appeared in this mutant was the development of an elongated gynophore/elongated internode in 45 % of dissected flowers (Table 3.11, Fig. 3.3 g, i).

At 27 °C the *sep1sep3sep4* triple mutant showed more severe phenotypes. The number of sepals and petals was dramatically decreased compared to flowers at 22 °C. Flowers often had only two or no sepals and petals (Table 3.11, Fig. 3.4 f) and 84 % developed secondary flowers (Table 3.10, Fig. 3.4 f, g, h). The carpeloid sepal phenotype was not observed, but 74 % of these mutants developed elongated gynophores/elongated internodes (Table 3.11, Fig. 3.4 h). In addition to the phenotype affecting perianth organ number and carpel morphology, the number of stamens was also affected. A deviation in both directions was observed (increase and decrease) from the six stamens normally observed in WT flowers (Table 3.11). Seed production was strongly decreased at 22 °C, with only a few seeds per plant, whereas at 27 °C, no seed production was observed. As a consequence, this triple mutant had to be maintained as heterozygous line (*sep1*$^{+/-}$*sep3sep4*, *sep1sep3*$^{+/}$*sep4*$^{+/-}$ or *sep1sep3sep4*$^{+/-}$) for future experiments.

To summarize; flowers of the *sep1sep3sep4* triple mutant are strongly affected at 22 and

27 °C, developing secondary flowers at a high frequency as was observed for *sep1sep2sep4* mutants. The number of perianth organs was reduced at 27 °C. The number of stamens deviates from the six usually observed in WT flowers at both conditions. The development of carpeloid sepals, seen in *sep1sep3sep4* triple mutants at 22 °C and the production of elongated internodes at 22 and 27 °C is a novel phenotype that was not observed in other single, double or triple mutants in this study.

Flowers of the *sep2sep3sep4* triple mutant

Compared to the *sep1sep2sep4* and *sep1sep3sep4* triple mutant flowers described above and the published phenotype of the *sep1sep2sep3* triple mutant, which shows a conversion of all floral organs into sepals (Pelaz S. et al., 2000) (Fig. 3.3 b, Fig. 3.4 b), flowers of the s*ep2sep3sep4* triple mutants had a mild phenotype. At 22 °C, 64 % of early arising flowers had sepaloid petals (Table 3.12, Fig. 3.3 j, k, l) and 15 % produced secondary flowers (Table 3.10, Fig. 3.3 j), but compared to the above-described mutants at a lower frequency (for comparison see Table 3.10). Perianth organ number was affected in these mutants as well; 28 % produced extra sepals and 32 % produced extra petals, while the majority of analyzed flowers had four sepals and petals (63 % had four sepals and 65.5 % had four petals, Table 3.10). Flowers very rarely developed fewer than four sepals or petals and the majority (60 %) of flowers had petals with folded tips (Table 3.12, Fig. 3.3 I). Stamen number was also affected in these mutants,
20 % developed five stamens and around 26 % had flowers with four stamens (Table 3.11). More than half of the analyzed flowers were not affected in the number of stamens. The most striking phenotype was visible at later stages of development and affected the carpels. At 22 °C around 17 % of all dissected carpels appeared bulged at stage 14 to 17 (Smyth D.R. et al., 1990) (Table 3.13, Fig. 3.5 a, c). Dissection of these carpels revealed that extra floral organs internal to the fourth whorl carpels were present in *sep2sep3sep4* triple mutant flowers. Ovules developed into carpel-like or leaf-like structures (Fig. 3.5 e, f) and nearly all ovules were aborted.

At 27 °C, the same phenotype was observed at a slightly higher frequency. Around 24 % of all dissected carpels revealed that phenotype (Table 3.13) and at later stages, carpels burst. Interestingly at 27 °C*, sep2sep3sep4* triple mutants did not develop secondary flowers in contrast to all other triple mutants. The majority of analysed flowers revealed no change in the number of perianth organs (Table 3.10) and 10 % had five sepals and ~ 8 % had five petals. Around one-tenth of dissected flowers developed three sepals and petals (15 % had three sepals and 7.8 % had three petals, Table 3.10). Flowers showed a decrease in the number of stamens (Table 3.11) and around 72 % had petals with folded tips that were also narrower

than those of WT (Table 3.12, Fig. 3.4 i, j). Early arising flowers revealed sepaloid petals at a high frequency (Table 3.13). Dissection of single flowers revealed carpels that were burst or split in flowers as seen in Fig. 3.4 k. Flowers of *sep2sep3sep4* triple mutants produced fewer seeds (only some seeds per plant) at 22 °C and no seeds at 27 °C. As a consequence, this triple mutant had to be maintained as heterozygous line (e.g. *sep2sep3sep4*$^{+/-}$) for further experiments.

To summarize the triple mutant combinations; all four triple mutants (*sep1sep2sep3*, *sep1sep2sep4*, *sep1sep3sep4*, *sep2sep3sep4*) were severely affected with regards to flower and reproductive development. Triple mutants show a variation concerning the strength of phenotypes starting with mild phenotypes, like changes in floral organ number and the development of secondary flowers to very strong phenotypes, especially at elevated temperatures, resulting in a loss of floral organ identity and fertility. Except for the *sep2sep3sep4* triple mutant, the other three triple mutant combinations developed secondary flowers at a high frequency. In the *sep1sep2sep4*, *sep1sep3sep4* and *sep2sep3sep4* triple mutants the development of all floral organs was affected in a variable manner, but generally mutants revealed a decrease in the number of floral organs, especially when grown at 27 °C. Interestingly, although triple mutants were severely affected, some showed unique phenotypes. Starting with the most obvious and previously characterized *sep1sep2sep3* triple mutant phenotype showing a conversion of floral organs into sepals (Pelaz S. et al., 2000). One common feature of all other triple mutants (*SEP1sep2sep3sep3, sep1SEP2sep3sep4*, *sep1sep2SEP3sep4*) was the ability to produce all four floral organs (sepals, petals, stamens and carpels), although in some combinations strongly affected. However, the lack of petals, stamens and carpels in the *sep1sep2sep3SEP4* mutant shows that *SEP4* is the only gene unable to confer petal, stamen and carpel identity alone. A unique phenotype for the *sep1sep3sep4* triple mutant was the development of an elongated internode/gynophore. The development of floral organs in the fourth whorl is characteristic of the *sep2sep3sep4* triple mutant. Except for the *sep1sep2sep4* triple mutant at 22 °C, all other triple mutants were severely affected in the production of seeds. Flowers of the *sep1sep2sep3* triple mutant did not produce any seeds under both growth conditions, whereas *sep1sep3sep4* and *sep2sep3sep4* triple mutants produce only a few seeds at 22 °C. At 27 °C, none of the described triple mutants was able to produce seeds. As a consequence, plants were not able to propagate under temperature conditions, 22 °C or 27 °C.

Table 3.10

	5 se (%)	4 se (%)	3 se (%)	2 se (%)	1 se(%)	0 se (%)	5 pe (%)	4 pe (%)	3 pe (%)	2 pe (%)	1 pe (%)	0 pe (%)	Sec. flowers (%)	Individual flowers analysed/ individual plants
sep1sep2sep3	1	99	-	-	-	-	-	-	-	-	-	-	28	25/5
sep1sep2sep3	1	99	-	-	-	-	-	-	-	-	-	-	40	30/4
sep1sep2sep4	26	31	33	9	-	-	26	31	32	10	-	-	64	77/7
sep1sep2sep4	-	4.2	9.5	40.5	23	21	-	4.3	7.4	19	24	21	16	42/5
sep1sep3sep4	11	51	24	22	-	-	8	8	22	61	8	-	76	45/7
sep1sep3sep4	-	5	11	31	26	35	-	-	2.5	15	14	69	84	58/8
sep2sep3sep4	28	63	5	3	-	-	32	65.5	2	-	-	-	15	50/6
sep2sep3sep4	10.5	69	15	2.6	2.6	-	7.8	78	7.8	2.6	-	2.6	-	38/5

Table 3.10: Phenotypic analysis of *sep1sep2sep3*, *sep1sep2sep4* and *sep2sep3sep4* triple mutants carried out at 22° C and 27 °C.

% = flowers having a defined number of perianth organs and developed secondary flowers, white rows = experiments carried out at 22° C, red rows = experiments carried out at 27° C.

Table 3.11

	7 or more stamens (%)	6 stamens (%)	5 stamens (%)	4 stamens (%)	3 stamens (%)	2stamens (%)	1stamen (%)	elongated gynophore (%)	individual flowers analyzed/individual plants
sep1sep2sep4	7	40.4	14	16.6	19	7	-	no	42/5
sep1sep3sep4	5.5	61.8	12.7	20	-	-	-	45	55/7
sep1sep3sep4	17	39.6	18.9	25.8	3.5	1.7	-	74	58/8
sep2sep3sep4	-	51.8	20.9	25.6	-	-	1.6	no	43/5
sep2sep3sep4	-	30	39.8	27.2	-	-	3	no	37/5

Table 3.11: Analysis of the three triple mutants regarding stamen number

Flowers of *sep1sep2sep4, sep1sep3sep4* and *sep2sep3sep4* triple mutants were analyzed with regards to the number of stamens in all analyzed flowers (given in %)and the development of an elongated gymnophore in *sep1sep2sep4* mutants.

Red rows = experiments carried out at 27 ℃, white rows = experiments carried out at 22 ℃

Table 3.12

	sepaloid petals, %	petals with folded tips, %	individual flowers anlalyzed/ individual plants
sep2sep3sep4 at 22 °C	64	60	50/6
sep2sep3sep4 at 27 °C	60	72	38/5

Table 3.12: Analysis of *sep2sep3sep4* triple mutants

Flowers were analysed at 22 °C and 27 C regarding the frequency of sepaloid petals and the development of folded tips at the edges of petals, % = number of analysed flowers that revealed sepaloid petals or folded petal tips.

Table 3.13

	Indeterminate or bulged carpels, in %	individual flowers anlalyzed/ individual plants
sep2sep3sep4 at 22 °C	17	343/5
sep2sep3sep4 at 27 °C	24	105/5

Table 3.13: Analysis of carpel morphology of *sep*2*sep*3*sep*4 triple mutants at 22 and 27 °C.

(% = number of counted carpels that appeared bulged and revealed indeterminate fourth whorl organs)

Table 3.14

	1 st whorl	2 nd whorl	3 rd whorl	4 th whorl	sec. flowers
sep1sep2sep3	normal	sepals	sepals	sepals	Yes
sep1sep2sep3	normal	sepals	sepals	sepals	Yes
sep1sep2sep4	↓	↓	normal	normal	Yes
sep1sep2sep4	↓	↓	normal	normal	Yes
sep1sep3sep4	↓	↓	↓	elongated internodes	yes
sep1sep3sep4	↓	↓	↓	elongated internodes	Yes
sep2sep3sep4	↑	↑, folded tips, sepaloid petals	↓	strongly affected (bulged)	Yes
sep2sep3sep4	↓	folded tips, sepaloid petals	↓	strongly affected (bulged)	No

Table 3.14: Summarizing table of floral whorls affected in all four triple mutants

Red rows = experiments carried out at 27 °C, white rows = experiments carried out at 22 °C, ↓ = decrease in organ number, ↑ = increase in organ number

Table 3.15

Mutant/organ number affected	1st whorl	2nd whorl	3rd whorl	4th whorl	sec. flowers	Seed production
sep1	normal	normal	normal	normal	no	yes
sep1	normal	normal	normal	short	no	yes
sep2	normal	normal	normal	normal	no	yes
sep2	normal	narrower	normal	bent	no	yes
sep3	normal	folded tips	normal	normal	no	yes
sep3	normal	folded tips	normal	normal	no	yes
sep4	↑	↑	normal	normal	no	yes
sep4	↑	↑	normal	normal	no	yes
sep1sep2	↓	normal	normal	shorter	no	yes
sep1sep2	↓	normal	normal	shorter	no	yes
sep1sep3	normal	folded tips	short	normal	normal	yes
sep1sep3	normal	conversion Into 1st whorl	short	short	no	no
sep1sep4	↑	↑	normal	normal	no	yes
sep1sep4	↓	↓	normal	normal	yes	yes
sep2sep3	normal	folded tips	normal	normal	no	yes
sep2sep3	normal	folded tips	short	bent	no	yes
sep2sep4	↑	↑	normal	normal	no	yes
sep2sep4	↑	↑	normal	normal	no	yes
sep3sep4	normal	folded tips	short	normal	no	yes
sep3sep4	normal	folded tips	short	normal	no	yes
sep1sep2sep3	normal	sepals	sepals	sepals	yes	no
sep1sep2sep3	normal	sepals	sepals	sepals	yes	no
sep1sep2sep4	↓	↓	normal	normal	yes	yes
sep1sep2sep4	↓	↓	↓	normal	yes	no
sep1sep3sep4	↓	↓	↓	elongated internode	yes	strongly ↓
sep1sep3sep4	↓	↓	↓	elongated internode	yes	no
sep2sep3sep4	↑	↑, folded tips, sepaloid	↓	strongly affected (bulged)	yes, rarely	strongly ↓
sep2sep3sep4	↓	folded tips, sepaloid	↓	strongly affected (bulged)	no	no

Table 3.15: Summarizing Table of affected organs in *sep* single, double and triple mutants

Red rows = experiments carried out at 27 °C, white rows = experiments carried out at 22 °C,

↓ = decrease in organ number, ↑ = increase in organ number

Fig. 3.3

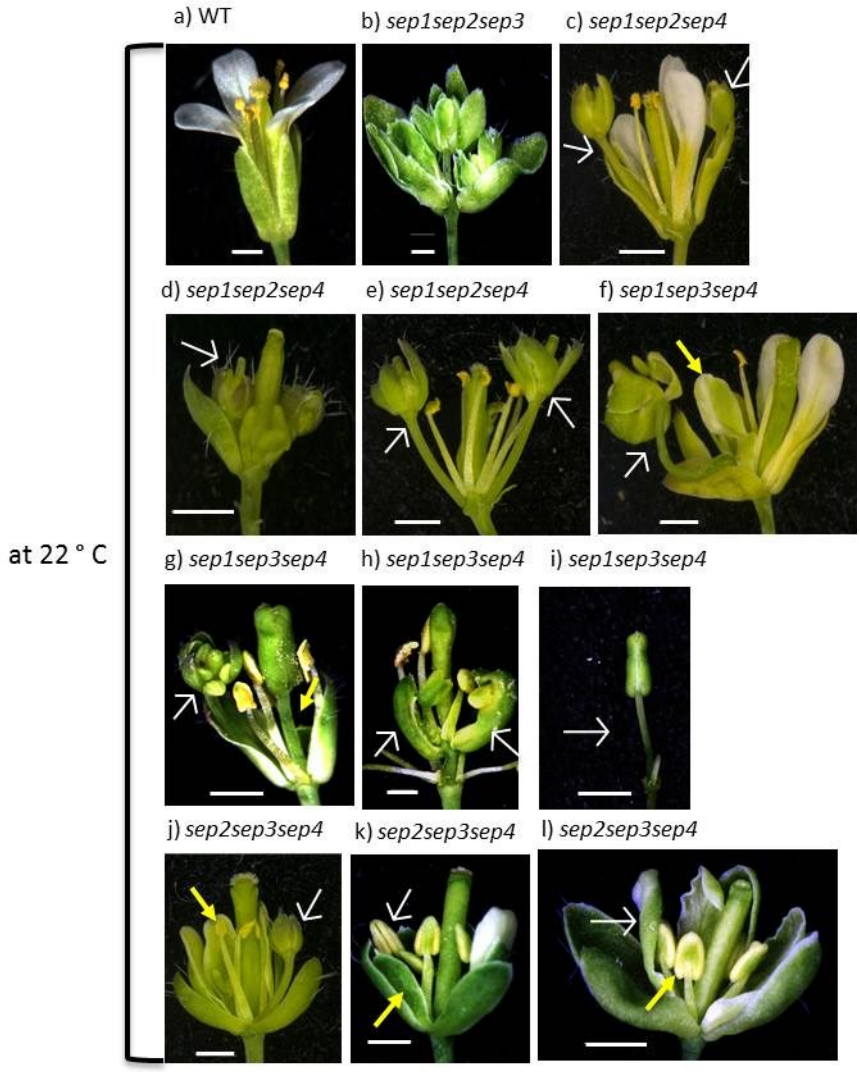


Fig 3.3: *SEP* triple mutants grown at 22 °C

a) WT flower, b) *sep1sep2sep3* triple mutant flower showing a conversion of all floral whorls to sepals, c) *sep1sep2sep4* triple mutant flower with two secondary flowers in the axils of the first whorl organs (white arrows), d) early arising *sep1sep2sep4* triple mutant flower showing a secondary flower (white arrow), e) *sep1sep2sep4* triple mutant flower with two secondary flowers (white arrows) that has no sepals and petals, f) *sep1sep3sep4* triple mutant flower that has a secondary flower (white arrow) and a sepaloid petal (yellow arrow),

Legend to be continued

Fig. 3.3 continued…

g) *sep1sep3sep4* triple mutant flower that has one secondary flower in the axils of the first whorl (white arrow) and an elongated internode (yellow arrow), h) *sep1sep3sep4* triple mutant flower having two carpeloid sepals (white arrows), i) *sep1sep3sep4* triple mutant carpel shows an elongated internode (white arrow), sepals, petals and stamens were removed, j) *sep2sep3sep4* triple mutants flower shows a secondary flower (white arrow) and sepaloid petals (yellow arrow), k) *sep2sep3sep4* triple mutant flower with greenish stamen (white arrow) and a sepaloid petal (yellow arrow), l) *sep2sep3sep4* triple mutant flower that has greenish stamen (blue arrow) and a sepaloid petal that has a folded tip (white arrow).

Bars= 1mm

Fig. 3.4

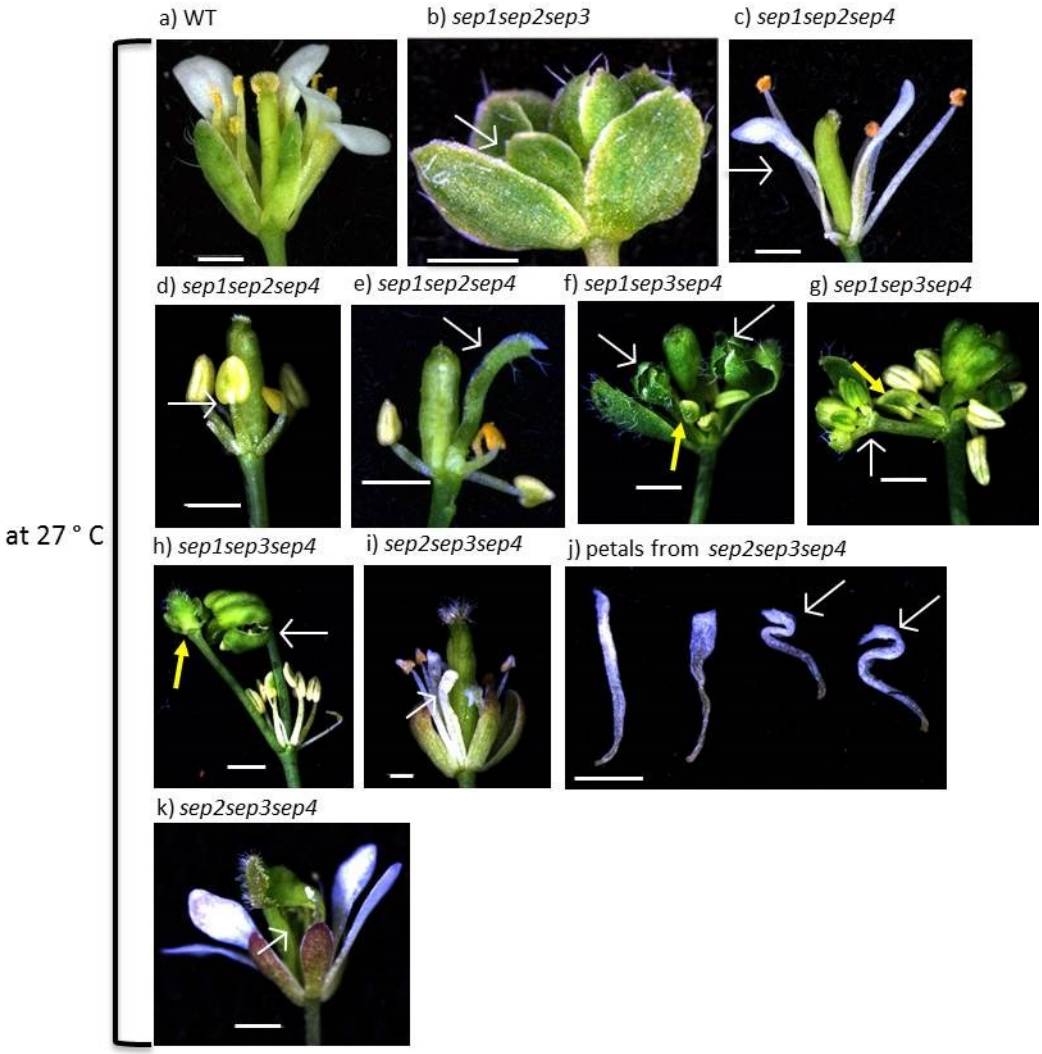


Fig. 3.4: *SEP* triple mutants grown at 27 °C

a) WT flower, b) *sep1sep2sep3* triple mutant that is composed of sepals (white arrow), c) *sep1sep2sep4* triple mutant flower that lacks sepals, has two petals (white arrow) and three stamens, d) *sep1sep2sep4* triple mutant flower that lacks sepals and petals and has greenish stamens (white arrow), e) *sep1sep2sep4* triple mutant flower that has a flower with one bract like sepal (white arrow), f) *sep1sep3sep4* triple mutant flower showing two secondary flowers (white arrows) and greenish stamens (yellow arrow), g) *sep1sep3sep4* triple mutant flower that has one secondary flower (white arrow) and greenish stamens (yellow arrow),

Legend to be continued

Fig. 3.4 continued

h) *sep1sep3sep4* triple mutant flower that has an elongated internode and a carpel that is burst (white arrow) and one secondary flower (yellow arrow). All flowers shown in Fig.3.4 f-h do not have petals, i) *sep2sep3sep4* triple mutant flower having narrower petal, with folded tips (white arrow), j) dissection of *sep2sep3sep4* triple mutant flower shows four petals that are narrower and two petals are strongly folded (white arrows), k) *sep*2*sep*3*sep*4 triple mutant flower that has burst carpels. Bars = 1mm

Fig. 3.5

Fig. 3.5: Comparison between WT and *sep2sep3sep4* siliques at 22 °C

a) Internodes from a *sep2sep3sep4* triple mutant plant showing bulged siliques (white arrow), b) silique from a WT flower, c) *sep2sep3sep4* triple mutant flower having a bulged silique (white arrow), d) open silique from a WT flower, e) open silique from a *sep2sep3sep4* triple mutant flower reveals leaf- like structures inside the fourth whorl (white arrow) and aborted ovules (yellow arrow), f) dissection of a *sep2sep3sep4* carpel shows a twisted leaf like structure (white arrow) with a stigma (yellow arrow).

Bars = 1mm, WT and *sep2sep3sep4* triple mutant siliques shown in Fig. 3.5 b- f were analyzed at the same stage

3.5 The expression of individual *SEP*s is independent of the other *SEP*s at 22 °C

As shown in section 3.4, the *sep1sep2sep3*, *sep1sep2sep4*, *sep1sep3sep4* and *sep2sep3sep4* triple mutants were severely affected at both 22 °C and 27 °C, but in contrast to the *sep1sep2sep3* triple mutant flowers, they were still capable of producing all floral organs. To test whether the expression of the non-mutated *SEP*s is affected in each triple mutant qPCR was performed on inflorescences (stage 1-12) of the triple mutants *sep1sep2sep3SEP4*, *sep1sep2SEP3sep4, sep1SEP2sep3sep4* and *SEP1sep2sep3sep4* grown at 22 °C and compared to WT. For each qPCR, four independent biological replicates were used and a two-tailed t-test was performed to check if the expression was significantly different compared to WT inflorescences, given as p-value in brackets. *Actin2* was used as a reference gene in each experiment. The expression of *SEP1* in the *sep2sep3sep4* triple mutant is not significantly different compared to WT (p=0.2) (Fig. 3.6 a), the same was observed for the expression of *SEP2* in the *sep1sep3sep4* triple (p=0.14) (Fig. 3.6 b) and for *SEP3* in the *sep1sep2sep4* triple (p=0.16), (Fig. 3.6 c). The only *SEP* gene to show any change in expression is *SEP4*, which shows a small increase compared to WT in the *sep1sep2sep3* triple mutant (p=0.043) (Fig. 3.6 d). These results suggest that expression of the *SEP* genes is largely independent.

Fig. 3.6

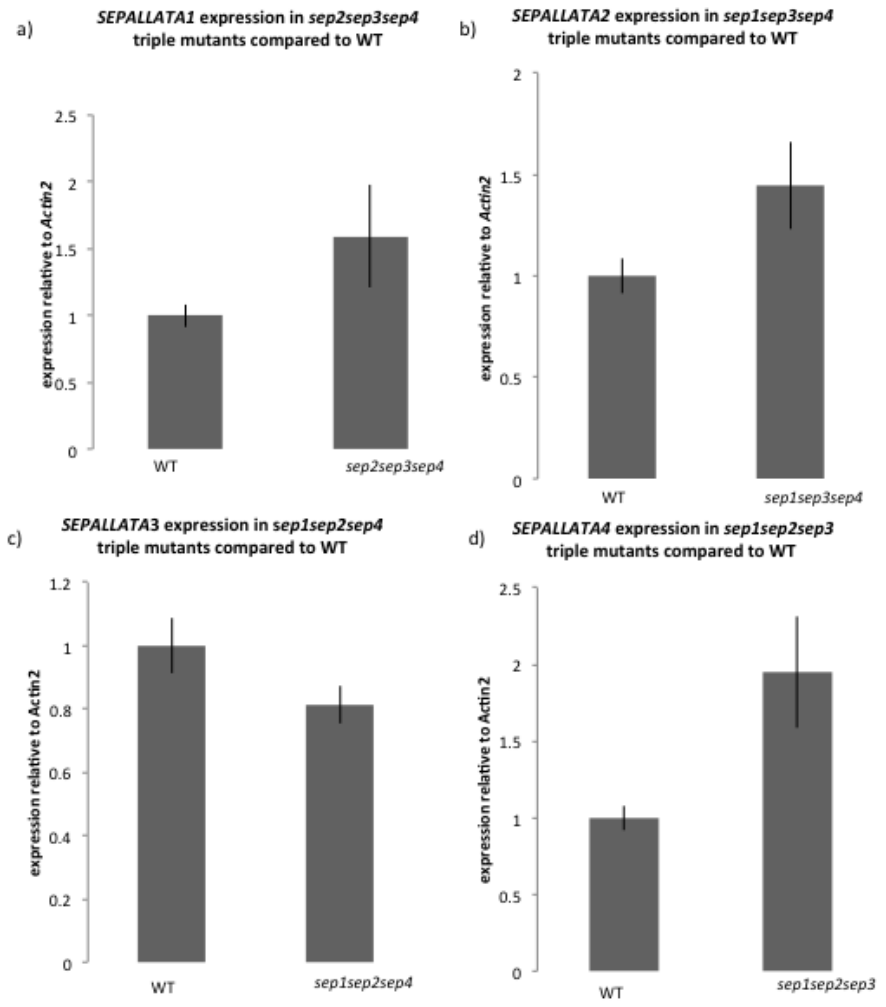


Fig. 3.6: Expression analysis for each non-mutated *SEP* in the triple mutants

a) *SEP1* expression in the *sep2sep3sep4* triple mutant compared to WT (p= 0.2), b) *SEP2* expression in the *sep1sep3sep4* triple mutant (p= 0.14), c) *SEP3* expression in the *sep1sep2sep4* triple mutant (p= 0.16), d) *SEP4* expression in the *sep1sep2sep3* triple mutant (p= 0.043). Four biological replicates were used and compared to WT inflorescences; significance was checked with a two-tailed t-test, results are given as p values in brackets.

Bars = Standard Error of the Mean (SEM), in every experiment, *Actin2* was used as a reference gene

3.6 Complementation of the extra perianth organ phenotype in *sep4-1* single and *sep1sep4* double mutants by g*SEP4*:GFP

To verify that the extra sepal and petal phenotype observed in *sep4*, *sep1sep4* and *sep2sep4* mutants is attributable to loss of the *SEP4* gene, a C-terminal *SEP4*:GFP tagged 4290 bp genomic construct under the control of its native promoter (2239 bp upstream of the start codon) (Fig. 3.7) was cloned and transformed into *sep4-1* (hereafter named g*SEP4:*GFP;*sep4*) and *sep1sep4* mutants (hereafter named g*SEP4*:GFP;*sep1sep4*). Multiple independent transgenic T1 lines were screened for g*SEP4:*GFP;*sep4.* 25 independent lines were analysed, from which 17 revealed a complementation in a variable manner. For g*SEP4*:GFP;*sep1sep4* 35 independent lines were screened and 20 revealed complementation. This confirms that the perianth organ phenotype is caused by the loss of the *SEP4* gene. After identification of all complementing lines the aim was to find lines with flowers that are nearly indistinguishable from WT for SEP4 target gene analysis. For that purpose several individual T2 lines were analysed and those selected that revealed good complementation results.

As summarized in Table 3.16, around 30 % of *sep4-1* single mutants revealed extra perianth organs at 22 °C. Screening of individual T2 lines revealed that in Line 1, 13 % of flowers had five sepals and 13 % had five petals, whereas in Line 2, 20 % of analysed T2 flowers developed five sepals and 20 % showed five petals. 10 % of all analyzed flowers revealed five petals and sepals for Line 5. In Line 8, 12% of analysed flowers had extra sepals and 12 % had extra petals and finally in Line 9, 10% of the flowers developed an extra sepal and 10 % developed extra petals. Based on this complementation analysis, Line 1, Line 2 and Line 9 were selected for further experiments.

As shown in Table 3.17, around 50 % of flowers from the *sep1sep4* double mutant developed extra perianth organs. As described for the *sep4-1* single mutant complementation in Table 3.16, individual transgenic lines for g*SEP4*:GFP;*sep1sep4* were screened for complementation. In Line 1 from g*SEP4:*GFP;*sep1sep4,* 14% of analysed flowers had an extra sepal and
10% had an extra petal, whereas in Line 2, 11 % had an extra first whorl organ and 9 % had extra second whorl organs. 33 % of flowers in Line 8 had five sepals and 33 % had extra petals and in Line 17, 11 % had five sepals and 14 % had five petals.

In summary: Line 2 from g*SEP4:*GFP;*sep1sep4* showed a good complementation for floral organs, 11 % developed extra perianth organs. An intermediate complementation was observed in Line 1 (14% extra sepals and 10 % extra petals) and Line 17 (11 % etxtra sepals and 14 % extra petals). Line 8 showed the worst complementation from all above described lines 33 % extra sepals and 33 % extra petals). Thus, for further analysis Line 1 and Line 2 were selected, as they have shown the best results for the complementation.

In addition confocal scanning microscopy was used to check the expression of the GFP-tagged SEP4 protein in Line 2 (g*SEP4*:GFP;*sep1sep4*) to establish the level of SEP4 protein expression and its tissue specificity. The SEP4 protein was detected in sepals of young floral primordia at stage 4-6 (Fig. 3.8 a, b). A weak signal was noticed in the conical region within the central dome in whorl 4 (Fig. 3.8 b). No expression was detected in any other whorl. These results are in agreement with previous studies from RNA *in situ analysis* of *SEP4* experiments. *SEP4* is first detected and strongest during stage 2 but is not seen in the inflorescence meristem. By stages 3–4, expression is localized to a conical region within the central dome and can be weakly detected in sepals. Expression persists into stage 6 in carpels but is no longer visible in sepals (Ditta G.S. et al., 2004). To get a rough estimation of the strength of the expression of the SEP4 protein, this line was compared to previous studies from a g*SEP3*-GFP line (Kaufmann K. et al., 2009). Comparison revealed that the expression of the SEP4 protein is at an intermediate level. This is useful to know because this line will be used in ChIP experiments described in the next chapter.

Fig .3.7

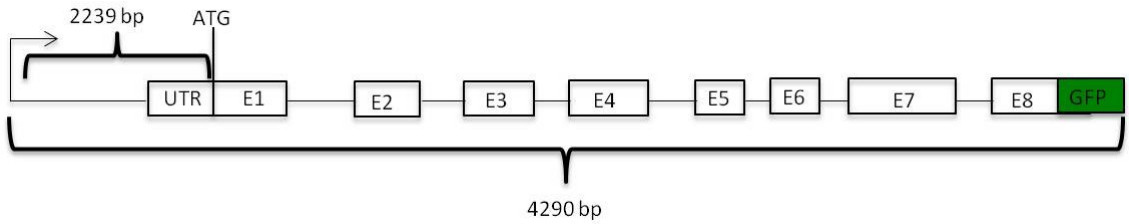


Fig. 3.7: Cloning strategy of the generation of the *SEP4* complementation construct to rescue the *sep4* mutant phenotype showing extra perianth organs.

The construct is under the control of its native promoter with a 2239 bp upstream region and is fused at the C-terminus to a Green Fluorescent Protein (GFP). UTR= translated region, E1 = Exon 1, ATG= start codon

Table 3.16

	4 sepals, %	5 sepals, %	6 sepals, %	4 petals, %	5 petals, %	6petals, %	individual flowers analyzed / individual plants
WT at 22 °C	100	0	0	98.4	1.6	0	60/4
sep4-1 at 22 °C	69.5	28.5	1.9	68.5	29.5	1.6	105/9
g*SEP4*:GFP;*sep4* Line1	87	13	-	87	13	-	15/1
g*SEP4*:GFP;*sep4* Line2	80	20	-	80	20	-	20/1
g*SEP4*:GFP;*sep4* Line5	90	10	-	90	10	-	20/1
g*SEP4*:GFP;*sep4* Line8	88	12	-	88	12	-	33/1
g*SEP4*:GFP;*sep4* Line9	90	10	-	90	10	-	38/1

Table 3.16: Complementation analysis of T1 lines carrying a g*SEP4*:GFP;*sep4* construct

%= flowers having a defined number of sepals and petals, - = not observed

Table 3.17

	4 sepals, %	5 sepals, %	6 sepals, %	4 petals, %	5 petals, %	6 petals, %	individual flowers analyzed / individual plants
WT at 22 °C	100	0	0	98.5	1.5	0	65/4
sep1sep4-1 at 22 °C	45	55	1-	53	42	5	65/13
g*SEP4*:GFP;sep1*sep4* Line1	86	14	-	90	10	-	29/1
g*SEP4*:GFP;sep1*sep4* Line2	89	11	-	89	11	-	18/1
g*SEP4*:GFP;sep1*sep4* Line8	67	33	-	67	33	-	18/1
g*SEP4*:GFP;sep1*sep4* Line17	89	11	-	86	14	-	28/1

Table 3.17: Complementation analysis of T1 lines carrying a g*SEP4:*GFP;*sep1sep4* construct

%= flowers having a defined number of sepals and petals, - = not observed

Fig. 3.8

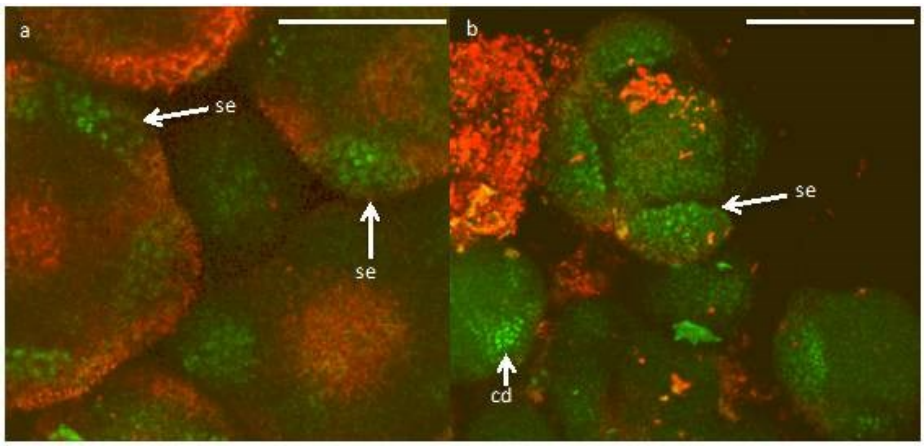


Fig. 3.8: Expression analysis of g*SEP4*:GFP; *sep1sep4* in floral primordia

Scanning confocal microscopy was used to test the expression of the SEP4 protein in a complemented g*SEP4*:GFP;*sep1sep4* line (Line 2). As reported from *in situ* hybridization studies, the SEP4 protein is localized in sepals (se) of early arising floral meristems (Ditta G.S. et al., 2004). The green signal shows the expression of the SEP4 protein while the red signal represents autofluorescence, a) GFP signals in sepals (se) marked with white arrows, b) signals in the sepals (se) and a signal in the central dome (cd), (both marked with a white arrows). Bars= 100 μm.

3.7 Meristem size is not altered in *sep4* single mutants

The finding that *sep4* single and the s*ep1sep4* and *sep2sep4* double mutants had a phenotype with extra perianth organs (summarized in Table 3.15) prompted us to test whether meristem size is affected in *sep4-1* single mutants (hereafter named *sep4* single mutants). Therefore Scanning Electron Microscopy (SEM) was applied on young floral primordia (stage 1-8) (Smyth D.R. et al., 1990) from *sep4* single mutants and compared to those of WT. Dissection of single samples at different stages revealed no obvious change in the size of the meristems . As seen in Fig. 3.9 a and b, both flowers (WT and *sep4*) have a similar meristem size at very early stages and also at stage 4 (Fig. 3.9 c and d), flowers do not reveal a significant difference in meristem size. Fig. 3.9 d and f show that the *sep4* flowers have an extra sepal (marked with a white arrow). However it should be noted, that these are preliminary results and for a final conclusion further experiments and a more detailed analysis are necessary. Interestingly, when comparing individual floral primordia of WT and *sep4* single mutants, changes in the morphology of sepals were seen. Individual flowers of *sep4* single mutants showed a cellular outgrowth on the tips of sepals (Fig. 3.9 g, marked with a white arrow) that were never seen on WT flowers.

Fig. 3.9

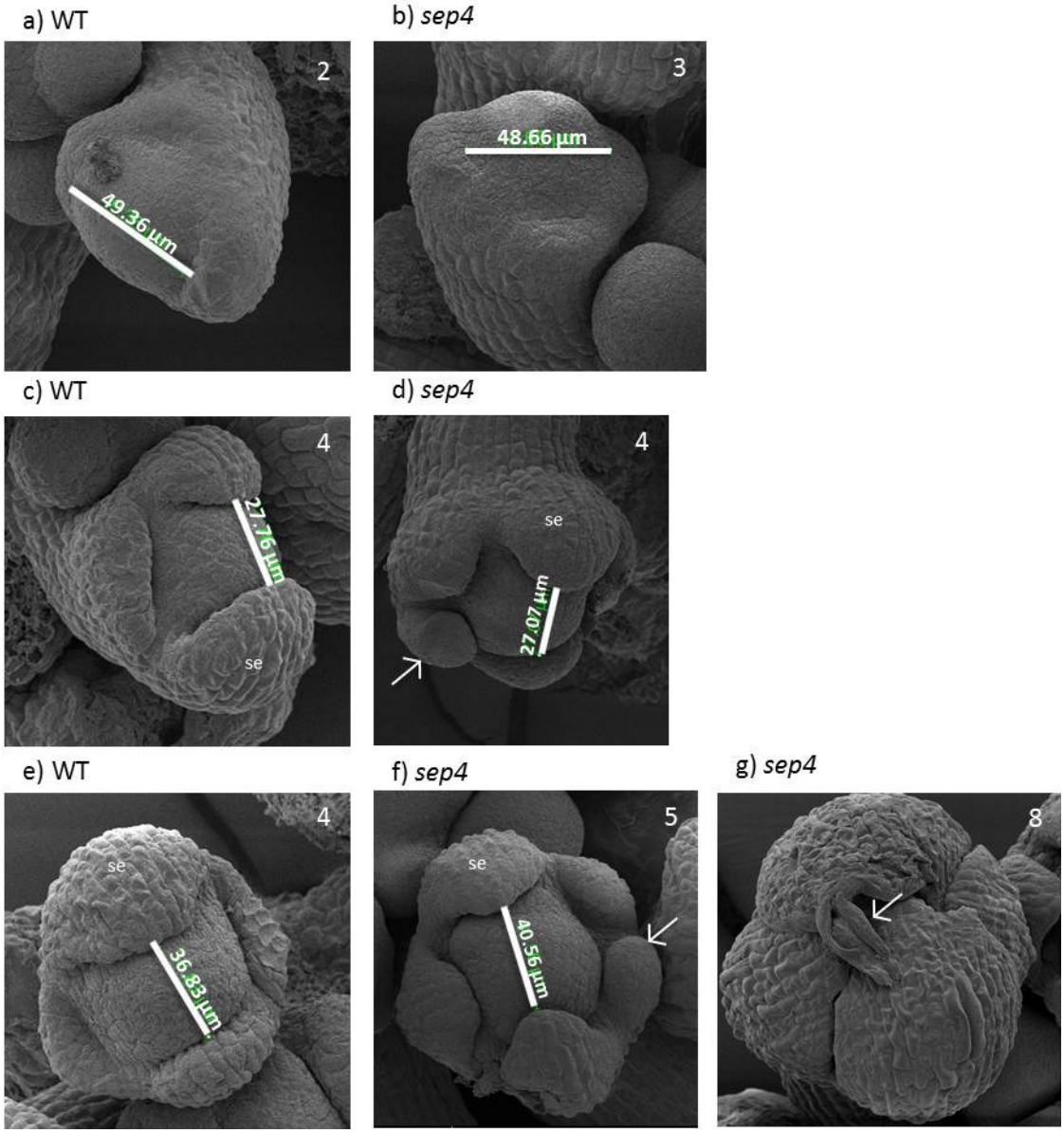


Fig. 3.9: Measurement of inflorescence meristem size in WT and *sep4* single mutants using SEM.

a) WT stage 2 flower, b) *sep4* stage 3 flower c) WT stage 4 flower, d) *sep4* stage 4 flower with a fifth sepal (white arrow), e) WT stage 4 flower ,f) *sep4* stage 4 flower with a fifth sepal (white arrow), g) *sep4* stage 8 flower showing a cellular outgrowth on the sepal (white arrow) numbers at the edges indicate the developmental stage of the flowers, se = sepal, green lines show the aperture of the primordia, measured in µm, all pictures were taken at the same magnification (1000 x). Sizes of bars: a) 49.36 µm, b) 48.44 µm, c) 27.76 µm, d) 27.07 µm, e) 36.83 µm, f) 40.56 µm.

3. 8 Discussion

3.81 Non-redundant organ-specific functions for single *SEP* genes are enhanced under changing environmental conditions

Comparative studies among different plant species revealed that multiple *SEP*s are common across angiosperms, since they have expanded due to gene duplication events. An early duplication prior to diversification of all present-day angiosperms produced two *SEP*-clades, the *AGL9* clade (*SEP3* clade) and the *AGL2*/*3*/*4* clade (*LOFSEP* clade) (Zahn L.M. et al., 2005). SEP proteins are necessary for all ABC floral organ identity functions by providing the "floral state", since flower development is abolished in *SEP* quadruple mutants (Honma T. and Goto K., 2001; Ditta G.S. et al., 2004). The occurrence of this gene family only in angiosperms suggests that the evolution of this gene family might be associated with the evolution of angiosperms (Zahn L.M. et al., 2005). Going one step further, the coincidence between the radiation of angiosperms and the origin of *SEP* genes suggests that these genes contributed to a large degree to the innovation of the flower and significantly added to the success of angiosperms.

Multiple members of this gene family are found in all angiosperms and these genes act similarly in specifying meristem and floral organ identity. They show a subtle differences in protein-protein interaction and reveal phenotypes in different plant species like in *Gerbera hybrid* (Kotilainen M. et al., 2000; Uimari A. et al., 2004), *petunia* (Vandenbussche M. et al., 2003) or tomato (Pnueli L. et al., 1994; Vrebalov J. et al., 2002; Liu D. et al., 2013). Surprisingly *SEPs* were reported to act redundantly in *A. thaliana* with only a specific phenotype in triple and quadruple mutant combinations. Flowers of *sep1sep2sep3* triple mutants have indeterminate flowers composed of sepals and floral organ identity is completely abolished in *sep1sep2sep3sep4* quadruple mutants with leaf-like structures (Pelaz S. et al., 2000; Ditta G.S. et al., 2004). In contrast single mutants affecting *SEP* genes were reported to be indistinguishable from WT flowers or have only a mild phenotype, as described for *sep3*, with a partial conversion of petals into sepals (Pelaz S. et al., 2000; Pelaz S. et al., 2001b; Ditta G.S. et al., 2004). The single mutant characterization described in section 3.2 clearly disproves this functional redundancy. These results show that all four single mutants show organ specific phenotypes (Table 3.5, Fig. 3.1) at 22 °C and 27 °C. The finding that the *sep4-1* and *sep4-2* single mutants affect perianth organ number (usually five instead of four) (Table 3.4, Fig. 3.1 g, h, i, s, t) and *sep3-2* developing folded petal tips at both temperature conditions (Table 3.3,

Fig. 3.1 f, r) reveals individual functions. The *sep1* and *sep2* single mutants show phenotypes at 27 °C affecting the carpel length (Table 3.1, Fig. 3.1 k, l) and carpel morphology (Table 3.2, Fig. 3.2 m, n), while both mutants are indistinguishable from WT at 22 °C. These described phenotypes for all *sep* single mutants affecting floral organs show that the reported redundancy for *SEP* genes does not exist in *A. thaliana*.

One reason, why the *sep3* and *sep4* single mutants reveal a phenotype at 22 °C, but the *sep1* and *sep2* single mutant are aphenotypic at that growth conditions could lie in their evolution. The first WGD duplication about 300 million years ago produced two clades**,** *SEP3* and *AGL2*/*3*/*4*. Within the *AGL2*/*3*/*4* clade*, SEP1* and *SEP2* are believed to be the result of recent gene duplication (Ermolaeva M.D. et al., 2003). Thus, *SEP3* and *SEP4* evolved much longer than *SEP1* and *SEP2* and might have acquired functions during this time that distinguishes them from the other two *SEP* genes (*SEP1* and *SEP2*). Interestingly similarities can be seen between *SEP1* and *SEP2* and *SHATTERPROOF 1* (*SHP1*) and *SHATTERPROOF 2* (*SHP2).* The *SHP* genes are also described as redundantly acting genes that are involved in the regulation of seed shattering. Single mutants cannot be distinguished from WT but s*hp1shp2* double mutants fail to disperse their seeds because they are unable to differentiate a dehiscence zone at the valve margin, the part of the silique where cell separation occurs in fruit development for seed dispersal (Liljegren S.J. et al., 2000). Both gene pairs (*SEP1*/*SEP2* and *SHP1*/*SHP2*) originated approximately 26 MYA ago and are paralogs, not in the process of becoming a pseudogene (Moore R.C. et al., 2005). Both gene pairs show heterogeneity at their C-terminal region, the part of MADS-Box genes that is considered to give transcriptional activation for downstream genes and for the formation of ternary or quaternary protein complexes (Egea-Cortines M. et al., 1999; Honma T. and Goto K., 2001). A similar diversification of the C-terminus was observed in the two MADS-Box genes *AP3* and *PI*, but these differences in the B-function floral organ identity genes resulted in a functional diversification (Litt A. and IrishV. F., 2003). In contrast to the *SEP1* and *SEP2* and *SHP1* and *SHP2* gene pairs, the duplication event producing *AP3* and *PI* occurred around 120 MYA and these genes have had more time to sub- or neo-functionalize (Kramer E.M. et al., 1998). It could be that the *SEP1* and *SEP2* or the *SHP* genes have not yet reached the point of diversification as observed for *AP3* or *PI.* The finding that different phenotypes were observed at 27 °C for *sep1* and *sep2,* affecting the carpel in different ways might suggest that the two genes are in the progress of sub- or neo-functionalization and their roles will become more defined in the future. This is supported by the finding that SEP1 and SEP2 have different interaction partners in yeast two hybrid analysis, leading to the conclusion that at the protein level there must be a functional difference between the two genes (Fan H.Y. et al., 1997; de Folter S. et al., 2005) SEP1 has more interaction partners than the SEP2 protein, for example both interact with AG, AGL16, AGL20,

AGL21, TT16, but SEP1 also interacts with FRUITFULL, SEEDSTICK, SHP1 and SHP2, AGL6, AGL24, AGL42, SVP, PI, AP1, OVEREXPRESSOR OF CATIONIC PEROXIDASE 3 and MODIFIER OF SNC1 (Fan H.Y. et al., 1997; de Folter S. et al., 2005).

These results support the idea that all four *SEP* genes have individual functions and contribute to the robustness of plant reproduction. Some might have a minor effect under standard conditions, but as soon as the environmental conditions change, their function in organ development becomes clearer and reveals the importance of *SEP*s for floral organ development.

The phenotypic analysis described in section 3.2, which showed individual phenotypes for each single mutant, fits with the function of *SEP* like genes in other plant species that have acquired specific and unique roles during flower development. Loss of function of *TM5*, the tomato *SEP3* homolog, causes severe alterations to floral organs affecting the inner three floral whorls. Flowers develop sepaloid petals, stamens show sepaloid identity and carpels incompletely fuse. However complete organ transformation was not seen in these tomato lines (Pnueli L. et al., 1994). The tomato gene *TM29* has the highest predicted protein identity to the *AtSEP1* gene respectively (Ma H et al., 1991; Mandel M.A. and Yanofsky M.F., 1998; Ampomah-Dwamena C. et al., 2002). Co suppression of *TM29* has a phenotype affecting the inner three whorls of the flower and the fruit. Petals acquire a partially sepaloid identity, third and fourth whorl organs are infertile and parthenocarpic fruits develop. Flowers of WT tomatos require pollination and fertilization to set fruits, whereas parthenocarpic fruits develop without pollination (Ampomah-Dwamena C. et al., 2002). Floral meristem identity was also affected, as these transgenic plants develop ectopic shoots with partially developed leaves and secondary flowers emerging from the fruit. These shoots continued to produce parthenocarpic fruits (Ampomah-Dwamena C. et al., 2002). *LeMADSRIN,* the tomato *SEP4* homolog , affects fruit development, since down-regulation results in fruits that fail to ripen (Vrebalov J. et al., 2002). *LeMADS1* and *SLMBP21*, belong to the *FBP9*/*FBP23* subclade of the *SEP* genes that is not present in *A. thaliana.* Down-regulation of *LeMADS1* causes only subtle phenotypes with elongated sepals. *SLMBP21* is involved in the development of the abscission zone as transgenic lines that down-regulate the gene abolished the development of the abscission zone (Liu D. et al., 2013).

Two *SEP* like genes have been reported in *Gerbera hybridia* to date, *Gerbera Regulator of Capitulum Development 1* (*GhGRCD1*) and *Gerbera Regulator of Capitulum Development 2* (*GhGRCD2*) (Kotilainen M. et al., 2000; Uimari A. et al., 2004). The flowers of *Gerbera hybridia* are different to those of *A. thaliana* as they have three different flower types, ray and trans flowers that are only female and disc flowers that contain male and female organs. Ray flowers contain in the third whorl sterile staminoide structures, because stamen development

starts normally but progresses slowly and as a consequence ends in abortion of the male organs before the flower opens. (Teeri T.H. et al., 2006). Additionally, these plants have an indeterminate inflorescence, although they produce a fixed number of flowers. The inflorescence meristem is consumed and replaced by floral meristems leading to a terminal symmetrical flower in the centre of the inflorescences (Harris E. M. , 1995; Teeri T.H. et al., 2006). Down-regulation of *GhGRCD1*, the *Arabidopsis SEP3* homolog affected the third whorl in female ray flowers with a homeotic transformation of sterile staminoides into petals. The disc flower, that normally has male and female organs had only subtle defects in the third whorl (Kotilainen M. et al., 2000). *GhGRCD2*, the *Arabidopsis SEP1* homolog is involved in meristem identity. Down-regulation showed flowers that had inflorescence with bracts, floral primordia and flowers growing in place of the ovules. Thus, fourth whorl identity was affected in transgenic *GRGD2* lines with meristems reverting back to inflorescence meristems (Uimari A. et al., 2004).. Another role of this gene is the control of the determinacy of the inflorescence meristem. Transgenic *GhGRCD2* lines produced nearly two fold more flowers than WT plants and the inflorescence meristems remained undifferentiated until flower production ended due to senescence (Uimari A. et al., 2004). These two genes provide examples for diversification as well, *GhGRCD1* affects the third whorl and *GhGRCD2* affects the fourth whorl and has an additional role in controlling the determinacy of inflorescences meristems (Kotilainen M. et al., 2000; Uimari A. et al., 2004).

Table 3.18

Species	**Gene name / Gene orthologue**				
Gene or Clade	*SEP1*	*SEP2*	*SEP3*	*SEP4*	*FBP9/23*
A. thaliana	*SEP1*	*SEP2*	*SEP3*	*SEP4*	Not present in *A. thaliana*
A.majus	*DEFH49*	-	*DEFH200, DEFH72*	-	Not present in *A.majus*
Gerbera hybridia	*GhGRC2*	-	*GhGRCD1*	-	Not present in *Gerbera hybridia*
Lycopersicon esculentum	*LeTM29*	-	*LeTM5*	*LeMADSRIN*	*LeMADS1, SLMBP21,*
Petunia	*PMADS12, FBP5*		*FBP2*	*FBP4*	*FBP9, FBP23*

Table 3.18 *SEPALLATA* genes and their orthologues in different species.

SEPALLATA genes in *A. thaliana* and their orthologues described. Genes were identified according to their expression profiles. The two Petunia genes *PMADS12, FBP5* belong to the *SEP1/SEP2* and are not further divided as *SEP1* or *SEP2* orthologue. The *FBP9/23* clade is not present in all species, only in *Lycopersicon esculentum* and *Petunia.*- = not known
Table modified after Zahn L. M. et all., 2005 and Malcomber S. T. and Kelogg E. A., 2005.

3.8.2 *sep* double mutants are severely affected in flower development, their robustness and reproductive fitness to environmental perturbation

As Table 3.15 summarizes, double mutants revealed phenotypes specific for loss of individual *SEP*s, as reported for the previous single mutants. *sep3-2* single mutants had folded petals and *sep4* single mutants showed an increase in the number of perianth organs (Table 3.9, Fig.3.1 g, h, i, s, t). The *sep3-2* single mutant phenotype affecting petal blade was seen in all double mutant combinations carrying this allele at 22 °C. Carpel morphology was affected in *sep1* and *sep2* mutants at 27 °C (Table 3.9, Fig. 3.1 k, l, m, n). In addition *sep2* mutants were affected in their petal size (Fig. 3.1 p). Novel combinatorial phenotypes also appeared exclusively in certain mutant combinations, especially at 27 °C. This shows that *SEP* genes regulate flower development in a redundant and non-redundant way and suggests that SEP proteins regulate common and individual target genes. One explanation for novel phenotypes appearing exclusively in certain double mutant combinations at 27 °C, such as the secondary flowers in *sep1sep4* or the reduction in sepal number in *sep1sep2* (Table 3.15), could lie in the perturbation of the complex assembly or a shift in the targets. It is known that SEP proteins form multimeric complexes with the ABC floral organ identity genes to regulate target gene expression (Honma T. and Goto K., 2001; Urbanus S.L. et al., 2009). Exposure of the mutants to elevated temperature could weaken the stability of the protein complexes and thus affect activation or repression of target genes. The enhanced phenotypes in triple mutant could be explained by the instability of these complexes too.

Interestingly some single or double mutants revealed similarities to phenotypes observed in other reported mutants. For example the extra perianth organs seen in *sep4* single and *sep1sep4* and *sep2sep4* double mutants at 22 °C has also been observed in mutants affecting the D-class bZIP transcription factor *PERIANTHIA* (*PAN*). Flowers of plants mutant for the *PAN* gene are pentameric in the first three whorls, with five sepals, five petals and five stamens and two carpels. Other parts of the plant or the number of meristem cells are not affected in these mutants (Running M.P. and Meyerowitz E.M., 1996). Therefore it has been suggested that the *PAN* gene affects the spacing mechanism that determines the relative position of floral organs (Meyerowitz E.M., 1997; Chuang C.F. et al., 1999). *PAN* acts downstream of the floral meristem identity gene *LFY*, *AP1*, *AP2* and independently of *AP3*, *PI, AG* and the meristem size genes *CLAVATA 1* and *CLAVATA 3* (Running M.P. and Meyerowitz E.M., 1996) in specifying pattern formation of flowers. *PAN* also regulates stem cell fate by directly controlling *AG* expression (Das P. et al., 2009). Another class of genes that affect floral organ number are

CLAVATA (*CLV)* genes (*CLV1*, *CLV2*, *CLV3*) that represent one of the key pathways in *A. thaliana*'s clavata pathway to regulate stem cell specification (Clark S.E. et al., 1993; Clark S. E. et al., 1995; Kayes J.M. and Clark S.E., 1998). *CLV1* and *CLV2* encode plasma-membrane receptors, while *CLV3* encodes a small secreted proprotein and releases a mature CLE protein to act as a multimeric ligand for a CLV1/CLV2 heteromeric signal transduction complex (Rojo E. et al., 2002). Mutation in any of these three genes cause an increase in organ number in all four whorls (average: five sepals and petals, eight stamens and five carpels), and also leads to additional whorls (Clark S.E. et al., 1993; Clark S. E. et al., 1995). Mutants accumulate massive populations of stem cells in shoot and flower meristems and thus an increase in organ number is associated with an increase in meristem size (Clark S.E. et al., 1993; Clark S. E. et al., 1995, Kayes J. M. and Clark S. E, 1998) .

ETTIN (ETT) a member of the auxin response factor family of TFs is another gene whose mutants affect the number of floral organs. Flowers of *ett* mutants have an increased sepal and petal number by approximately one and a decreased stamen number by the same amount. The number of fourth whorl organs is not affected but the morphology and shape differs compared to WT flowers. Mutants show a decreased ovary size and appear sterile due to failure of integument growth around the egg sac, additionally tapering of the gynoecium into a stalked like structure and outgrowths of tissues from the ovary in the medial plane is observed (Sessions R. A. and Zambrsci P. C. 1995, Sessions R. A. et al., 1997) . The increase in perianth organ number is not caused by an increase in early meristem size, whereas stamen and carpel phenotypes are associated with irregular elongated terminal floral meristems (Sessions R. A., 1997). Although all above described mutants have a similar phenotype with respect to perianth organ numbers seen in *sep4* single and *sep1sep4* and *sep2sep4* double mutants, they are unlikely to cause this phenotype. Flowers of *pan* and *ett* mutants reveal a decrease and *clv* mutants show an increase in the number of stamens. Flowers of the *sep4* single and *sep1sep4,* or *sep2sep4* double mutants were not affected in the number of stamens or in the carpel morphology and carpel number, whereas *clv* and *ett* mutants showed phenotypes for fourth whorl organs.

The perianth organ phenotype in *sep4* mutants is not caused by an increase in the size of (inflorescence) meristems, as shown in section 3.7 (Fig. 3.9), where SEM revealed no difference in the size of meristems at early stages compared to WT. These results fit with the observation that two of five petals often appeared smaller than the other three petals (Fig. 3.1 i, marked with two asterisks). Although no measurement was performed on *sep1sep4* and s*ep2sep4* double mutants regarding the size of meristems, it is unlikely that these double mutants would reveal a massive change in the size, especially when considering that the frequency in perianth organs in *sep4* single and *sep2sep4* double mutants is very similar (Table

3.6). Why the frequency of perianth organsin the *sep1sep4* double mutant at 22 °C is higher compared to the *sep4* single and *sep2sep4* double mutants remains elusive (Table 3.6). However, one possible explanation for the increase in sepals and petals might be an increase in the number of founder cells or a change in the spacing in floral organ primordia. This could be an interesting subject for further studies.

The high frequency of secondary flowers in *sep1sep4* double mutants at 27 °C (Table 3.6, Fig. 3.2 m, n) has also been reported for strong *ap1-1* alleles. *AP1* is a MADS-Box TF that promotes early floral meristem identity in synergy with *LEAFY* and acts at later stages during floral organ development as a floral organ identity gene (Irish V.F. and Sussex I.M., 1990; Coen E.S. and Meyerowitz E.M., 1991; Weigel D. and Meyerowitz E.M., 1993). *ap1-1* mutants develop bracts instead of sepals in the first whorls, loss of petals in most flowers and secondary flowers in the axils of first whorls. Secondary flowers have the same developmental pattern and eventually form tertiary flowers. The secondary flowers can be explained by a partial reversion of the floral meristem to an inflorescence meristem suggesting that *AP1* is necessary for the transition of vegetative to floral meristems (Mandel M.A. et al., 1992; Mandel M.A. and Yanofsky M.F., 1995). One explanation for the increased frequency of secondary flowers in *sep1sep4* double mutants might be that genes are affected that are necessary for floral meristem identity. This could be e.g. that SEP1 and SEP4 act in complexes and regulate downstream targets necessary for meristem identity. Why this phenotype only occurs at 27 °C is an interesting question. It might be that the assembly of protein complexes changes at higher temperature and as a result, the *AP1* gene or other genes, such as *LFY* and *FUL*, involved in meristem identity, cannot be targeted and regulated. Therefore it would be a good experiment to test the expression of the genes *AP1*, *CAL* and *FUL* in the *sep1sep4* double mutant with qPCR and compare them to WT. A second possibility is that those meristem identity genes rely on direct interactions with e.g. SEP1 and SEP4. If both genes are missing the genes *AP1, CAL* or *FUL* are activated but cannot activate their target genes and that in turn causes the observed phenotype with secondary flowers. To check this theory, a good experiment would be to test the expression of *AP1*, *CAL* or *FUL* in the *sep1sep4* double mutant, e.g. with qPCR experiments. If the expression is similar to WT, it is more likely that the phenotype with secondary flowers in the *sep1sep4* double mutant is caused by the failure of establishing direct protein protein interactions between the meristem identity proteins and the two SEP proteins. The observation that this phenotype was seen in a double mutant carrying a *sep4* mutant allele underscores the meristem function of the *SEP4* gene and its role in establishing meristem identity (Ditta G.S. et al., 2004) .

Remarkably extra perianth organs were almost never seen in the *sep3sep4* double mutant (Table 3.6). One explanation for this could be that SEP3 and SEP4 act differentially on a common target gene. For example, SEP3 could activate a common target gene involved in specifying perianth organ number, whereas SEP4 represses it later or *vice versa.* When *SEP3* is functional and *sep4* is non-functional, a phenotype is seen with an increase in sepal and petal number. When both *SEP*s are non-functional, this phenotype is seen only extremely rarely, indicating that functional *SEP3* is required to see the s*ep4* phenotype. However based on the idea that both of the *SEP*s target a gene involved in specification of perianth organ number, one should see a decrease in the number of sepals and petals if *SEP4* is functional and *sep3* is non-functional. This is not the case, as *sep3-2* single mutants and mutant combinations carrying this allele did not reveal a deviation from the four sepals and petals. However, both genes are necessary to set the right organ number as the double mutant shows a deviation (either up or down) in the number of perianth organs (Table 3.6, Table 3.9). Thus, *SEP3* and *SEP4* are involved in a mechanism to regulate the development of the correct number of floral organs. What kind of mechanism that is remains elusive and could be an interesting topic for future studies.

In summary, two things can be concluded from these mutant combinations: firstly *SEP*s do not act fully redundantly, as seen in different mutants and mutant combinations, especially at 27 °C. The four *SEP*s in *A. thaliana* are collectively necessary to maintain the robustness of plant reproduction to environmental perturbations. Although plants were able to produce seeds, a decrease in the amount of seeds was noticeable at 27 °C (e.g. in *sep1sep3* or *sep2sep3* double mutants), suggesting that multiple *SEP*s are maintained to enhance plant fitness, especially in unpredictable environmental conditions. Secondly, different mutants and mutant combinations affect different floral organs and some mutant combinations revealed novel phenotypes, suggesting different protein interaction partners or individual targets.

3.8.3 *SEP4* alone cannot confer 2nd, 3rd and 4th whorl organ identity

As described in chapter 3, section 3.4 (Fig. 3.6) the expression of the non-mutated *SEP*s in each triple mutant is not affected, suggesting that *SEP*s do not depend on each other for expression. The only gene that showed a slight increase in expression was *SEP4* in the *sep1sep2sep3SEP4* triple mutant (Fig. 3.6 d). One explanation for this slightly higher expression might be that the

SEP4 gene is expressed in sepals (Ditta G.S. et al., 2004) and the flower of the s*ep1sep2sep3* triple mutant is composed entirely of sepal-like organs (Pelaz S. et al., 2000). Therefore the amount of *SEP4* might be higher than in a WT flower simply because of this homeotic change.

One of the most obvious findings was that all individual *SEP*s, apart from *SEP4*, are capable of promoting floral organ identity, as seen in their ability to produce sepals, petals, stamens and carpels in the respective mutant combinations (*SEP1sep2sep3sep4*, *sep1SEP2sep3sep4*, *sep1sep2SEP3sep4* and *sep1sep2sep3SEP4*) (Table 3.14, Fig. 3.3, 3.4). As previously reported and confirmed here, the *sep1sep2sep3* triple mutant is composed of sepals and mutants occasionally develop secondary flowers (Pelaz S. et al., 2000). Thus *SEP4* is able to confer sepal identity and provide the floral context, or the basis for floral development, because *sep1sep2sep3sep4* quadruple mutants are completely composed of leaf like organs. What makes *SEP4* so different from all the other *SEP*s? Could this failure to make floral organs be explained by the expression of *SEP4*? All *SEP* genes are expressed in the floral buds and in mature seedpods, but subtle differences are reported. The three *SEP* genes (*SEP1*, *SEP2*, *SEP4*) have a slightly different expression pattern compared to *SEP3* in the floral organ primordia. *SEP1*, *SEP2* and *SEP4* are expressed in all four whorls, whilst *SEP3* starts slightly later and is expressed in whorls 2-4 (Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Pelaz S. et al., 2000; Ditta G.S. et al., 2004). In contrast to the other three *SEP* genes that are reported not to be expressed in the vegetative tissue, *SEP4* expression can also be detected in cauline leaves (Ma H et al., 1991; Ditta G.S. et al., 2004). Our studies have shown that *SEP4* expression is detected in seedlings and rosette leaves as well (Supplementary data S7, S8). This broad expression pattern affecting vegetative and reproductive phase suggests that *SEP4* has a more widespread function than the other three *SEP*s and can be regarded as the “green” or “vegetative” *SEP* gene. It has been previously shown that *SEP4* contributes to the development of all four floral organs, as shown in genetic titration experiments with *sep1 sep2 sep3*$^{+/-}$ mutant flowers that did not show massive morphological changes except a reduction in the number of stamens. In contrast, flowers of *sep1 sep2 sep3*$^{+/-}$*sep4* mutants were affected in organ number and identity as for example loss of fourth-whorl determinacy or the reduction in the number of sepals (Ditta G.S. et al., 2004). In addition, *SEP4* is reported to have a role in maintaining meristem identity, as shown in intermediate phenotypes affecting meristem identity defects in *ap1 sep4* double mutants compared to *ap1* single and *ap1 cal* double mutants (Ditta G.S. et al., 2004). Strong *ap1* mutants show partial conversion of flower meristems into inflorescence meristems, evidenced with the development of flowers in the axils of first whorl organ (Bowman J.L. et al., 1993). In *ap1 cal* double mutants, flower meristems are converted in inflorescence meristems, producing continuously inflorescence meristems that have a “cauliflower “structure. Single

mutants of the gene *cal* are aphenotypic, suggesting that the function of *CAL* is encompassed by *AP1*. In contrast, flower development is abolished in *ap1 cal ful* triple mutant (Ditta G.S. et al., 2004). *FUL* is a close relative to *AP1* and *CAL* that specifies meristem identity (Ferrandiz C. et al., 2008). Flowers of *ap1 sep4* double mutants show an intermediate phenotype between *ap1* and *ap1 cal* mutants, with inflorescences that reveal a "cauliflower" phenotype in early arising flowers, but soon start to produce *ap1* like flowers, suggesting that *SEP4* contributes to maintain meristem identity and is necessary for *CAL* function (Ditta G.S. et al., 2004). Furthermore a mutation in the *sep4* gene can enhance the meristem identity effect in *ap1 cal* mutants, as the *ap1 cal sep4* triple mutant takes longer before starting to produce flowers. A similar "cauliflower" phenotype has been observed in the *ap1 sep1 sep2 sep4* quadruple mutant, suggesting that SEP proteins are necessary for *CAL* activation (Ditta G.S. et al., 2004). These phenotypes could be either explained by a direct activation of meristem identity genes by SEP proteins. For example could SEP4 regulate the expression of *FUL* what in turn could explain the intermediate phenotype of *ap1 cal sep4* triple mutants in between *ap1 sep4* double mutants (slowly production of flowers) and *ap1 cal ful* triple mutants (no production of flowers). To test thistheory, one experimental approach could be to check the expression of the gene *FUL* in a *sep4* mutant. A significant downregulation of *FUL* would provide an indication that SEP4 directly regulates this gene. Another possibility could be that SEP proteins and meristem identity proteins like CAL rely on direct protein protein interactions. If SEP proteins are missing, the protein protein interaction is disturbed and as a consequence, the meristem identity genes can't activate their target genes. To test this, the expression of the meristem identity genes could be checked. If their expression is similar to WT it is more likely that SEPs and meristem identity proteins rely on direct interactions, whereas a change in expression could be interpretated as a direct regulation. Furthermore, genetic experiments have shown that the *SEP4* gene has the most prominent role for meristem identity, as other mutant combinations with *sep* allele (e.g *ap1 sep3, ap1 sep1 sep2*) never caused a "cauliflower" phenotype (Ditta G.S. et al., 2004). Based on their expression pattern, *SEP1*, *SEP2* and *SEP3* are highly specific for flower and ovule development and single copies of these genes have enough transcriptional activity to maintain the identity of each floral whorl. In contrast, based on its earlier expression, one of the main roles of *SEP4* is to contribute to meristem identity and establish the ground state of the flower, which means providing the environment that the other *SEP* genes can act and regulate organ identity. This floral ground state includes the development of sepals, whereas the transcriptional activity might be too weak to specify organ identity of petals, stamens and carpels. Thus, *SEP4* is not able to specify the identity of all floral organs but only sepals and can be regarded as floral meristem identity gene with a minor function in the regulation of all four floral whorls.

3.84 Triple mutants reveal distinct phenotypes and massively affect the robustness of plant reproduction

As above described, the most striking phenotype affecting floral organ identity is seen in the s*ep1sep2sep3SEP4* triple mutant with floral organs composed of sepals. Nevertheless, the other three triple mutants showed individual characteristics. One example is the *SEP1sep2sep3sep4* triple mutant revealing flowers or flower like structures inside the fourth whorl (Table 3.13). This is known as floral indeterminacy and can be explained by a compromised function of the C-class gene *AG* (Mizukami Y. and Ma H., 1995), since similar phenotypes were observed in intermediate AG RNAi lines (Chuang C.F. and Meyerowitz E.M., 2000). Maybe the other *SEP*s are more strongly involved in the regulation of ovule specific proteinsthan SEP1. It has been previously reported that *SEP*s are necessary for ovule specification as shown in *sep1*$^{+/}$ *sep2 sep3* mutant flowers that produce a similar phenotype (Favaro R. et al., 2003). Ovule development is disrupted in *stkshp1shp2* mutants with some ovules showing a conversion to leaf-like or carpel-like structures (Favaro R. et al., 2003). It could be that be that SEP2 and SEP3 can form stronger complexes with ovule specific proteins than SEP1, or ovule specific proteins rely more on interactions with SEP2 and SEP3 to regulate their downstream targets. In addition it has been reported that SEP3 can add transcriptional activation to these protein complexes (Honma T. and Goto K., 2001; Castillejo C. et al., 2005) and maybe SEP2 can also add more transcriptional activation activity to ovule specific protein complexes than SEP1.

One of the largest common effects observed in all triple mutants was their effect on propagation. Apart from the *sep1sep2sep4* triple mutant at standard growth conditions, *sep1sep3sep4* and *sep2sep3sep4* triple mutants showed a massive decrease in the production of seeds and no seeds were produced in the *sep1sep2sep3* triple mutant. None of the analyzed triple mutants was able to set seeds at 27 °C (Table 3.15). Thus, one of the conclusions is that multiple copies of *SEP* genes enhance the robustness and fitness of the plant to environmental perturbations. As plants are sessile organisms, they have to evolve mechanisms to withstand changing environmental conditions. One of these seems to be the maintenance of multiple gene copies that have undergone a functionalization process, enabling them to respond to changes in their environment. Based on our studies, it seems that the maintenance of multiple *SEP* genes has allowed the plant to propagate under various temperature conditions and presumably other environmental pertubations. Therefore it would be worth conducting field studies with *SEP* or other reported redundant gene mutants (e.g. *SHP1, SHP2*, due to their role in pod shatter, another important process for plant reproduction) to investigate whether

single, double or triple mutants reveal a selective disadvantage under more natural conditions. This could be extended to the study of important crop species (e.g. rice, wheat) to measure the crop yield or seed production in mutants lacking one or more duplicated genes. Another interesting question is how these genes affect the metabolism of a plant, either under standard or impeded growth conditions in terms of nutrient production (vitamin and mineral content, starch content, polyphenols). So far experiments were only performed dealing with the question how these genes affect flowering (Pelaz S. et al., 2000; Pelaz S. et al., 2001a; Ditta G.S. et al., 2004; Lopez-Vernaza M. et al., 2012), however recent genome-wide studies have shown that floral organ identity or flowering time genes are not only involved in flower specific pathways, but also for example in pathogen responses (Winter C.M. et al., 2011). Therefore it would not be a surprise if *SEP*s affect more processes in the plant than flower development. Ideally, novel functions could be employed into economically important aspects, for example to increase seed or nutrient production.

Some mechanistic questions remain to be solved. Considering their important role in the formation of multiprotein complexes (Honma T. and Goto K., 2001; Immink R.G. et al., 2009), it will be interesting to unravel how the individual functions can be linked to targets for individual SEPs. Based on the phenotypes, *SEP*s have individual as well as common functions. Looking at the molecular level raises the question “what can we expect in terms of targets”? Would it not be likely to see common as well as individual targets for all SEPs? The next chapter will deal with an approach to identify genome wide targets with main focus on the SEP4 protein.

Chapter 4

Molecular identification of direct targets of SEP4

4.1 Advanced molecular methods and their impact on unravelling genetic redundancy and Gene Regulatory Networks (GRNs)

One of the biggest questions in biology is how complex organisms with hundreds of different tissues develop from single progenitor cells? This is linked to the question how genes are activated or repressed at different developmental stages, in different tissues and in response to exogenous cues. *Arabidopsis thaliana* contains roughly 25000 protein coding genes including approximately 2000 transcription factors TFs (Guo A. et al., 2005; Mitsuda N. and Ohme-Takagi M., 2009). TFs regulate the expression of their target genes by binding to recognition sites in promoter regions to either increase or decrease their expression. The complete set of genes in the genome can be seen as a gene regulatory network (GRN) that coordinates differentiation processes throughout development. Often TFs bind and regulate multiple targets simultaneously and these targets are controlled by multiple factors. GRNs can be constructed to describe these interactions and to represent interactions at multiple TF-target levels. The first approaches have been undertaken to unravel these GRNs in plants, (Bassel G.W. et al., 2012) but our understanding of the interplay between different TFs is still rudimentary. Thanks to progress in global gene expression analysis (Aleksic J. and Russell S., 2009), more and more data is becoming available that could be integrated into the GRNs. What is now becoming clear is that GRNs act as a web of crosstalk, feedback and redundancy bound tightly with other developmental processes by common TFs (Posé D. et al., 2012). As chapter 3 has shown, complete functional redundancy does not exist in the *SEP* family and this seems to be a feature of reported genetic redundancy in general. Our mutant analysis has shown that is it just a question of finding the right experimental conditions to detect an effect in a reportedly redundant gene and there is almost no limit for experimental set-ups. As Louis Wolpert once remarked about no-phenotype knockout mice, “But did you take it to the opera”? (Tautz D., 2000). As well as exposing an organism to enhanced stress levels, another possibility to assess redundancy is the use of molecular methods to identify direct targets of

individual *SEP*s. This allows the dissection of genetic redundancy at a molecular level. Using this approach could give more information about commonly regulated and distinct targets and thereby shed light into individual functions. A long-term goal is to integrate these data into mathematical models to obtain a more detailed picture of GRNs.

This chapter will describe approaches using methods like Chromatin immunoprecipitation (ChIP), Chromatin immunoprecipitation followed by deep sequencing (ChIP-SEQ) and microarrays to identify direct targets of SEP4 and compare them to published data for SEP3 (Kaufmann K. et all., 2009).
Two questions will be addressed: firstly what is the role of SEP4 during flower development and secondly, can we explain the non-redundant functions reported in chapter 3 by differences in preferred targets between the SEPs? To address the first question, ChIP experiments were established to identify direct targets of SEP4. After successful implementation of this method the next step was the identification of genome-wide targets of SEP4 in different mutant backgrounds. To address the second question, I compared the newly identified targets of SEP4 with previously characterised SEP3 targets.
In this chapter, a short introduction will explain the methods used, followed by the results of ChIP and ChIP-SEQ experiments from *SEP4*:GFP lines in different mutant backgrounds and gene expression studies.

4.2 ChIP and ChIP-SEQ

Protein-DNA interactions are essential for living organisms, for example in the recognition of *cis*-regulatory elements (CREs) by TFs. Gene expression is mainly controlled by CREs that recruit TFs to modulate transcription. It is helpful to understand which of the many TFs expressed in the plant recognizes which CRE on specific promoters, to get a better understanding of developmental processes and how transcriptional networks occur. ChIP is widely used to identify direct TF binding sites *in vivo* in the genomes of yeast, animals and plants (Bowler C. et al., 2004). The ChIP protocol consists of several steps (Fig. 4.1), starting with chemical crosslinking of DNA-protein interactions followed by fragmentation of the cross-linked chromatin e.g. by sonication and subsequent immunoprecipitation of the TF and its bound DNA fragment using a specific antibody (AB). Cross-linked DNA-protein complexes are reversed, the DNA is purified and finally the enrichment of a particular DNA sequence is

checked with quantitative real-time PCR (ChIP-qPCR). This method measures the abundance of a particular DNA sequence, enriched by a protein-specific immunoprecipitation versus an immunoprecipitation with a non-specific antibody control. The level of enrichment is expressed as fold enrichment above the background, which means enrichment relative to that of a non-specific antibody control.

The disadvantage of this method is that only a few genes and promoters can be analysed in one experiment. The discovery of novel, unexpected targets in such an experiment is nearly impossible. More recently, advances in sequencing technologies have resulted in ChIP followed by deep sequencing (ChIP-SEQ) becoming available. This technology allows Protein-DNA interactions to be mapped at a genome wide scale (Johnson D.S. et al., 2007; Robertson G. et al., 2007; Kaufmann K. et al., 2010a). In this work, Illumina Sequencing technology was used. This method can be divided into several steps: after satisfactory ChIP, the samples are processed to generate libraries for subsequent sequencing. The ends of the ChIP DNA need to be repaired before adapters are ligated to the samples followed by low cycle amplification. These amplified PCR products are denatured and one end of the single-stranded product attaches to the surface of the flow cell. The flow cell is a channel sealed glass microfabricated device that allows bridge amplification of fragments on its surface. The “free” end is allowed to anneal to a primer on the surface of the flow cell that has a complementary sequence to the adapter resulting in double stranded bridge structures. These double stranded “bridges” are denatured producing two single strand products. This cycle is repeated to form clusters also called cluster formation. Subsequently adding polymerase, primer and fluorescently labelled nucleotides that prevent extension performs sequence synthesis. As a result one nucleotide is added to each DNA fragment. After every synthesis round, an image of the flow cell is captured using fluorescence microscopy followed by the next synthesis step (Mardis E.R., 2008). After finishing the process, image analysis is performed to determine the DNA fragments in each cluster and to generate the so called “base calls”. Subsequently the obtained reads are computationally mapped against the reference genome using a sequence alignment (Li H. et al., 2008). Deep sequencing relies on DNA regions that are enriched by the ChIP being more often sequenced than expected by chance, therefore sequence alignment can be used to find over-represented regions revealing a “peak” at a certain position that marks the position where DNA and protein interact.

Fig. 4.1

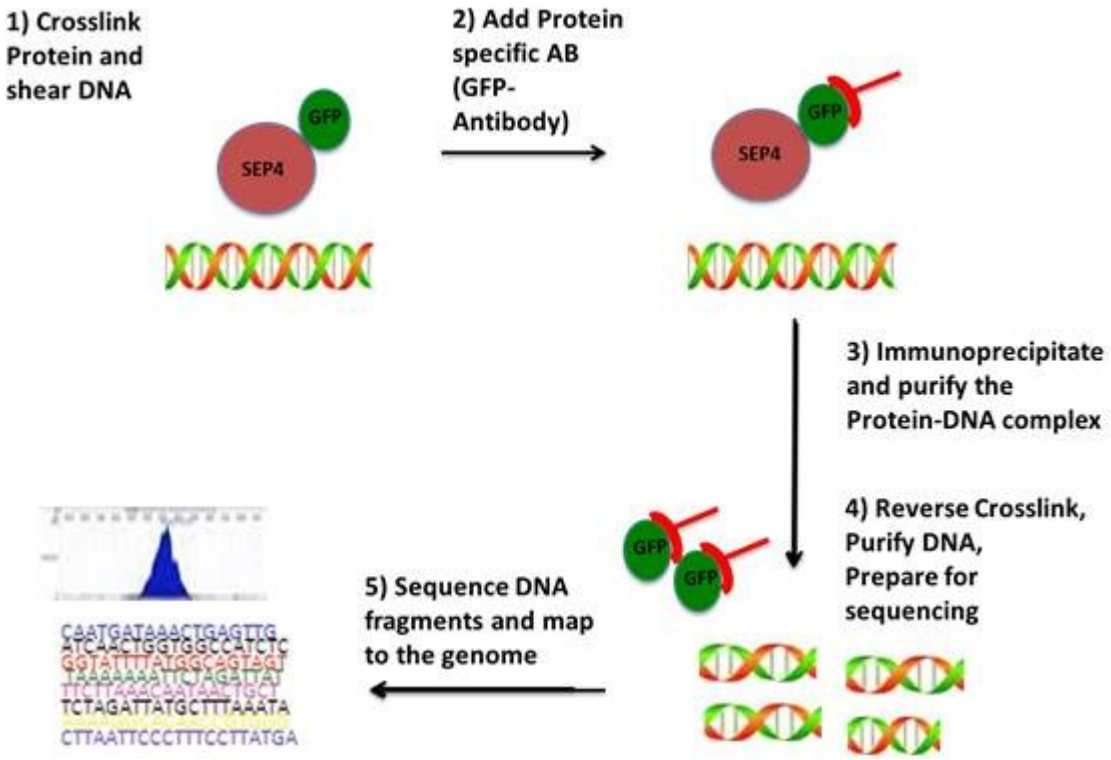


Fig. 4.1: Diagrammatic representation of ChIP-SEQ protocol for the SEP4 protein.

1) The SEP4 protein is fused to a green fluorescent protein (GFP). Cells are fixed in a formaldehyde containing solution (1 %) to preserve *in vivo* DNA-protein interactions. Afterwards nuclei and chromatin are released by cell lysis. DNA is sheared into up to 500 bp fragments by sonication. 2) A specific GFP antibody is added to the sheared DNA-Protein fragments. 3) The Protein bound DNA is immunoprecipitated. 4) Proteins are released from the DNA by reverse-crosslinking and digestion. Finally DNA is purified and qPCR experiments are performed to test the enrichment of the ChIP sample using positive and negative control genes. After checking for enrichment, samples are prepared and processed into DNA libraries. 5) Libraries are sequenced and resulting reads are mapped against the *A. thaliana* genome

4.3 Microarrays

DNA Microarrays enable us to analyse the expression of thousands of genes simultaneously. Two methodologies of arrays are available, firstly the so-called cDNA array, where PCR-generated cDNA probes are robotically spotted on solid supports (glass slides or nylon membranes). The samples to compare are labelled with 2 different fluorescent dyes and co-hybridize to the same array. The (relative) abundance of control vs. sample is measured (Schena M. et al., 1995).

Affymetric developed the so-called “GeneChip®” arrays where several short oligonucleotides represent one gene. They are synthesized “*in situ”* by photolithogrpahy and samples under comparison are labelled with the same dye and hybridised to different arrays (Chee M. et al., 1996). The absolute mRNA content is measured. This study used ATH1 arrays (also known as 23K or 25K array) that included probe sets representing approximately 23000 Arabidopsis genes.

4.4 Identification of *FD*, *SEPALLATA3*, *SEPALLATA4* and *LEUNIG* as direct targets of SEP4 in g*SEP4*:GFP;*sep4*

As described in section 3.6, g*SEP4:*GFP lines were generated, which rescued the extra perianth organs of the *sep4* and *sep1sep4* mutants. Lines of g*SEP4*:GFP;*sep4* that showed good complementation (e.g. Lines 2, 7 and 9 of g*SEP4*:GFP;*sep4,* Table 3.16) and of g*SEP4:*GFP;*sep1sep4* (Line 1 and line 2 of g*SEP4*:GFP;*sep1sep4*, Table 3.17), were used to perform multiple independent ChIP experiments. To identify targets of SEP4, the Lines g*SEP4*:GFP;*sep4* 2, 7 and 9 were used for ChIP experiments because they revealed good complementation results (Chapter 3, section 3.7, Table 3.16). In parallel ChIP was performed on WT plants that did not express the GFP coupled version as a control sample.

To verify that the SEP4 ChIP was successful, qPCR was employed to measure the relative abundance of particular DNA sequences enriched by a protein-specific immunoprecipitation versus the WT control. The level of enrichment of the target region can be calculated as the ratio of amplified product from the ChIP material (anti-GFP) to that from the WT control. The results between the two samples were normalized using sequences of two reference genes, *Heat Shock Factor1* (*HSF1)* and *RESPONSE REGULATOR 6* (*ARR6*) that were previously used in a genome wide binding profile study of SEP3 (Kaufmann K. et al., 2009). Based on the fact that no direct target of SEP4 was known, but the genes *APETALA1 (AP1*), *SEPALLATA3* (*SEP3*) and *FD* were identified as direct targets of SEP3 (Kaufmann K. et al., 2009; Kaufmann K. et al., 2010b), a close homologue of *SEP4*, primers specific for these genes were used to check whether they are also direct targets of SEP4. In addition, *SEP4* and *LEUNIG* (*LUG*) primers were designed to test whether SEP4 binds to its own promoter and whether *LUG* is a direct target of SEP4.

As seen in Fig. 4.2 a, Line 2 showed good enrichments for *SEP3*, *FD* and *SEP4*. *LUG* was not tested as a candidate for this line. Line 7 revealed strong enrichments for *LUG* and *SEP4* but no increased fold change was detected for *SEP3*, *AP1* or *FD* (Fig. 4.2 b). A similar result was detected for Line 9 with increased enrichments for *SEP4* and *LUG* but no enrichments for *SEP3*, *FD* and *AP1* (Fig. 4.2 c). As from previous studies known, the genes *AP1* and *FD* were identified as direct SEP3 targets (Kaufmann et al., 2010), and Line 2 revealed enrichment for these two genes as well. For further experiments, Line 2 from g*SEP4*:GFP;*sep4* will be used because it shows greater fold enrichments for *FD* and *SEP3* compared to Line 7 and 9 (Fig. 4.2 a compared to Fig. 4.2 b and c).

Fig. 4.2

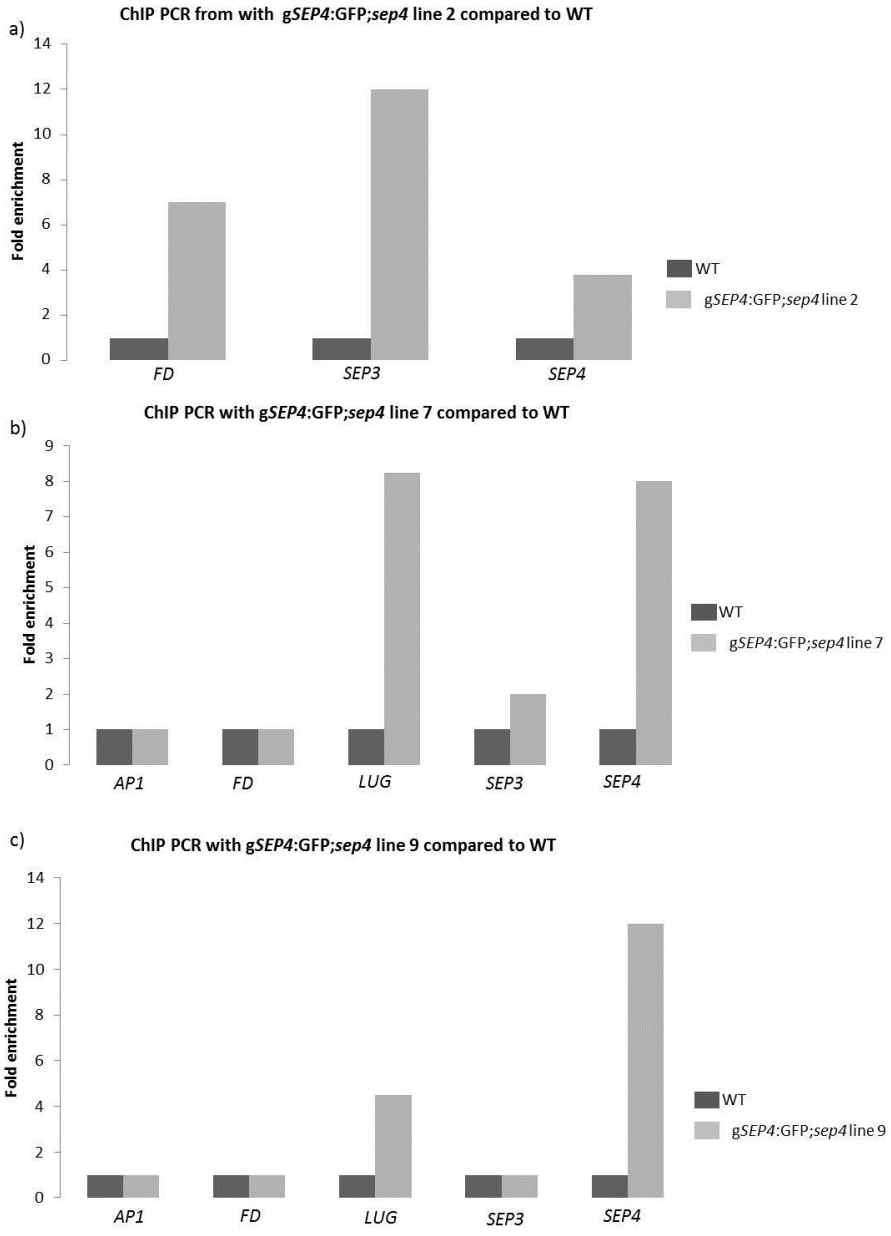


Fig. 4.2 ChIP PCR analysis to identify direct targets of g*SEP4*:GFP;*sep4*

Three different lines were checked, a) qPCR results from g*SEP4:*GFP;*sep4* Line 2 with *FD*, *SEP3* and *SEP4*, b) qPCR results from Line 7 g*SEP4:*GFP;*sep4* with *AP1*, *FD*, *LUG*, *SEP3* and *SEP4,* c) qPCR results from Line 9 g*SEP4*:GFP;*sep4* with *AP1*, *FD, LUG*, *SEP3* and *SEP4*. Two reference genes *ARR7* and *HSF* were used in each experiment

4.5 Identification of *APETALA1*, *FD*, *SEPALLATA3* and *SEPALLATA4* as direct targets of SEP4 in g*SEP4*:GFP;*sep1sep4*

The complementation analysis described in section 3.6 showed that the Lines 1 and 2 of g*SEP4*:GFP;*sep1sep4* (Table 3.17) complemented the perianth organ phenotype well. These lines were selected for ChIP. As described in section 4.4 , ChIP experiments were performed on WT plants that did not express the GFP coupled version as a control sample. After each ChIP experiment, qPCR was employed to measure the relative abundance of a particular DNA sequence enriched by a protein-specific immunoprecipitation versus the WT control. Recently, a genome wide target identification study of SEPALLATA3 was published (Kaufmann K. et al., 2009; Kaufmann K. et al., 2010b). We decided to use following primer pairs from this study to test if they are direct targets of SEP4 as we assume that some of them share common targets. Those were: *APETALA1 (AP*1), *APETALA2* (*AP*2), *APETALA3* (*AP*3), *AGAMOUS* (*AG)*, *SEPALLATA3* (*SEP*3), *SEPALLATA4* (*SEP4*), *FD*, *AGAMOUS-LIKE 20* (*SOC1)* and *LEAFY* (*LFY)*. Both lines gave similar results revealing enrichments for *AP1*, *FD*, *SEP3* and *SEP4* (Fig. 4.3 a-c). However g*SEP4*:GFP;*sep1sep4* Line 2 showed greater fold enrichment for the genes *AP1*, *FD*, *SEP3* and *SEP4* (Fig. 4.3 a, b). These results suggest that *AP1*, *SEP3, SEP4* and *FD* are direct targets of SEP4, at least in a complemented g*SEP4*:GFP;*sep1sep4* background. Line 2 is the preferred line as it showed better enrichments for the tested candidate genes *AP1*, *FD, SEP3*, *SEP4*, subsequently this line will be used for further experiments to obtain genome wide targets of SEP4

Section 4.4 and 4.5 describes experiments that were the basis for the next steps, namely generating ChIP-DNA libraries followed by subsequent sequencing (Ilumina/Solexa sequencing, ChIP-SEQ) to obtain genome-wide binding sites of SEP4 in two mutant backgrounds.

Fig. 4.3

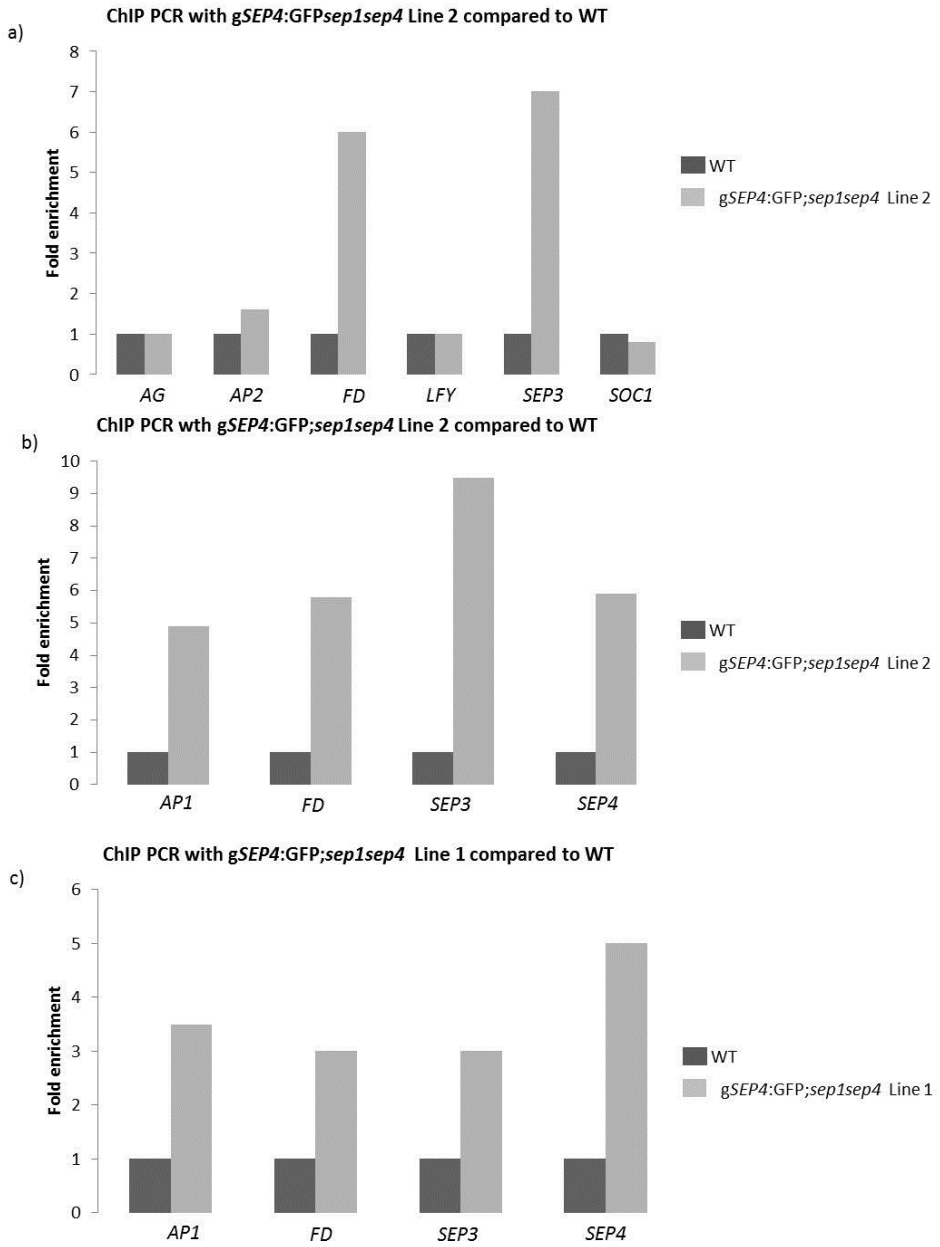


Fig. 4.3 : ChIP PCR analysis to identify direct targets of SEP4 in g*SEP4*:GFP;*sep1sep4.*

Two independent lines were used and tested by qPCR, a) qPCR results from g*SEP4*:GFP;*sep1sep4* Line 2 with *AG*, *AP1*, *FD*, *LFY*, *SEP3 and SOC1* compared to WT, b) qPCR results from g*SEP4*:GFP;*sep1sep4* Line 2 with *AP1*, *FD*, *SEP3* and *SEP4* compared to WT, c) qPCR results from g*SEP4:*GFP;*sep1sep4* Line 1 with *AP1*, *FD*, *SEP3* and *SEP4* compared to WT. Two reference genes, *ARR7* and *HSF* were used in each experiment

4.6 Identification of genome-wide targets of SEP4 using Next generation sequencing

ChIP-DNA libraries were generated from g*SEP4:*GFP;*sep1sep4* Line 2 and g*SEP4*:GFP;*sep4* Line 2 using the NEXTflex™ DNA Sequencing Kit (see Material and Methods, section 2.62). In addition to the SEP4 ChIP-DNA libraries, a library from a WT sample was prepared as a control. The first sample from g*SEP4*:GFP;*sep1sep4* Line 2 was sequenced using the Illumina® GAIIx platform (MPI, Tuebingen, Germany) and hereafter named g*SEP4:*GFP;*sep1sep4* rep1. All Bioinformatic analysis was performed by Pedro Madrigal (Prof. Paweł Krajewski, Institute of Plant Genetics PAS, Poznań, Poland) and Jose Munjos (Plant Research International, Wageningen). This analysis included the mapping of the sequnces against *A. thaliana* genome as well as all statistical analysis to identify target genes. From this run performed using the Illumina® GAIIx platform (MPI, Tuebingen, Germany), 36051224 unpaired 35-bp reads for the SEP4 sample were obtained of which 54.69 % exactly mapped one time to the *Arabidopsis* genome (overall alignment 78.59 %, Tair 9 version used as reference, Table 4.1). It is important that these reads map one time to the *A. thaliana* genome to obtain specific enriched regions. If reads for example map several times to the genome, mapping of those reads will result in a number of unspecific reads distorted over the genome and not reveal specific regions. The reads were extended to 300 bp in order to recover the average original DNA fragments that were subjected to sequencing similar as described by Robertson et al. (Robertson G. et al., 2007). This allows positioning of the maximum of enrichment present in the samples at high resolution and means specific enriched regions. To test for enrichment at each nucleotide position in the sample compared to the control, a score based on the Poisson distribution was used, as used for statistical modelling of tag counts (Robinson M.D and Smyth G.K:, 2007). For each genomic region representing a candidate peak, the maximum score value was used to test the significance of the peak (defined as peak score). The false discovery rate (FDR) was used to control the error rate of the testing procedure.

In this study, all other samples (WT rep1, WT rep2, Line 2 from g*SEP4*-GFP*sep1sep4* rep2, Line 2 from g*SEP4*-GFP*sep4* rep1 and Line 2 g*SEP*4-GFP*sep4* rep2) were sequenced using the Illumina® HiSeq 2000 platform (Plant Research International, Wageningen, NL). From this run, for WT rep1, 69740220 total reads were obtained of which 66.56 % exactly mapped one time to the *Arabidopsis* genome, the second replicate from the WT sample revealed 30428194 total reads with 44.32 % exactly mapping one time to the *Arabidopsis* genome (Table 4.1). For the second replicate from g*SEP4*:GFP;*sep1sep4* Line 2, 38950350 total reads were obtained of which 20.86 % exactly mapped one time to the *Arabidopsis* genome (Table 4.1). The two replicates from g*SEP4*:GFP;*sep4* Line 2 revealed for rep1, 21602587 total reads with 14.16 %

reads that uniquely mapped one time to the genome and for rep2, 13392508 total reads of which 20.21 % exactly mapped one time to the genome (Table 4.1

The *SEP4* gene is fused at the N-terminus to a GFP protein (Fig. 3.7). In this experiment, a GFP antibody was used in the ChIP experiments to pull down the SEP4 protein and its targets. In contrast, the ChIP protocol should not pull down targets of the negative control (a WT sample that is not fused to GFP). Therefore, an abundance of DNA from the target genes that were bound by SEP4 compared to the WT control sample can be expected. The sequencing of the DNA fragments from a ChIP experiments can be used to quantify the occurrence of specific DNA sequences in the sample and identify genomic regions that are enriched. High throughput sequencing of DNA fragments from ChIP experiments gives millions of reads that are mapped against a reference genome. A significant accumulation of reads to a region in the genome is called a peak. Based on the idea that SEP4 binds specifically to target genes whereas the negative control does not, a successful sequencing experiment should result in significant peaks localized close to a gene or in the *SEP4* gene, whereas the negative control should not give significant peaks but only randomly distorted reads in the genome. The sequencing results can be visualized using the Integrated Genome Browser (http://bioviz.org/igb/) (see Fig. 4.5).

As seen in Table 4.1 the number of reads that exactly mapped one time on the *A. thaliana* genome for all SEP4 samples is very low, except for the first replicate from the g*SEP4*:GFP;*sep1sep4* sample. Less than 20 % of all samples mapped exactly one time to the genome and as a consequence, these results cannot be included in further analysis (personal recommendation Pedro Madrigal). Accordingly, the first replicate from g*SEP4*:GFP;*sep1sep4* was used for further analysis and will be referred as SEP4 targets. In this target list at FDR < 0.001, 1211 enriched regions (also called target genes) were found (Fig. 4.4 b) and analysed for Gene Ontologies (GOs) that were overrepresented in our SEP4 target list (target list: S2, electronic supplementary data, eS). I used the AGRIGO web based tool (Du Z. et al., 2010) to identify overrepresented GO terms and functional annotation clustering using a p-value < 0.05. Dissection of GOs according to biological functions revealed overrepresented terms involved in cellular process (GO:0009987, p-value 5e-10), biological regulation (GO:0065007, p-value 2.2e-09), regulation of cellular metabolic process (GO:0031323, p-value 7.4e-09), regulation of biological process (GO:0050789, p-value 6.7e-08), response to stimulus (GO:0050896, p-value 1.7e-06), reproductive developmental process (GO:0003006, p-value 3.1e-06), multicellular organismal process (GO:0032501, p-value 4e-06), reproductive process (GO:0022414, p-value 1.1e-05) and developmental process (GO:0032502, p-value 1.9e-05). Dissection into molecular functions revealed overrepresented GO terms in binding (GO: 0005488, p-value 2.1e-18), catalytic activity (GO:0003824, p-value 8.7e-12) and transcription regulator activity (GO:0030528, p-value 1.1e-09) (Table 4.2). Our SEP4 target list revealed that many genes

involved in flower development are direct targets. For example all the ABC genes *AP1*, *AP2*, *AP3*, *PI* (Fig. 4.5 b), *AG* and the two E -class genes *SEP3* and *SEP4* (Fig. 4.5 a) appeared as direct targets. The two genes *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*), both involved in meristem identity were also targeted by SEP4. In the GO group reproductive process, genes appeared that are associated with the (negative) regulation of *AG,* like the transcriptional co-repressors *LEUNIG* and *SEUSS*, two negative regulators of *AG* as well as *BELL 1* an ovule-specific negative regulator of the C -function gene (Western T.L. et al., 1999). Another target gene involved in the development of reproductive organs is *SUPERMAN* (*SUP*), a cadastral gene that maintains whorl boundaries and has been proposed to control the balance of cell proliferation at the boundary between stamens and carpels by regulating the transcription of genes that affect cell division (Hiratsu K. et al., 2002). The two *SHATTERPROOF (SHP1, SHP2)* genes that are necessary for pod shatter (Liljegren S.J. et al., 2000) also appeared in the GO group reproductive process.

Another group of genes detected were those involved in flowering time, such as *AGAMOUS-LIKE 20* (*SOC1*), *SHORT VEGETATIVE PHASE* (*SVP*), *AGAMOUS LIKE 24* (*AGL24*), *FLOWERING LOCUS C* (*FLC*), *EMBRYONIC FLOWER 1* (*EMF1*), *FIONA1* (*FIO1*) and *FD* (Fig. 4.5 c). The finding that all genes identified in the ChIP experiments (*AP1*, *FD*, *SEP3* and *SEP4*) as described in section 4.5 Fig. 4.3 a, b also appeared as targets in the ChIP-SEQ experiment confirmed that the ChIP protocol was successful in identifying targets of SEP4. Why genes like *AG* and *SOC1* were not enriched in the ChIP experiment but were targets in the sequencing might lie in design and specifity of the designed primer pairs.

Under the GO “response to stimulus”, transcription factors that are involved in hormone signalling like *Auxin response factor 2 (ARF2)*, *Auxin response factor 3 (ARF3)*, *Auxin response factor 7 (ARF7)*, *Gibberellin 2-oxidase* (*GA2OXO1*) or *ETTIN* (*ETT*) were identified.

As well as genes involved in floral organ development and hormone signalling, transcription factors involved in organ boundary formation were found, like the NAC domain containing transcription factor *CUP SHAPED COTYLEDON 3* (*CUC3*) Another group of identified direct targets are involved in Nitrogen signalling, for example *LATERAL ORGAN BOUNDARY 37 and 39* (*LBD37, LBD39*).

Kaufmann et al. (Kaufmann K. et al., 2009) performed a genome-wide binding study on SEP3 using a native antibody raised against a C-terminal peptide of SEP3 for their ChIP experiments. These publically available data (Muiño J.M. et al., 2011) revealed enriched GO terms in biological regulation (GO: 0065007, p-value 7.9e-39), response to stimulus (GO: 0050896, p-value 4.1e-36), postembryonic development (GO:0009791, p-value 2.7e-32), response to hormone stimulus (GO:0009725, p-value 1.9e-18), developmental process (GO:0032502, p-

value 2e-18), reproduction (GO:0000003, p-value 2.2e-10), flower development (GO:0009908, p-value 2.6e-06) and many more (Supplementary data S4, list of GOs, list of all targets available as electronic supplementary material, eS).

We were interested in common targets between SEP4 and SEP3 and used the published targets of SEP3 (Kaufmann K. et al., 2009) to compare them against the SEP4 targets using Gene Spring version 12.1 (Agilent Technologies). As above describe, the AGRIGO web based tool (Du Z. et al., 2010) was used to identify overrepresented GO terms. From 3437 targets of SEPALLATA3 (Fig. 4.4 a), 309 were found that were also targeted by SEP4 (Fig. 4.4 c, S3, eS). From these common targets, overrepresented GO terms revealed genes involved in biological regulation (GO:0065007, p-value 1.5e-10), regulation of metabolic process (GO:0019222, p-value 9.3e-10), regulation of biological process, (GO:0050789, p-value 1.3e-09), regulation of cellular process (GO:0050794, p-value 4e-09), response to abiotic stimulus (GO:0009628, p-value 2.5e-07), reproductive process (GO:0022414, p-value 5.4e-07), multicellular organismal development (GO:0007275, p-value 7.9e-07) and positive regulation of biological process (GO:0048518, p-value 0.00037). Overrepresented GO terms involved in molecular functions were found for transcription regulator activity (GO: 0030528, p-value 1.8e-14) and binding (GO: 0005488, p-value 2.8e-07) (Table 4.3). Among those genes involved in developmental processes, many were involved in flower development. For example all ABC genes appeared as common targets (*AP1*, *AP2*, *AP3*, *PI, AG*), as well as genes like *SUP*, *SOC1*, *SVP*, *AGL24*, *FD*, *AUXIN RESPONSE FACTOR 3*, *5*, *7* (*ARF3*, *ARF5, ARF7)* and *SHATTERPROOF2* (*SHP2*) (S3).

Table 4.1

	Total number of reads	Number of reads (in %), overall alignment rate	Number of reads that aligned exactly 1 time, (in %)
WT , rep1	69740220	88.58	66.56
WT, rep2	30428194	66.35	44.32
g*SEP4*:GFP;*sep1sep4* Line2, rep1	36051224	78.59	54.69
g*SEP4*:GFP;*sep1sep4* Line2, rep2	38950350	30.58	20.86
g*SEP4*:GFP;*sep4* Line2, rep1	21602587	21.81	14.16
g*SEP4*:GFP;*sep4* Line2, rep2	13392508	31.35	20.21

Table 4.1: Summary of the sequencing results from ChIP-SEQ experiments

Sequencing results from WT, gS*EP4*-GFP*sep1sep4* Line 2 and gSEP4-GFP*sep4* Line 2 ChIP-SEQ experiments. The total number of reads is given for each replicate, next to the percentage of reads that aligned against the *A. thaliana* genome (Tair 9) and the percentage of reads that exactly mapped one time against the *A. thaliana* genome.

Table 4.2

Function	GO	Description	p-value
Biological	0009987	cellular process	5e-10
	0065007	biological regulation	2.2e-09
	0031323	regulation of cellular metabolic process	7.4e-09
	0019222	regulation of metabolic process	4.8e-08
	0050789	regulation of biological process	6.7e-08
	0050896	response to stimulus	1.7e-06
	0003006	reproductive developmental process	3.1e-06
	0032501	multicellular organismal process	4e-06
	0022414	reproductive process	1.1e-05
	0032502	developmental process	1.9e-05
Molecular	0005488	Binding	2.1e-18
	0003824	catalytic activity	8.7e-12
	0030528	transcription regulator activity	1.1e-09

Table 4.2: Overrepresented GO terms from SEP4 targets

GOs are dissected in biological and molecular functions, AGRIGO was used to identify GO terms, p-values < 0.005

Table 4.3

Function	GO	Description	p-value
Biological	0065007	biological regulation	1.5e-10
	0019222	regulation of metabolic process	9.3e-10
	0050789	regulation of biological process	1.3e-09
	0050794	regulation of cellular process	4e-09
	0009628	response to abiotic stimulus	2.5e-07
	0022414	reproductive process	5.4e-07
	0007275	multicellular organismal development	7.9e-07
	0048518	positive regulation of biological process	0.00037
Molecular	0030528	transcription regulator activity	1.8e-14
	0005488	Binding	2.8e-07

Table 4.3: Overrepresented GO terms for 309 common targets between SEP3 and SEP4

GOs are dissected in biological and molecular functions, AGRIGO was used to identify GO terms, p-values < 0.005

Fig. 4.4

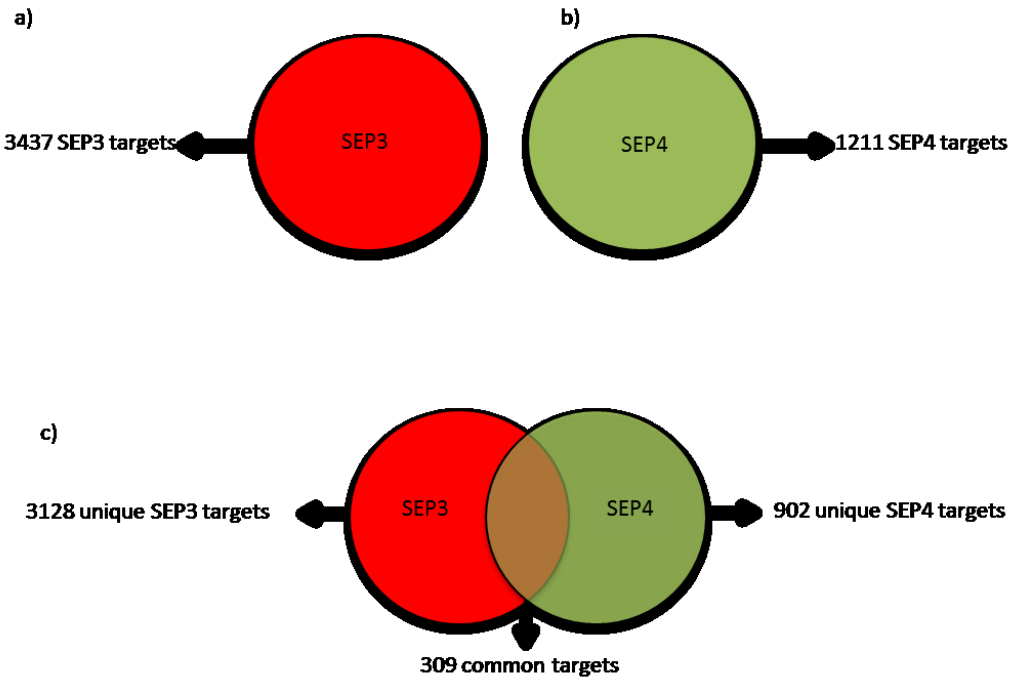


Fig. 4.4: Comparison between SEP3 and SEP4 ChIP SEQ targets

Venn Diagrams showing the number of targets for a) SEP3, b) SEP4 and c) overlapping and individual targets of SEP3 and SEP4. All targets were in the same tissues (inflorescences 1-12), FDR was set to <0.001

Fig. 4.5

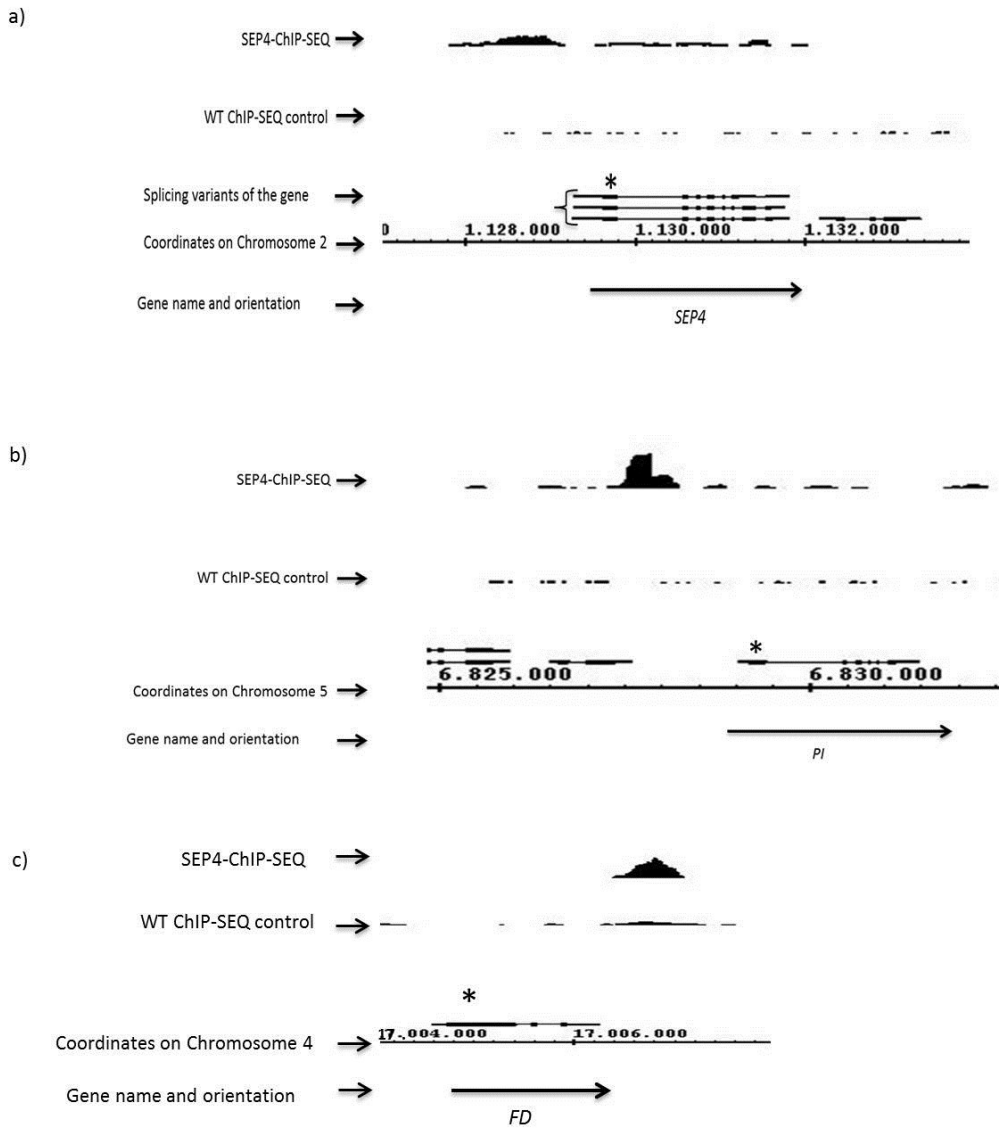


Fig 4.5: Binding profiles of gene loci from identified targets of SEP4.

The graphs in each panel show the local enrichment of SEP4 binding in g*SEP4*:GFP:*sep1sep4* over the WT control sample, asterisks indicate the translational start codon. The graphs in each panel are from a) *SEPALLATA4*, b) *PISTILLATA*, c) *FD*. Note that the peaks for *SEP4* and *PI* are located in the up-stream promotor region whereas the *FD* peak is in the downstream promotor region. Gene models are taken from TAIR 9, for visualisation of the peaks, Integrated Genome Browser was used.

Fig. 4.6

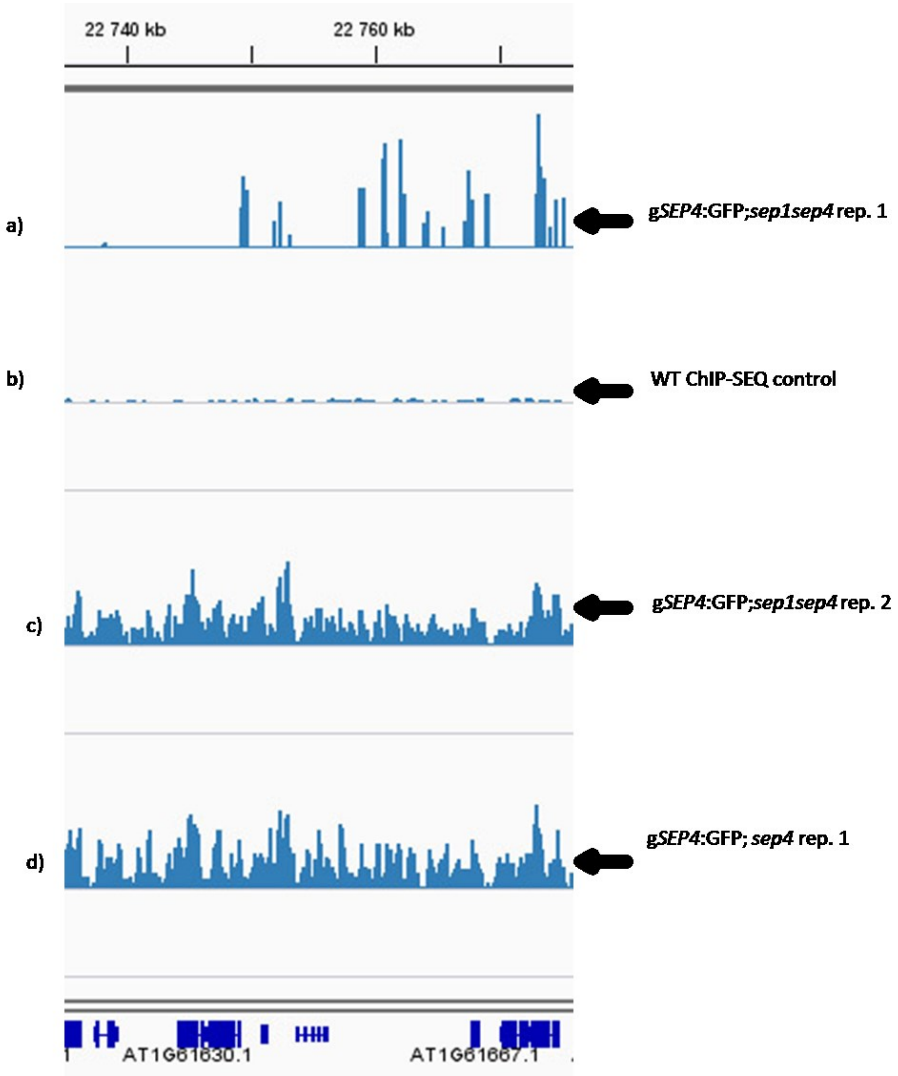


Fig. 4.6: Comparison between different ChIP SEQ experiments

Shown is a cut out from Chromosome 1, where peaks from different experiments were compared to WT and each other regarding their peak quality, a) g*SEP4:*GFP;*sep1sep4* rep. 1*,* reveals specific peaks for a region on the genome that was used in the cut out, b) WT, showing randomly distorted peaks over a genomic region c) g*SEP4:*GFP;*sep1sep4* rep. 2, reveals low peaks that are randomly distorted on the region of the genome used in the cut out, d) g*SEP4*:GFP;*sep4* shows unspecific peaks all over the region of the chromosome that was used for the cut out and a high background signal.

Thus, experiments shown in c) and d) could not be used for further experiments due to the unspecific peaks and high background signals compared to those shown in a).

Models of the genes (Tair 10) flanking the genes are shown at the bottom, the Integrated Genome Browser was used for visualisation. Bioinformatic analysis was done by Pedro Madrigal, this cut out was provided by Pedro Madrigal.

4.7 Identification of independent targets for SEP3 and SEP4

Analysis of the direct targets of SEP3 and SEP4 revealed that, although they share many common targets, the majority of their identified targets are distinct and only bound by either SEP3 or SEP4. I will refer to these targets as unique SEP3 or unique SEP4 targets in further analysis. Comparison between direct SEP3 and SEP4 targets showed that the following genes were uniquely bound by SEP3. The *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) family (*SPL3, SPL5, SPL9, SPL12*) are regulated by miRNA156 and have a role in regulating flowering. The three genes *SCHNARCHZAPFEN* (*SNZ*), *SCHLAFMUETZE* (*SMZ*) and *TARGET OF EAT 1* (*TOE1*) belong to the AP2-domain containing TF family appeared also in the list of SEP3 targets but were not targeted by SEP4. All three genes are regulated by miR172 and act as floral repressors (Zhu Q.H. and Helliwell C.A., 2011). Another overrepresented group of genes targeted by SEP3 were genes involved in Auxin signalling, for example the genes Auxin response factor 4, 6, 8, 10, 11, 16 and 17 and the Auxin-responsive proteins 2, 3, 5, 6, 7 8 and 27 (*IAA2, IAA3, IAA5, IAA6, IAA7, IAA8,* and *IAA27*) appeared as direct targets. Another group of unique SEP3 targets were members of the R2R3 factor gene family encoding TFs like the MYB domain proteins 70, 76, 88, 106, 109 (MYB 70, 76, 88, 106, 109). Furthermore the gene *CLAVATA*1 (*CLV1*), involved in controlling meristem size in the SAM appeared as a target (Clark S.E. et al., 1993; Clark S. E. et al., 1995; Kayes J.M. and Clark S.E., 1998). *JAGGED (JAG)*, a putative transcription factor with a single C_2H_2 zinc-finger domain involved in the formation of lateral organs (Dinneny J.R. et al., 2004) was another unique gene targeted by SEP3 that is known to have a role during plant development.

Analysis of the unique SEP4 targets revealed overrepresented GOs in post-embryonic development (GO:0009791, p-value 6.2e-08), floral organ development (GO:0048437, p-value 0.00046) and response to stimulus (GO:0050896, p-value 0.00088). Genes in the GO post-embryonic development that are targets of SEP4 but not of SEP3 include for example *Nuclear Pore Anchor* (*NUA*), a gene involved in the suppression of premature exit from cell division at the *Arabidopsis* root meristem (Xu X.M. et al., 2007), *BARELY ANY MERISTEM 3* (*BAM3*) which is involved in leaf and meristem development (DeYoung B.J. et al., 2006), *ARABIDOPSIS THALIANA COLD SHOCK PROTEIN 2* (*GRP2*), a negative regulator of cold acclimation and seed germination (Fusaro A. F. et al., 2007), *ERECTA-like 1* (*ERL1*), *FLOWERING LOCUS C* (*FLC),*both involved in flowering time. Another unique SEP4 target is *LEUNIG* (*LUG)*, a negative regulator of *AG* (Sridhar V.V. et al., 2006). The genes *EMBRYO DEFECTIVE 1513* (*EMB1513*), *Maternal Effect Embryo Arrest maternal 23* and *27* (*MEE23* and *MEE27*), homeobox protein BEL1 (*BEL1)* are all involved in ovule development. For example *BEL1* is an ovule-specific negative regulator

of *AG* (Western T. L. and Haughn G. W., 1999). The gene *CLAVATA3/ESR-RELATED 44* (*CLE44*) is involved in leaf development and flowering (Strabala T. J. et al., 2006). NUCLEAR FACTOR Y, SUBUNIT B13 (*NF-YB13*) belongs to a TF family known to regulate developmental phenotypes and stress responses, such as photoperiod-dependent flowering (Ben-Naim O. et al., 2006; Wenkel S. et al., 2006). *LATERAL ORGAN BOUNDARY 39* (*LBD39*), a repressor of anthocyanin biosynthesis and nitrogen signalling (Rubin G. et al., 2009) appeared as a unique SEP4 target as well as the TF *MYB 90*, (eS).

4.8 *AGAMOUS*and *PISTILLATA* are upregulated by SEP4

As the ChIP-SEQ experiments showed many genes involved in flower development are targets of both SEP4 and SEP3. All the ABC genes (*AP1*, *AP2*, *AP3*, *PI*, *AG*) and the two *SEP* genes SEP3 and SEP4 were identified as common targets between SEP3 and SEP4. It has been shown that SEP3 activates the ABC floral organ identity genes (Kaufmann K. et al., 2009). In order to verify whether the potential direct targets of SEP4 are affected in their expression, qPCR was performed on *sep4-1* mutant inflorescences (stage 1-12) and compared to WT. The following genes were tested: *AP1*, *AP2*, *AP3*, *PI*, *AG* and *SEP3.* The genes *PI* and *AG* showed a change in expression as evidenced by a significant down regulation in the *sep4-1* mutant (Fig. 4.7). The other genes tested were not affected in their expression. As a result it can be suggested that SEP4 is able to regulate the transcription of the B and C-function floral organ identity genes *PI* and *AG.* Thus SEP4 is able to activate floral organ identity genes. This is in agreement with genetic experiments from Ditta et al. and the conclusion that *SEP4* is necessary for a critical threshold for proper flower development and contributes to the development of all four floral organs (Ditta G.S. et al., 2004).

Fig. 4.7

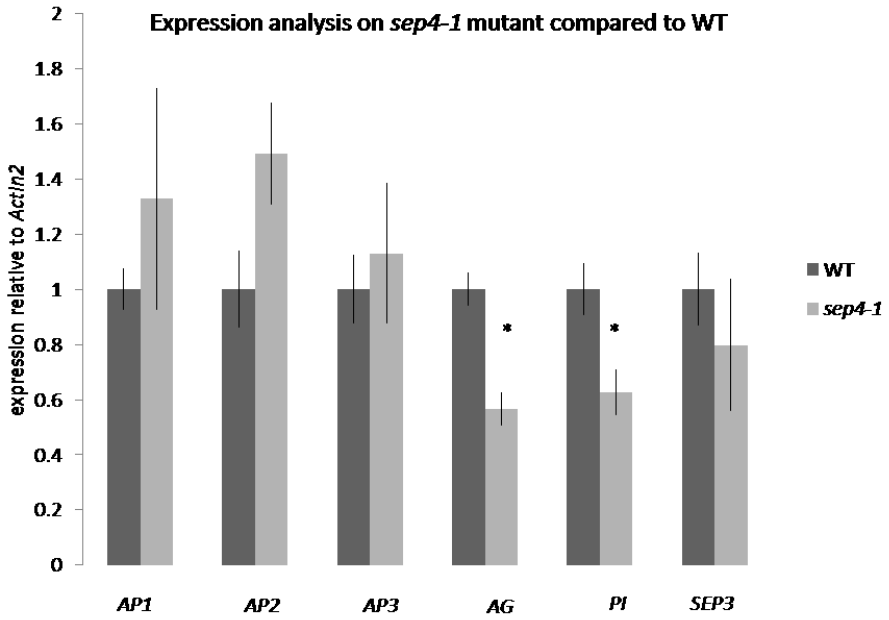


Fig. 4.7: **Expression analysis on floral organ identity genes in *sep4-1* mutants.**

The ABC genes *AGAMOUS* (*AG*), *APETALA1* (*AP1*), *APETAL2* (*AP2*), *APETALA3* (*AP3*), *PISTILLATA* (*PI*) and the MADS-Box gene *SEPALLATA3* (*SEP3*) were tested by qPCR, if their expression is affected in the *sep4-1* mutant and compared to WT.

Bars= Standard Error of the Mean (SEM), asterisks indicate if expression is significantly different to WT expression with p= <0.05, Four biological and two technical replicates were used for each experiment, Inflorescences from stage 1-12 was used as tissue.

4.9 Global expression analysis of *sep3-2* and *sep4-1* mutants using ATH1 Microarrays

Due to the number of target genes identified in the SEP4 ChIP-SEQ experiment and the already published SEP3 data, microarray experiments were performed to obtain an overview of how many transcripts are affected in their expression in *sep3-2* and *sep4-1* mutants (up regulation or down regulation in the mutant). These experiments will also give an idea how many of the direct targets of SEP3 and SEP4 are affected in their expression. The array data were analysed using Gene Spring software (Agilent Technologies, version 12.1). Scatter plot analysis was used to compare the expression values of all ATH1 microarray experiments. Logarithmic expression values (log2) of each mutant sample were plotted versus WT samples. Genes were considered as enriched when they showed at least a fold change of 1.5 up or down compared to the WT.

4.91 Overrepresented GOs in *sep3-2* microarrays

For *sep3-2,* 973 up-regulated (S5, eS) and 317 down-regulated transcripts (S6, eS) were found (Fig. 4.8 a). The enrichment of gene ontology (GO) terms among these up- and down-regulated transcripts was investigated using AGRIGO (Du Z. et al., 2010), filtering for a p value less than 0.01 ($p<0.01$). GOs in terms of biological functions revealed that up regulated transcripts for *sep3-2* were enriched in processes involved in sexual reproduction (GO :0019953, p-value 3.1e-08), response to stimulus (GO:0050896, p-value 2.2e-07), multi-organism process (GO:0051704, p-value 0.0003), developmental growth (GO:0048589, p-value 0.001) and localization (GO:0051179, p-value 0.0011) (Table 4.4).

Down-regulated transcripts for *sep3-2* were involved in reproductive processes (GO:0022414, p-value 1.4e-08), reproduction (GO:0000003, p-value 2.2e-08), multicellular organismal development (GO:0007275, p-value 5e-07), developmental process (GO:0032502, p-value 1e-05), cellular processes (GO:0009987, p-value 1.9e-05) and biological regulation (GO:0065007, p-value 0.00012) (Table 4.5).

4.92 Overrepresented GOs of *sep4-1* microarrays

For *sep4-1*, 517 transcripts were up- and 904 down-regulated (Fig. 4.8 b, S5, S6, eS). Genes that were up regulated were involved in metabolic process (GO:0008152, p-value 2.3e-07), post-embryonic development (GO:0009791, p-value 9.3e-07), response to stimulus (GO:0050896, p-value 4.7e-05), negative regulation of developmental process (GO:0051093, p-value 0.00054) and regulation of biological process (GO:0050789, p-value 0.00091) (Table 4.4).

Down-regulated transcripts were enriched in terms of biological functions in localization (GO:0051179, p-value 3e-11), response to stimulus (GO:0050896, p-value 3.2e-10), establishment of localization (GO:0051234, p-value 4.8e-10), sexual reproduction (GO:0019953, p-value 7.6e-10), multicellular organismal process (GO:0032501, p-value 0.0011) and cellular developmental process (GO:0048869, p-value 0.0012) (Table 4.5).

4.93 Common transcripts between *sep3-2* and *sep4-1*

Amongst those two microarray experiments for *sep3-2* and *sep4-1*, 63 transcripts were commonly up-regulated in both mutants (Fig. 4.8 c, Table 4.4, S5) and involved in metabolic process (GO:0008152, p-value 4.3e-05), establishment of localization (GO:0051234, p-value 0.00023), localization (GO:0051179, p-value 0.00032), response to stimulus (GO:0050896, p-value 0.0024) and cellular process (GO:0009987, p-value 0.0041). 82 transcripts appeared commonly down regulated between both mutants (Fig. 4.8 d, Table 4.5, S6) and showed enriched GOs in lipid metabolic process (GO:0006629, p-value 0.00047), post-embryonic development (GO:0009791, p-value 0.00042) and reproduction (GO:0000003, p-value 0.004).

Table 4.4

↑ *sep3-2*	GO	description	p-value	↑ *sep4-1*	GO	description	p-value
	0019953	sexual reproduction	3.1e-08		0008152	metabolic process	2.3e-07
	0050896	response to stimulus	2.2e-07		0009791	post-embryonic develop-ment	9.3e-07
	0051704	multi-organism process	0.0003		0050896	response to stimulus	4.7e-05
	0048589	develop-mental growth	0.001		0051093	negative regulation of develop-mental process	0.00054
	0051179	localization	0.0011		0050789	regulation of biological process	0.00091
Common ↑ (*sep3-2* and *sep4-1*)	GO	description	p-value				
	0008152	metabolic process	4.3e-05				
	0051234	establish-ment of localization	0.00023				
	0051179	localization	0.00032				
	0050896	response to stimulus	0.0024				
	0009987	cellular process	0.0041				

Table 4.4: Summary overrepresented GOs of up-regulated transcripts in *sep3-2* and *sep4-1* microarrays

Table 4.5

↓ *sep3-2*	GO	description	p-value	↓ *sep4-1*	GO	description	p-value
	0022414	reproductive processes	1.4e-08		0051179	localization	3e-11
	0000003	reproduction	2.2e-08		0050896	response to stimulus	3.2e-10
	0007275	multicellular organismal development	5e-07		0051234	establishment of localization	4.8e-10
	0032502	developmental process	1e-05		0019953	sexual reproduction	7.6e-10
	0009987	cellular processes	1.9e-05		0032501	multicellular organismal process	0.0011
	0065007	biological regulation	0.00012		0048869	cellular developmental process	0.0012
Common ↓ (*sep3-2* and *sep4-1*)	GO	description	p-value				
	0006629	lipid metabolic process	0.00047				
	0009791	post-embryonic development	0.00042				
	0000003	reproduction	0.004				

Table 4.5: Summary overrepresented GOs of down-regulated transcripts in *sep3-2* and *sep4-1* microarrays

Fig. 4.8

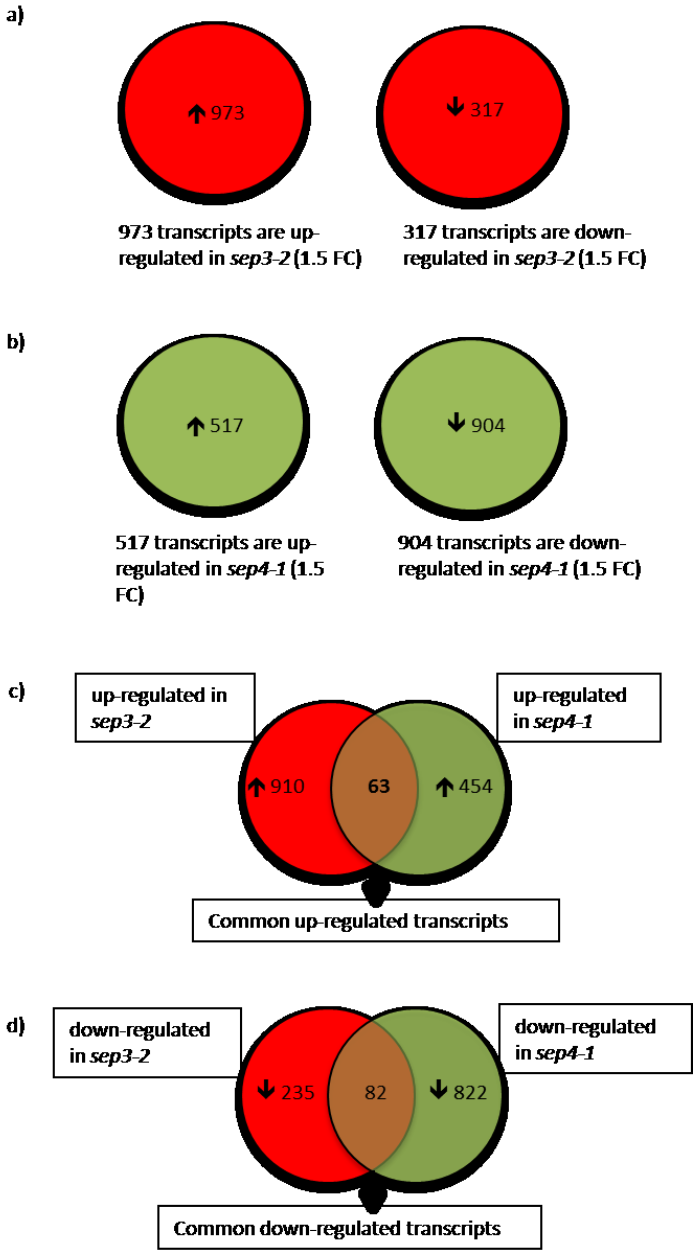


Fig. 4.8: Venn diagrams showing up- and down regulated transcripts for *sep3-2* and *sep4-1* microarrays

a) up- and down-regulated transcripts from *sep3-2* microarrays, b) up- and down-regulated transcripts from *sep4-1* microarrays, c) overlapping up-regulated transcripts between *sep3-2* and *sep4-1* microarrays and d) overlapping down-regulated transcripts between *sep3-2* and *sep4-1* microarrays, Fold Change (FC) for microarrays= 1.5, p = 0.05

4.10 89 SEP4 targets show a changed expression in *sep4-1* microarrays

Comparing the SEP4 targets from the ChIP-SEQ experiments with the microarray experiments from *sep4-1* mutants revealed 89 genes were targeted by SEP4 and also affected in their transcription. 55 transcripts were down-regulated (FC >1.5) and 34 transcripts were up-regulated (FC > 1.5) in the *sep4-1* mutant compared to WT (Fig. 4.9 a), (eS). GO analysis showed no significant enrichment in terms of biological function but looking at individual genes revealed that transcription factors involved in developmental processes were down regulated including *NUCLEAR FACTOR Y SUBUNIT A10 (NF-YA10)* a CCAAT-binding transcription factor (Sieffers et al., 2009), the transcriptional repressor myb domain protein 4 (*MYB4)* (Riechmann J. L., et al., 2000), homeobox-leucine zipper protein 22 (*HAT22*) (Riechmann J. L., et al., 2000), *SEPALLATA4*, showing the validity of the data and *GIBBERELLIN 2-OXIDASE 1* (*ATGA2OX1*). The up-regulated transcripts included transcription factors involved in growth such as *RAV1* (ETHYLENE RESPONSE DNA BINDING FACTOR 4) (Kagaya J. et al., 1999), *GROWTH-REGULATING FACTOR 2* (*ATGRF2)*, *AGAMOUS-LIKE 24* (*AGL24*), *AGAMOUS-LIKE 20* (*SOC1*), *FD* (all involved in the regulation of flowering time) (Lee J. and Lee I., 2010) and *CUP SHAPED COTYLEDON3* (*CUC3*) involved in organ boundary formation (Hibara K. et al., 2006), *LOB DOMAIN-CONTAINING PROTEIN 39* (*LBD39*) involved in Nitrogen signalling (Rubin G. et al., 2009) and *PATHOGEN AND CIRCADIAN CONTROLLED 1* (*PCC1*), a gene that is up regulated by the circadian clock and in response to avirulent and virulent strains of *Pseudomonas syringae* (Sauerbrunn N. and Schlaich N.L., 2004).

Comparison between the microarray data and the common targets of SEP3 and SEP4 showed that 28 transcripts were affected in their transcription (Fig. 4.9 b, S9). Amongst the common targets of both SEP3 and SEP4 which are up-regulated in the *sep4-1* microarrays are *AGL20*, *AGL24*, *CUC3, RAV1, AtGRF2, FD* and unknown genes (*AT4G37240*, *AT4G27450, AT5G52960*, *AT5G53160*). In the list of down-regulated transcripts the following genes appeared: *NUCLEAR FACTOR Y*, *SUBUNIT A10* (*NF-YA10*), *HAT22*, *SEP4*, a Rho GDP-dissociation inhibitor family protein, *beta-galactosidase 7* (*BGAL7*), *ATGA2OX1*, *BETA-XYLOSIDASE 1* as well as unknown genes.

4.11 61 transcripts of 902 unique SEP4 targets show a changed expression

Comparing transcripts showing altered microarray expression, which were also unique targets in the SEP4 ChIP-SEQ revealed 61 transcripts (Fig. 4.9 c, eS). These transcripts showed GOs for response to stimulus (GO 0050896, p-value 0.05), metabolic process (GO 0008152, p-value 0.06). No gene was directly involved in flower development as e.g. no GO was found for flower developmental processes. Genes that appeared were for example involved in response to herbivore (*AT3G16400*), response to wounding (*AT4G22880*, *AT4G02260*), response to auxin stimulus (*AT2G04160*) and response to bacterium (*PCC1, AT2G44490*) or in Nitrogen signalling *(LBD39)*, as well as unknown genes (*AT5G02710*, *AT3G50900*, *AT4G19240*, *AT1G09310*, *AT1G10090*, *AT1G10090*, *AT1G10090*).

Fig. 4.9

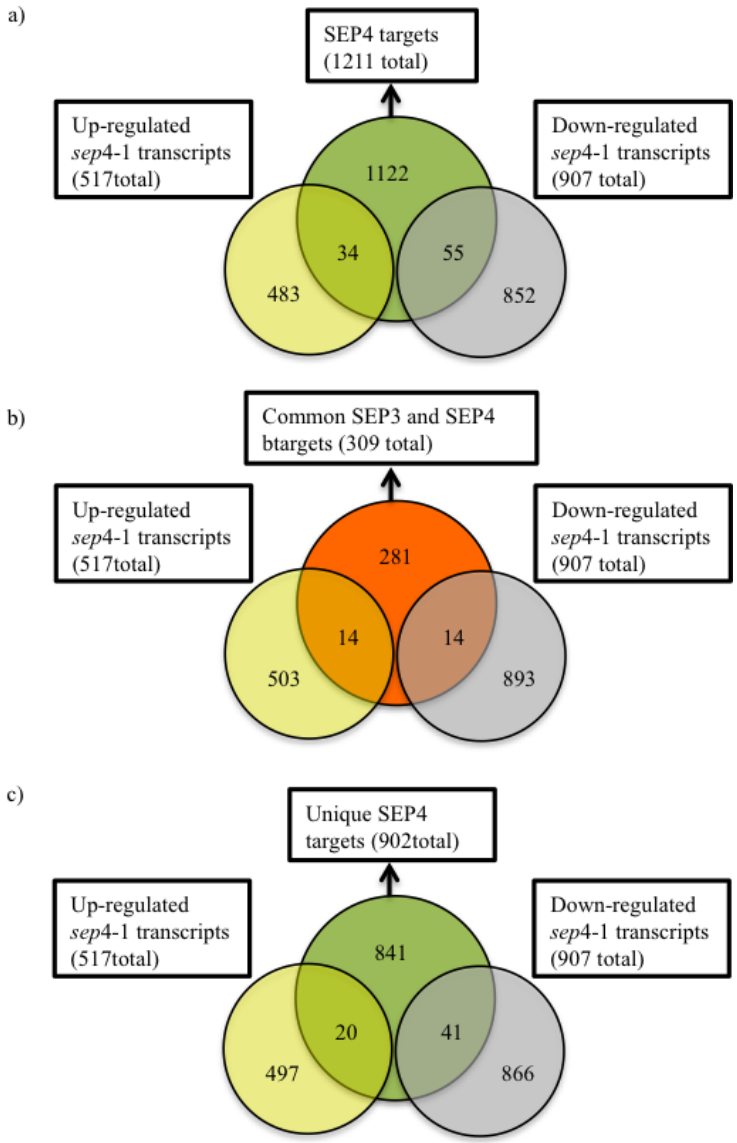


Fig. 4.9: Venn diagrams showing overlapping genes between ChIP-SEQ and microarray experiments for SEP3, SEP4 and for *sep4-1* microarray experiments.

a) genes that were SEP4 targets and affected in the *sep4-1* microarrays, b) transcripts that were affected in the *sep4-1* microarray and were common targets between SEP3 and SEP4 ChIP-SEQ, c) unique SEP4 targets and affected in the *sep4-1* microarrays,

FDR for ChIP SEQ = 0.001, Fold Change (FC) for microarrays= 1.5, p = 0.05

4.12 *SEP4* represses floral meristem and flowering time genes

ChIP-SEQ and microarray experiments identified genes involved in flowering time or in the transition from the vegetative to reproductive phase. The genes *AGL24*, *SOC1,* and *FD* appeared as SEP4 targets and all showed increased expression in the *sep4* mutant compared to WT (described in the previous section 4.10). These three genes were also identified in the previous SEP3 genome-wide binding study and were confirmed as being affected in their expression as shown in a down regulation (Kaufmann K. et al., 2009). *SVP* and *FLC* appeared also as SEP4 targets, but did not show a change in expression in the *sep4* mutant compared to WT in the microarray experiments. All these SEP4 target genes were tested by qPCR. Although expression of *SVP* and *FLC* was unaltered compared to WT in the *sep4* microarrays, we decided to test them due to their important roles in flowering. The gene *FLC* was particularly interesting, because it is a unique SEP4 target and not targeted by SEP3. As seen in Fig. 4.10, the genes *AGL24*, *SOC1*, *FD*, and *SVP* are up-regulated in *sep4-1* mutants compared to WT. Thus, these genes are direct targets, which are repressed by SEP4. However, expression of *FLC* is unaltered in the *sep4* mutant compared to WT (Fig. 4.10), confirming the microarray data.

As well as floral transition genes, the following confirmed SEP3 target genes (Kaufmann K. et al., 2009) were also identified as direct SEP4 targets in this study: *CAULIFLOWER* (*CAL*), *FRUITFUL* (*FUL*) and *ETTIN (ETT).* Although their expression was unaltered in the *sep4* microarrays, we decided to test them based on their important roles during flower development. The two MADS-Box genes *CAULIFLOWER* (*CAL*), *FRUITFUL* (*FUL*), are closely related to *AP1* and are functionally redundant with *AP1.* All of these three genes have a function in specifying floral meristems (Ferrándiz C. et al., 2000). *SEP4* is known to play a role in determining floral meristem identity (Ditta G. et al., 2004), therefore these genes were particularly interesting to test. *ETTIN (ETT)* has a reported phenotype with extra perianth organs, that is similar to the *sep4* phenotype reported here (section 3.2, Table 3.4) (Sessions A. et al., 1997). As seen in Fig. 4.11 expression of *FUL* is significantly altered in the *sep4* mutant compared to WT, shown in a down-regulation, a finding that was not seen in the *sep4* microarray experiments. The two genes *CAL* and *ETT* were unaltered in their expression compared to WT, confirming the *sep4* microarray data.

4.13 Individual SEP4 targets are affected in their expression

As well as these common targets, *LATERAL ORGAN BOUNDARY 39* (*LBD39*) and *LEUNIG* (*LUG*) appeared as targets of SEP4 that are not targeted by SEP3. *LBD39* was up-regulated in the *sep4-1* microarrays and this result was confirmed by qPCR (Fig. 4.12). Although *LUG* expression was unaltered in the *sep4* mutant microarrays, it was significantly up-regulated in the *sep4* mutant compared to WT (Fig. 4.12) in separate qPCR analysis.

To summarize the ChIP, ChIP-SEQ, microarray and qPCR experiments: genes involved in floral organ development (*AG*, *PI*), the meristem identity gene *FUL* and genes involved in the transition from the vegetative to reproductive phase and flowering time (*FD*, *AGL24*, *SOC1*, *SVP*) are direct targets of SEP4 and their expression is affected in a *sep4-1* mutant background. Those genes have also been identified as SEP3 targets and are therefore common targets of both TF factors.

The genes *LBD 39* and *LUG* are direct targets of SEP4 and are affected in their expression. Neither gene is targeted by SEP3 and thus they seem to be distinct targets of SEP4.

SEP4 can function as an activator or repressor for different genes as shown in expression analysis experiments in *sep4* mutants compared to WT. The flowering time genes *AGL24*, *FD, SOC1* and *SVP* are up-regulated in the *sep4* mutant compared to WT. Furthermore, the two genes *LBD39* and *LUG* are up-regulated in the *sep4* mutant compared to WT, showing that *SEP4* has a repressive function on these genes under standard conditions. The floral organ identity genes *AG* and *PI* and the meristem identity gene *FUL* were significantly down-regulated in the *sep4* mutant compared to WT. Therefore *SEP4* has an activating function on these genes. This suggests that direct targets of SEP4 are either activated or suppressed, presumably as a result of SEP4 regulating their expression as part of different complexes.

Fig. 4.10

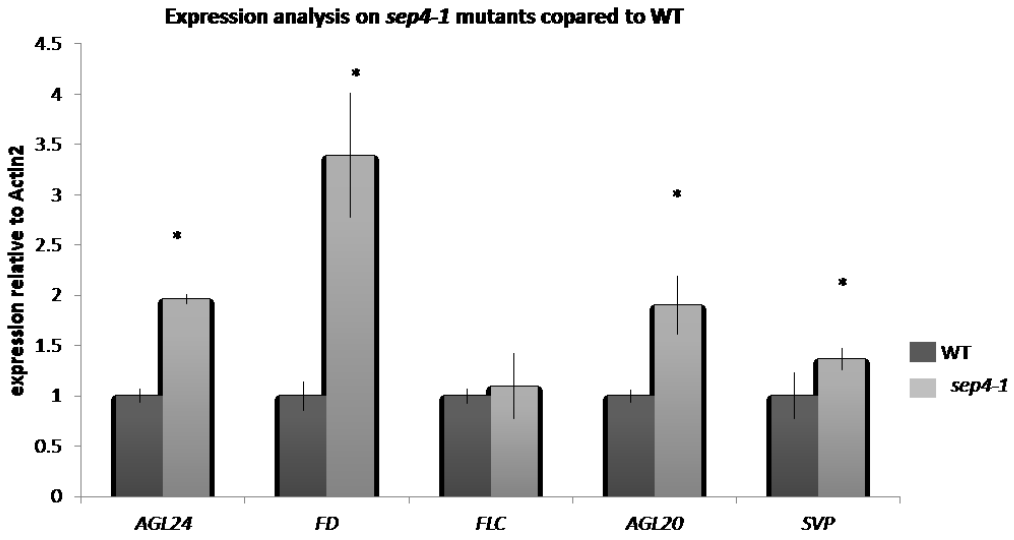


Fig. 4.10: Expression analysis of selected target genes in *sep4-1* mutant inflorescences.

The genes *AGL24*, *FD*, *FLC, SOC1*, *SVP* were tested by qPCR in the *sep4-1* mutant and compared to WT. Bars = Standard Error of the Mean (SEM), asterisks indicate if expression is significantly different to WT expression with $p < 0.05$, Four biological and two technical replicates were used for each experiment, Inflorescences from stage 1-12 were used as tissue

Fig. 4.11

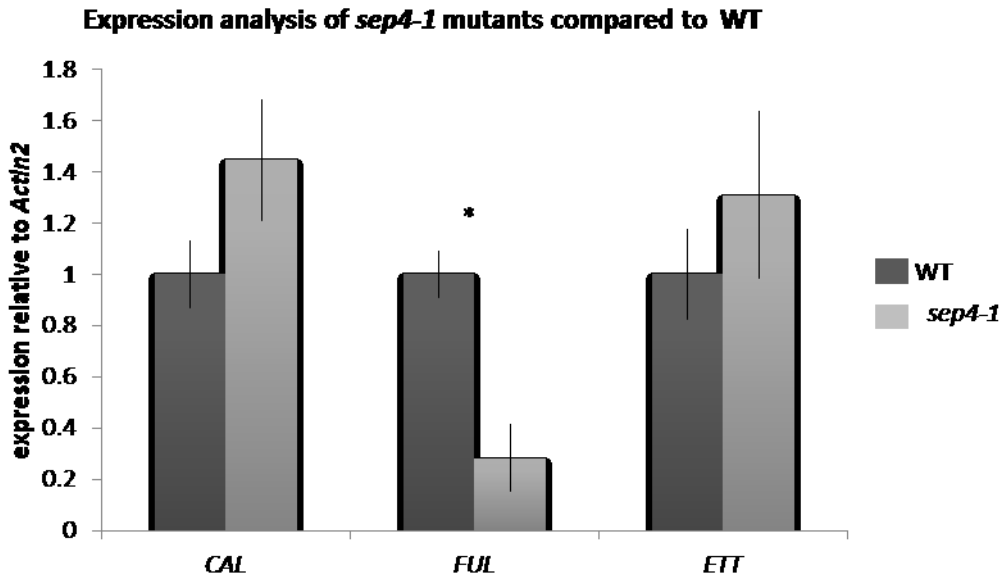


Fig. 4.11: Expression analysis on *sep4-1* mutant inflorescences of selected target genes.

The genes *CAL*, *FUL, ETT* were tested by qPCR in the *sep4-1* mutant and compared to WT. Bars = Standard Error of the Mean (SEM), asterisks indicate if expression is significantly different to WT expression with $p < 0.05$, Four biological and two technical replicates were used for each experiment, Inflorescences from stage 1-12 were was used.

Fig. 4.12

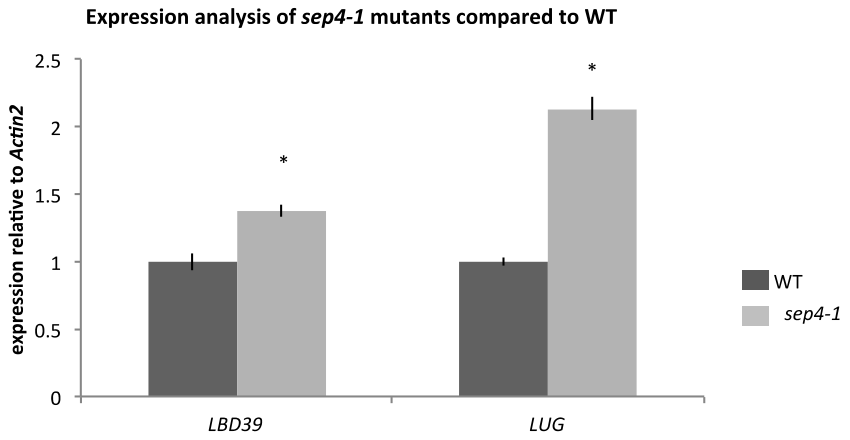


Fig. 4.12: Expression analysis on *sep4-1* mutant inflorescences on individual SEP4 target genes.

The genes *LBD39* and *LUG* were tested by qPCR in the *sep4-1* mutant and compared to WT. Bars = Standard Error of the Mean (SEM), asterisks indicate if expression is significantly different to WT expression with $p < 0.05$, Four biological and two technical replicates were used for each experiment, Inflorescences from stage 1-12 were was used.

4.14 Flowering time is affected in *sep4* mutants

The finding that *SEP4* represses the flowering time genes *AGL24*, *SOC1*, *FD*, and *SVP* (Fig. 4.10) prompted us to test whether *sep4* mutants are affected with respect to flowering time. Mutants were grown under long day (LD) conditions at 22 °C and compared to WT plants in terms of the number of rosette leaves, cauline leaves and the time before plants started to bolt. Compared to WT plants, *sep4* mutants, on average, flower earlier (*sep4*: 17.4 days ± 0.168, WT: 22 days ± 0.223, Fig. 4.13 a, c), and with fewer rosette leaves (RL) (*sep*4: 6.94 ± 0.130, WT: 10.05 ± 0.168, Fig. 4.13 b) and cauline leaves (CL) (*sep4*: 2.8 ± 0.068, WT: 2.95 ± 0.078).

Fig. 4.13

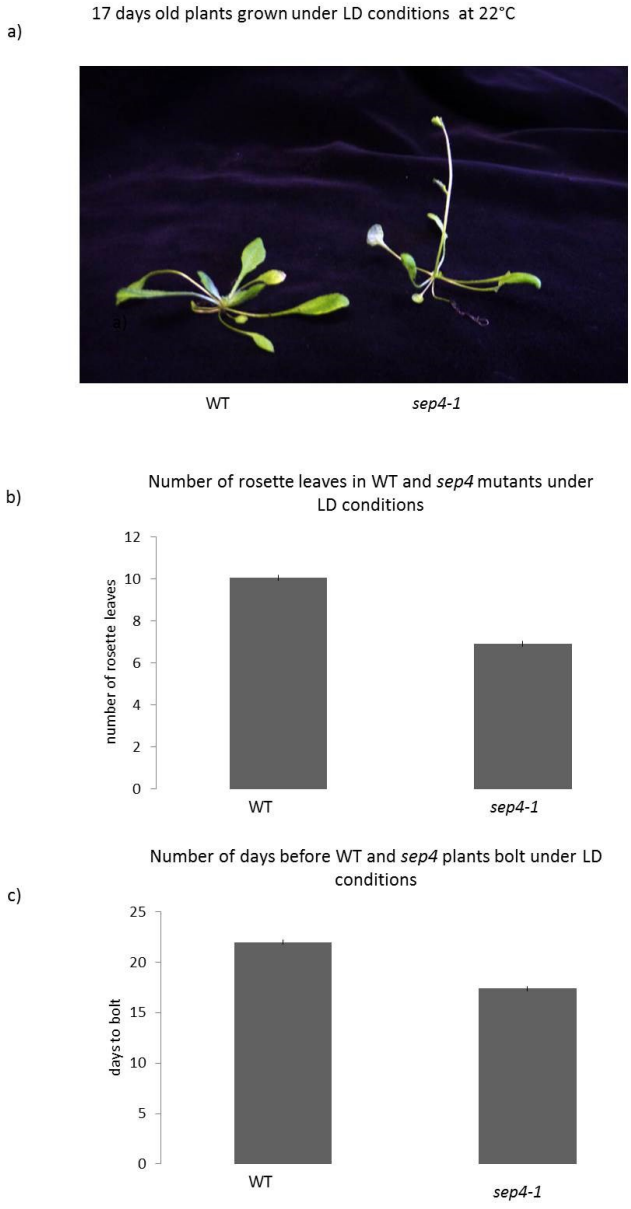


Fig. 4.13: Measurement of flowering time in WT and *sep4-1* mutants.

a) picture of a 17 day old WT plant compared to a *sep4-1* mutant plants grown under LD conditions at 22 °C, b) number of rosette leaves before plants bolt, c) number of days until plants start to bolt, Bars = Standard Error of the Mean (SEM), $p < 0.05$, experiments were performed under long day (LD) conditions at 22 °C, 16h light/8h dark cycle.

4.15 6 common targets show opposing changes in gene expression in *sep3-2* and *sep4-1* mutants

When comparing genes targeted by both SEP3 and SEP4 which show differences in expression in *sep3-2* and *sep4-1* microarray experiments, six genes were found that were antagonistically expressed. Five of these genes were down-regulated in *sep4-1* and up-regulated in *sep3-2*. Those were *NF-YA10*, a TF that is proposed to regulate target genes via binding to CCAAT-boxes (Mantovani R., 1999), the two genes *AT5G52960* and *AT1G68875*, coding for unknown proteins, *BETA-GALACTOSIDASE 7* (*BGAL7*), involved in catabolic processes (Hu W. et al., 2003), and *AT1G12070*, a protein coding for Rho GDP-dissociation inhibitor activity. *CUC3,* a TF involved in postembryonic shoot meristem and organ boundary formation (Hibara K. et al., 2006) was the only gene that was up-regulated in *sep4-1* microarray and down-regulated in *sep3-2*. In order to confirm differential expression of these genes, qPCR was performed on inflorescences of *sep3-2* and *sep4-1* mutant flowers and compared to WT. As seen in Fig. 4.14 the genes *AT1G68875* (unknown protein), *BGAL7* and *CUC3* were confirmed as being antagonistically expressed in qPCR experiments. The remaining three genes, *AT5G52960, NF-YA10* and *AT1G12070* were not confirmed as being antagonistically expressed but were significantly down-regulated in *sep4-1* mutants compared to WT, confirming the *sep4* microarray results. In order to check whether one of these 6 genes might cause the extra perianth organ phenotype observed in *sep4* mutants (Chapter 3.2, Table 3.5), the full length cDNA of the genes that were significantly down-regulated in *sep4-1* mutants compared to WT, *NF-YA10*, *AT5G52960, BGAL7*, *AT1G68875* and *AT1G12070* was fused to the CaMV *35S* promoter (35S::NF-YA10, 35S::*AT5G52960, 35S::BGAL7*, 35S::*AT1G68875, 35S::AT1G1207)* and introduced into *sep4-1* mutant backgrounds using the floral dip method (Clough S.J. and Bent A.F., 1998). Assuming that a decreased expression of one of these genes might cause the perianth organ phenotype, increased expression might rescue the *sep4* organ number phenotype. Therefore several independent lines of the first generation of transgenic plants were screened regarding the phenotype for additional perianth organs. However, none of these lines could rescue the *sep4* phenotype. As seen in Table 4.6, all lines showed a variation in the number of additional sepals and petals.

The NAC-domain transcription factor *CUC3* (Vroemen C.W. et al., 2003) was antagonistically expressed in *sep3-2* and *sep4-1* mutants. *CUC3* was significantly up-regulated in *sep4-1* mutants and if this causes the increased perianth organ number in *sep4*, then a *cuc3* mutation should rescue the perianth organ number phenotype. Therefore, a *cuc3-105 sep4-1* double mutant was generated to test whether a mutation in *CUC3* can rescue the *sep4* phenotype of

extra sepals and petals. Homozygous *cuc3-105 sep4-1* double mutants (hereafter named *cuc3sep4* double mutants) were grown at standard growth conditions (22.°C/16 h light/dark cycle) and analysed for perianth organ number. The extra perianth organ number phenotype was also not rescued in *cuc3sep4* double mutants (Table 4.7), but interestingly, double mutants showed an even further increase in perianth organ number. Single mutants of the *CUC3* gene are described having a role in embryonic shoot meristem formation and cotyledon boundary formation, producing heart shaped cotyledons (Vroemen C.W. et al., 2003; Hibara K. et al., 2006). The *sep4* phenotype, described in this work shows extra sepals and petals. As seen in Table 4.7, the *cuc3sep4* double mutant showed an enhanced perianth organ phenotype compared to *sep4-1* single mutants. 19 % of analysed flowers of *cuc3sep4* double mutants had four sepals and 21 % had four petals, whereas the majority had 5 sepals and petals. From all analysed flowers of this double mutant, 65 % had flowers with 5 sepals and 61 % had five petals. Furthermore, 15 % developed six sepals and 17 % developed six petals (Table 4.7, Fig. 4.15 c). Compared to the *sep4-1* single mutant that showed around 30 % extra perianth organs, the *cuc3sep4* double mutant showed an enhancement of the phenotype (Table 4.7). Surprisingly, these double mutants revealed additional phenotypes that were not seen in any of the two single mutants. Organ fusions affecting the second and third whorl were noticed, as evidenced by stamenoid petals with pollen sacs fused to the boundary region between petal base and the petal lobe along the margin (Fig. 4.15 f, g). 25 % of analysed flowers of *cuc3sep4* double mutants showed petals that were fused with a stamen (Table 4.7), usually one or two petals were affected in flowers showing that phenotype. Rarely, petals having a trumpet shape were observed as in Fig. 4.15 g (marked with red arrow). Another organ boundary effect, noticeable at a low frequency, was the fusion between petals at the base as seen in Fig. 4.15 e. Stamen number was affected in these double mutants, plants produced on average one stamen fewer than WT (Table 4.7). Petals were affected in their morphology as they had strongly folded tips (Fig. 4.15 d, white arrow) and showed a sepaloid identity (Fig. 4.15 d, red arrow).

Fig. 4.14

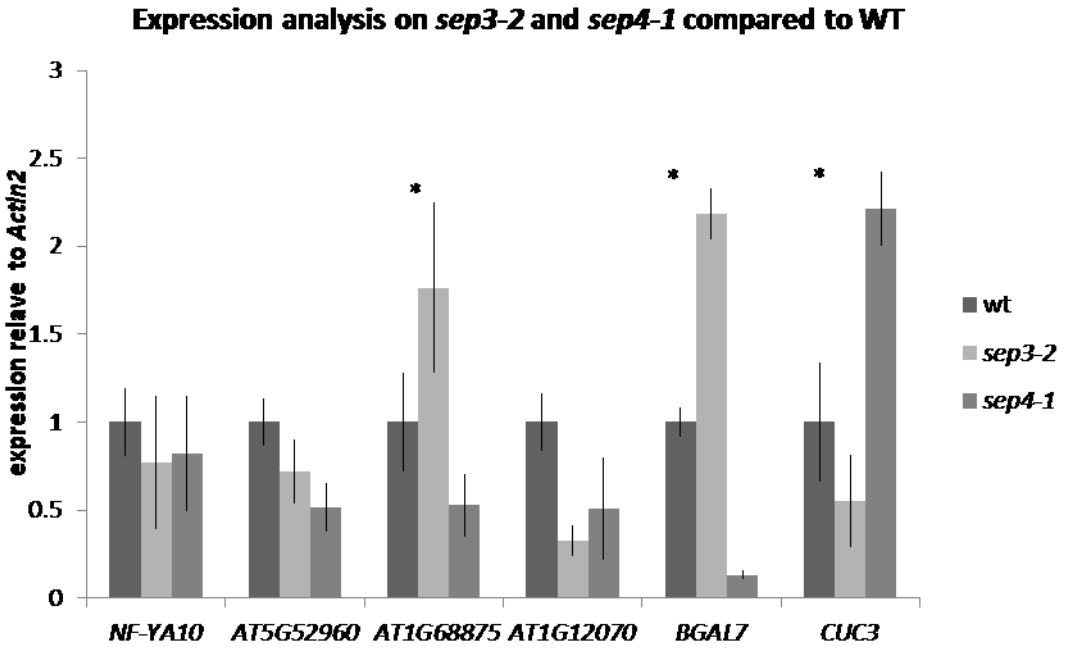


Fig. 4.14: Expression analysis of common ChIP-SEQ targets of SEP3 and SEP4 which are antagonistically expressed in *sep3-2* and *sep4-1* mutants.

qPCR was performed on the genes *NF-YA10*, *AT5G52960*, *AT1G68875*, *AT1G12070*, *BGAL7*, *CUC3,* Bars = Standard Error of the Mean (SEM), asterisk indicate if expression is significantly different to *sep3-2* expression with $p < 0.05$, Four biological and two technical replicates were used for each experiment, inflorescences from stage 1-12 were used.

Table 4.6

	Line	fowers having an additional perianth organ, in %		Line	fowers having an additional perianth organ, in %
sep4-1		~ 30			
35S::*NF-YA1*0 in *sep4-1*	1	25	35S:: *AT5G52960* in *sep4-1*	1	30
	2	36		2	43
	3	21		3	50
	4	26		4	35
	5	43		5	18
35S::*AT1G68875* in *sep4-1*	1	50		6	27
	2	63		7	50
	3	25		8	45
	4	41	*35S::AT1G12070* in *sep4-1*	1	33
	5	30		2	50
	6	40		3	35
	7	16		4	58
35S::*BGAL7* in *sep4-1*	1	30		5	18
	2	33		6	19
	3	33		7	20
	4	47		8	21
	5	26			
	6	37			
	7	25			
	8	36			

Table 4. : Results of T1 generation of overexpressor lines in a *sep4-1* background.

Phenotypic analysis of individual lines constitutively expressing *NF-YA10, AT1G68875, AT5G52960, AT1G121070* and *BGAL7* in a *sep4-1* mutant background regarding the percentage of analysed flowers that show one extra sepal and petal compared to *sep4-1* single mutants, all flowers were grown and analysed at 22 ° C

Table 4.7

	3 sepals in %	4 sepals in %	5 sepals in %	6 sepals in %	3 petals in %	4 petals in %	5 petals in %	6 petals in %	stamenoid petals in %	Average stamen number	individual flowers analyzed/individual plants
WT	0	100	0	0	0	98.4	1.6	0	0	6	60/4
sep4-1	0	69	30	0	0	67	33	0	0	6	33/4
cuc3-105	0	100	0	0	2	98	0	0	0	6	28/5
cuc3sep4	0	19	65	15	0	21	61	17	25	4.9	38/5

Table 4.6: Phenotypic characterization of *cuc3sep4* double mutants.

Flowers of *cuc3sep4* double mutants grown at 22 ° C were analysed and compared to those of WT, *sep4-1* and *cuc3-105* flowers, %= flowers having a defined number of sepals and petals.

Fig. 4.15

Fig. 4.15: Phenotypic analysis of *cuc3sep4* double mutant flowers.

a) WT flower, b) *sep4* mutant having 5 petals instead of four (marked with a white arrow), c) *cuc3 sep4* double mutant flower having 6 petals (marked with a white arrow), d) *cuc3sep4* double mutant flower showing strongly folded petal tips (marked with a white arrow) and a sepaloid petal (marked with a red arrow), e) *cuc3sep4* double mutant showing two petals fused at the base (marked with a white arrow), f) stamenoid petal from a *cuc3sep4* double mutant flower (marked with a white arrow), g) a stamenoid petal from a *cuc3sep4* double mutant flower (marked with a white arrow) that has a trumpet shape (marked with a red arrow). Bars = 1mm

4.16 Discussion

4.16.1 Improvement of the ChIP-SEQ method

The ideal ChIP-SEQ experiment aims to map the binding sites of a target protein with maximal signal-to-noise ratio and completeness across the genome. However, our experiments have shown that this goal is not that easy to achieve as seen in section 4.6 (Fig. 4.6). Sequencing results of most samples of the SEP4 ChIP-SEQ were not successful, as evidenced by a low percentage of reads that exactly mapped one time to the Arabidopsis genome (Table 4.1). Only one of the ChIP-SEQ experiments could be used to identify DNA binding sites of SEP4. To ensure a reliable quality of the data, biological replicates are necessary and although there is no consensus on the number of replicas, experiments should be performed at least in biological duplicates (Kidder B.L. et al., 2011). There are already some technical guides available to improve the ChIP protocol and enhance the chances for success for the sequencing results (Kaufmann K. et al., 2010a; Kidder B.L. et al., 2011) . Before sending off samples for sequencing, one should be sure that the ChIP experiment was successful. This can be tested, for example with genes that are known targets. In case of a good ChIP sample, the tested gene should show a higher enrichment compared to the control. A recommendation is to use at least two genes to test the ChIP samples and these samples should show at least an 8 to 10 fold enrichment compared to the control sample (personal communication K. Kaufmann). For example section 4.5 Fig. 4.3 a shows that *FD* and *SEP3* and Fig. 4.3 b shows that *AP1*, *FD, SEP3* and *SEP4* were identified as direct target with increased enrichments compared to the WT. All of these genes were identified as direct targets in the genome wide binding study of SEP4, reported in this work. Another critical step is the library preparation. A typical library preparation is described in Chapter 2, section 2.62 and includes end repair, adapter ligation, and size selection followed by gel purification and PCR with primers specific to the sequencing platform. For the PCR step it is important that adaptor-ligated DNA products are not over amplified, as can happen if too many cycles are run. Over amplification can cause a loss of specific signal, bias or redundancy in the number of sequencing tags. This can be one explanation why most of our sequencing experiments failed. Fig 4.6 shows peaks from sequencing experiment that failed compared to the one SEP4 sample (g*SEP4:*GFP*;sep1sep4* rep1, Fig. 4.6 a) that was used for further studies in this work. A high heterogeneity and strong background signals as seen in Fig. 4.6 c, d for the two samples (g*SEP4:*GFP*;sep1sep4* rep2,

g*SEP4:*GFP*;sep4* rep1) made it impossible to work out individual, strongly enriched peaks compared to WT samples (personal communication Pedro Madrigal, Kerstin Kaufmann). Another recommendation is to check the DNA after the library preparation again for enrichment using specific primer pairs that were used in the ChIP before. This step was done in our experiments as well and appropriate enrichments were detected for specific genes. A great variation in the fragment size, caused by unequal sonication can also result in problems with the subsequent library preparation, because smaller fragments, which are easier to PCR amplify, end up over-represented. This might lead to an increased background noise ratio in the sequencing results. This source of error can be also excluded in our case, because samples were checked on a gel after sonication to ensure they are fragmented to the optimal size.

Taken together, there are many critical steps in the ChIP protocol and library preparation and in this study, library preparation seemed to be the most critical step in these experiments. For future experiments it is necessary to improve this procedure. Based on the fact that Illumina multiplex sequencing is becoming the standard method for sequencing and allows running many samples in one flow cell, one approach could be to do multiple libraries with different PCR cycles to find the right conditions and give results with less background noise. However, ChIP-SEQ is still a quite new technology and therefore it susceptible to errors. The more frequently this technology is used, the better the chances are to find the right conditions and avoid errors.

As already mentioned, an ideal experiment should have two biological replicates (Kidder B.L. et al., 2011) to obtain a reliable target list, for example confirmed by significant statistical analysis. Nevertheless, this study has shown that one experiment is a good starting point for further analysis, as long as the replicate has a good quality. It was possible to confirm the regulatory effect of SEP4 for several target genes (*AG, PI, AGL24, FD, SOC1, FUL, CUC3*, *LBD39, LUG*, Fig. 4.7, 4.10, 4.11, 4.12, 4.14) and link this direct binding to a biological process. This study has shown that SEP4 affects different genes in different developmental steps (repressive function flowering time genes, section 4.12). Thus, one good ChIP-SEQ experiments can be a powerful tool for further studies and biological experiments.

4.17 Floral organ identity genes are targeted by SEP4

Our ChIP-SEQ experiments have shown that SEPALLATA4 binds more than a thousand regions in the Arabidopsis genome. GO revealed that 118 of these putative targets had transcription

factor activity. The genome of *A. thaliana* encodes roughly for 25000 genes (Initiative, 2000) with at least 1533 transcriptional regulators (Riechmann J.L. et al., 2000). Around 8 % should encode for a TF by chance. In this study 1211 direct targets were identified from which 118 encoded for TFs, which is about 9 % and in agreement with the prediction. In *A. thaliana,* 107 MADS-Box genes were identified (de Folter S. et al., 2005), by chance 7 % of the TFs encode for MADS-Box TF. In this study, 12 out of 118 identified TFs belong to the MADS-Box family (~ 10 %), which shows this family of TFs is enriched. From these 107 MADS-Box genes, 43 belong to the Type II family that is known to have various roles during flower development (Nam J. et al., 2004) and in this study 10 out of 12 targeted TFs were Type II MADS-Box TFs, whereas two belong to the Type I MADS-Box TFs which shows that type II MADS-Box genes are an enriched group. In this list of Type II MADS-Box genes, the floral organ identity genes *AP1*, *AP3*, *PI*, *AG* and the flowering time genes *SOC1*, *FLC* or *AGL24* were targeted. The two genes *AGL23* and *AT1G17310* were targeted and belong to the Type I MADS-box TF family. Other TFs that appeared as putative targets and were overrepresented belong to the Zinc finger TF family. In *A. thaliana*, 176 members of the H2C2-zinc finger protein family are identified and they represent one of the largest families of transcriptional regulators in *Arabidopsis* (Englbrecht, C. C., et al., 2004). 16 genes of that family (~ 9 %) were identified and *SUPERMAN (SUP)* is one of those. *SUP* is a cadastral gene that maintains the inner boundary of *AP3* expression (Bowman J.L. et al., 1992). Another overrepresented group of TF were MYB TFs. A genome wide expression study revealed that 198 genes in the *A. thaliana* genome encode for these TFs (Yanhui C. et al., 2006). 14 identified targets in this study belong to this family (~ 7 %). One of these 14 identified MYB TFs is *ASYMETRIC LEAVES1,* a gene that is necessary for normal cell differentiation and promotes leaf adaxial fate (Xu L. et al., 2003). 12 genes were identified that belong to the AP2 family of TFs, the floral homeotic gene *AP2* represents one of them and is involved in perianth organ formation and a repressor of C-function gene *AG* (Bowman J.L. et al., 1991).

The expression of the floral organ identity genes *AP1, AP2, AP3, PI, AG* and *SEP3* was tested by different approaches, such as microarrays and qPCR and two of them (*PI* and *AG)* were down-regulated in the *sep4* mutant (Fig. 4.7), although their expression was not affected in the microarrays. The other genes were unaltered in their expression in *sep4* mutants compared to WT. For example the expression of *SEP3* was not affected in *sep4* mutants (Fig. 4.7), although this gene was identified in previous ChIP experiments as direct target with one of the best enrichments (section 4.5, Fig. 4.3). These results are in agreement with in section 3.5 described expression analysis (Fig. 3.6); the expression of individual *SEP*s is independent in the triple mutants. One explanation why SEP4 targets *SEP3* but has no regulatory effect on it might lie in its evolutionary history. The first WGD event in *A. thaliana* produced the *SEP3* clade and

LOFSEP clade (with *SEP1, SEP2, SEP4*) and maybe SEP4 has still the ability to bind *SEP3* but does not regulate the gene, to avoid a perturbation in the balance as described in the gene balance theory (Birchler J.A et al., 2005). The *SEP4* gene itself was also identified as a putative target (Fig. 4.2, 4.3), suggesting a feedback regulation by its own gene product. This observation seems to be a common feature for MADS-Box genes, as similar observations were made for *SOC1* (Immink R. et al., 2012), *SEP3* (Kaufmann K. et al., 2009) and *AG* (ÓMaoiléidigh D.S. et al., 2013).

According to this study, SEP4 regulates the two floral organ identity genes *PI* and *AG,* suggesting that in the *sep1sep2sep3sep4* quadruple mutant, all ABC genes could be down-regulated. This is an interesting finding, as it has been shown that the expression of the B- and C- function genes *AP3*, *PI* and *AG* is not affected in the *sep1sep2sep3* triple mutant (Pelaz S. et al., 2000), although this triple mutant resembles the phenotype of the bc-function double mutants, concluding *SEP*s do not transcriptionally activate B- and C -function genes. The reverse was observed in B and C -function mutants, with *SEP*s that were not altered in their expression (Mandel M.A. and Yanofsky M.F., 1998; Pelaz S. et al., 2000). However, no information is available if transcription of ABC–function genes is affected in the *sep1sep2sep3sep4* quadruple mutant, and this is certainly a necessary experiment to do in the future. The results described here predict an altered expression of ABC - function genes in the *sep1sep2sep3sep4* quadruple mutant and undermine the extraordinary role of *SEP4* compared to the other *SEP* genes. Firstly, *SEP4* has a different expression pattern as its expression starts earlier, from the seedling stage (Supplementary S7, S8) onwards until to the inflorescences, whereas none of the other *SEP*s is expressed in vegetative tissues (Ma H. et al 1991.; Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Pelaz S. et al., 2000; Ditta G.S. et al., 2004). Secondly, all other *SEP*s (*SEP1-3*) are independently able to confer floral organ identity in all four whorls as shown in the phenotypic analysis of the triple mutant combinations in this work (section 3.4). In contrast, *SEP4* is able to give sepal identity and provide the floral context, whereas petal, stamen and carpel identity are abolished as shown in the *sep1sep2sep3* triple mutant, composed of sepals (Pelaz S. et al., 2000) and *sep1sep2sep3sep4* quadruple mutant, composed of leaf like organs (Ditta G.S. et al., 2004). However, it is worth to note that the *sep1sep2sep4* triple mutant develops sepals (at least at 22 °C, Table 3.10, 3.13, 3.14, Fig 3.3 c, 3.3 d), although *SEP3* is not expressed in the sepals. This could be explained by a partial redundancy, where *SEP3* might change the expression pattern if the other three *SEP*s are missing and thus provide sepal identity. Therefore it would be interesting to test whether *SEP3* expression can be detected in sepals in the *sep1sep2sep4* triple mutant. This could be done by *in situ* experiments. As our analysis has shown the ability to provide sepal identity is no longer possible at enhanced growth conditions (27 °C). Thirdly, B

- and C- function expression is affected in *sep4* mutants compared to WT, suggesting that *SEP4* might transcriptionally activate *PI* and *AG.* The unaltered expression of the A - and B -class genes *AP1*, *AP2*, *AP3* (section 4.7, Fig. 4.7) in *sep4* mutants compared to WT can be explained by a degree of redundancy.

Conversely, recent studies have shown that SEPALLATA3 can directly bind and activate ABC genes (Kaufmann K. et al., 2009) . In particular, *AP3*, *AG*, and *AP1* have been shown to be strongly activated by SEP3 (Kaufmann K. et al., 2009). It has been previously shown that gene products of these three ABC genes represent major protein interaction partners of SEP3 (Immink R.G. et al., 2009). One explanation between these different results regarding the ability of *SEP* genes to activate (A)BC genes could lie in the experimental approach. Pelaz et. al (Pelaz S. et al., 2000) did *in situ* experiments in *sep1sep2sep3* triple mutants to test whether the expression of the B - and C -class genes is affected. In this study, all ABC genes were tested by qPCR in inflorescences of *sep4* mutants and compared to WT. A completely different approach was used by Kaufmann et. al. (Kaufmann K. et al., 2009), who used the GR induction system and a promoter constitutively over-expressing the *SEP3* gene (35S::*SEP3*::GR) to characterize the regulatory effects of SEP3 on the potential target genes identified by a genome-wide binding study (Kaufmann K. et al., 2009). This is an artificial system, the glucocorticoid receptor (GR) is a member of the family of vertebrate steroid receptors (Aoyama T. and Chua N.H., 1997). In the presence of the steroid hormone as for example Dexamethasone (Dex), glucocorticoid (GR) is translocated from the cytoplasm to the nucleus and activates transcription from promoters containing glucocorticoid response elements (Picard D., 1994). In that sudy, a line that constitutively expressed the translational fusion of SEP3 to the rat glucocorticoid receptor hormone-binding domain (GR) was generated. After treatment with the steroid hormone Dexamethasone, the SEP3-GR fusion translocates from the cytoplasm to the nucleus and can influence transcription. Seedlings expressing this construct were treated at three different time points (8 h, 1 day, 10 days) and checked by qPCR (Kaufmann K. et al., 2009) In this study, some of the tested genes showed an early response to SEP3 induction whereas others were regulated only after prolonged SEP3 induction (e.g. the genes *FUL*, *PI*, *SEP1*, *SEP2*), suggesting that SEP3 alone was not sufficient to regulate these genes, but needs to interact with other partner proteins (Kaufmann K. et al., 2009). However it is worth to consider that experiments performed using constitutive expression of genes never reflect the native situation. In this case, it is likely that *SEP3* transcription was massively increased and also present at stages and tissues where the gene would not normally be expressed. Therefore, in these experiments it might be that some putative target genes show changed expression suggesting that SEP3 has a regulatory effect on that particular gene, but under native conditions this effect would not be detectable. This

could also be an explanation why *in situ* experiments have shown that the expression of B and C floral organ identity genes is not affected in the *sep1sep2sep3* triple mutant (Pelaz S. et al., 2000). Thus, SEP3 can bind these genes and has a regulatory effect on them but alone is not sufficient to activate them. To check the expression of putative SEP4 targets in this study, inflorescences from *sep4-1* mutants, stage 1-12 (Smyth D.R. et al., 1990) were used and expression of selected genes was determined by qPCR. The advantage of using inflorescences from the *sep4-1* mutant for expression analysis is the reflection of the native condition as there is no perturbation such as artificial promoters or induced and enhanced gene expression using hormones and chemicals. Another idea behind this approach was to use the same tissue and conditions as used in the ChIP-SEQ procedure to make the comparison between targets and the regulatory effects of SEP4 more reliable. The disadvantage of this method is that flowers of *A. thaliana* are small and difficult to dissect and initiate sequentially from the floral meristem. Thus the inflorescences of the flowers are at different developmental stages and the qPCR results represent the effect on the transcripts on a broad range of developmental stages. It is likely that some genes did not appear as affected in the microarray or qPCR experiments because they are activated or repressed only at limited stage and using a broad range of developmental stages will dilute the sample and lead to undetectable changes in expression. One way to avoid the problem with unequal distribution of developmental stages and obtain a homogenous population of floral tissue is to use the “floral induction system” (Wellmer F. et al., 2006). This system makes use of a fusion between the *AP1* gene and the hormone binding domain of the rat GR binding domain in an *ap1 ca*l double mutant background. *APETALA1 (AP1)* and *CAULIFLOWER (CAL),* regulate the initiation of flower development in a redundant manner (Ferrándiz C. et al., 2000) and double mutants of both genes (*ap1 cal*) undergo a massive over-proliferation of inflorescence-like meristems resulting in “cauliflower” like structures. Overexpression of *AP1* in WT plants leads to a transformation of vegetative and inflorescence meristems into floral meristems (Mandel M.A. and Yanofsky M.F., 1995) and activation of *AP1* in the inflorescence-like meristems of *ap1 cal* double mutants (35S::*AP1*-GR *ap1 cal*) leads to synchronous flower development (Wellmer F. et al., 2006). This approach facilitates the collection of tissues for experiments (e.g. ChIP-SEQ or microarrays) because the plants produce many flowers that are at the same stage. However this experimental approach was not considered as an alternative for our experiments because it is an artificial system and will not show the native situation, especially the use of 35S promoters can give a distorted image of the “normal” situation. Another approach to test whether the expression of putative targets is affected could be the use of *in situ* experiments. This is a “clean” experiment, reflecting the native situation, shedding more light into the question, if a certain gene is expressed in floral tissues. It will also give an indication how

strong the gene of interested is expressed at different developmental stages in different tissues. Therefore, this is definitely an experiment that should be considered for the future.
Another difference between the two experiments to identify targets of SEP3 and SEP4 was the use of different antibodies. However, compared to the artificial GR induction system using a 35S::*SEP3*::GR transgenic line, this is a minor difference, but should be mentioned at this point. To identify SEP3 targets, a native antibody was used, whereas in our study, the *SEP4* gene was coupled to a Green Fluorescent Protein (GFP). These different experimental approaches might already give differences with regards to target identification. Therefore one should be careful in the interpretation of the results in terms of unique and common targets because it is not known how the use of different antibodies affects the results.

4.18 *FRUITFULL*, a meristem identity gene is up-regulated by SEP4

The genes *AP1*, *CAL* and *FUL* were identified as direct targets in the SEP4 ChIP-SEQ experiments and tested using qPCR to determine, whether their expression is altered in *sep4* mutants compared to WT. All three are meristem identity genes, responsible for the formation of floral meristems that will differentiate into flowers (Ferrándiz C. et al., 2000; Jack T., 2004; Grandi V. et al., 2012). Although *FUL* was significantly down-regulated (Fig. 4.11), expression of the two other genes, *AP1* (Fig. 4.7) and *CAL* (Fig. 4.11) was unaffected. Thus *FUL* is up-regulated by SEP4. Previous studies have attributed a role for *SEP4* in meristem identity, as shown by an intermediate meristem identity defect in the *ap1 sep4* double mutant compared to *ap1* single and *ap1 cal* double mutants (Ditta G.S. et al., 2004). Flowers carrying a mutation for the *ap1* gene develop secondary flowers in the axils of first-whorl organs. This phenotype has been interpreted as a partial reversion of floral meristems into inflorescence meristems, and this conversion is almost complete in *ap1 cal* double mutants where flower meristems behave like inflorescence meristems and continuously elaborate new meristems, resulting in a "cauliflower" phenotype. Nevertheless, after a period, plants start to produce *ap1*-like flowers and eventually set seeds (Bowman J.L. et al., 1993). In contrast, flowers of the *ap1 cal ful* triple mutants never produce flowers (Ditta G.S. et al., 2004). Inflorescences of the *ap1 sep4* double mutant plants appear cauliflower-like early after bolting but soon start to produce *ap1*-like flowers on their periphery and still have a "cauliflower like" appearance in the centre for a considerable time before this "cauliflower" phenotype disappears (Ditta G.S. et al., 2004). The authors concluded from this experiment that a mutation in sep*4* has a similar but less severe

effect on maintenance of floral meristems than a mutation in the *cal* gene, suggesting that the SEP4 protein is necessary for CAL activity (Ditta G.S. et al., 2004). A direct regulatory effect on the *CAL* gene could not be confirmed. (Fig. 4.11). However, another possibility might be that CAL and SEP4 rely on protein protein-interactions and act in a complex regulating further targets. Another conclusion from this experiment was that *SEP4* is involved in determining floral meristem identity, supported by the finding that plants constitutively overexpressing *SEP4* have a terminal flower phenotype (Ditta G.S. et al., 2004). Mutations in the gene *TERMINAL FLOWER* (*TFL*), (a negative regulator of *LFY* and *AP1*) cause early flowering and premature conversion of the normally indeterminate shoot meristem into a flower meristem (Shannon S. and Meeks-Wagner D.R., 1991). An enhanced meristem identity defect was observed in *ap1 cal sep4* triple mutant with flowers that had the "cauliflower" phenotype for a longer period than the *ap1 cal* double mutant before producing flowers. A decrease in *CAL* activity cannot explain this enhanced meristem defect and therefore other genes must have a similar role to *CAL* in promoting flowering. One of these candidates was *FUL* and the loss of the *SEP4* gene could result in a decrease in *FUL* activity, explaining the enhanced phenotype in *ap1 cal sep4* triple mutants (Ditta G.S. et al., 2004). Thus, *SEP4* could have an activating role on *FUL* based on these genetic experiments with different mutant combinations. In this study, it was shown at a molecular level that *FUL* is a direct target of SEP4, and in a *sep4* mutant, *FUL* expression is decreased (Fig. 4.11), suggesting *FUL* is up-regulated by *SEP4* . However it could be that this meristem phenotype is not completely based on a feedback with the *FUL* gene and a change in expression, but also based on protein protein interactions. These meristematic defects might be partly caused by interactions and partly by a change in the expression of the *FUL* gene.

4.19.4 *SEP4* acts as an important repressor in floral meristems by directly repressing floral integrators

Floral transition is controlled by multiple environmental and endogenous signals. During the floral transition, the shoot apical meristem (SAM) changes its fate and becomes an indeterminate inflorescence meristem that develops multiple determinate floral meristems (FM). FMs produce a precise number of floral organs arranged in concentric whorls (Irish V.F., 1999; Jack T., 2004). Flowering time genes like *SHORT VEGETATIVE PHASE* (*SVP*) (Hartmann U. et al., 2000), *SUPPRESSOR OF CONSTANS 1* (*SOC1*) (Hepworth S.R. et al., 2002), *AGAMOUS LIKE*

24 (*AGL24*) (Michaels S.D. et al., 2003), *FD* (Abe M. et al., 2005) , *FLOWERING LOCUS C* (*FLC*) (Hepworth S.R. et al., 2002) and *FLOWERING LOCUS T* (*FT*) are involved in the floral transition. Our experiments have shown that *SEP4* directly represses *AGL24*, *SOC1*, *SVP* and *FD* (section 4.12, Fig. 4.10). These molecular data are confirmed with the observation of an early flowering phenotype in *sep4* single mutants compared to WT plants under long day conditions (section 4.14, Fig. 4.13). Thus, *SEP4* acts as a floral repressor, a function that has not been described for any of the *SEP* genes. By repressing flowering time genes, *SEP4* could abolish floral reversion, a process where inflorescence like structures develop within flowers caused by a loss of floral meristem identity in the meristematic cells (Tooke F. et al., 2005). It has been shown that ectopic expression of *SVP* and *AGL24* causes floral reversion (Yu H. et al., 2004; Trevaskis B. et al., 2007). The observation that the *sep2sep3sep4* triple mutant (Section 3.4, Table 3.12) showed sepaloid petals that have been described for plants constitutively expressing *SOC1* as well (Liu C. et al., 2007), support our finding that *SEP4* is a repressor of this flowering time gene. One explanation why *sep4* mutants have an early flowering phenotype but never show floral reversion might lie in partial redundancy. Other genes such as *LFY* or *AP1,* known to have a role in the prevention of floral reversion (Parcy F. et al., 2002) are still functional in the *sep4* mutant and can maintain the floral state. Mutations in the meristem identity genes *LFY* and *AP1* lead to a partial conversion of flowers into shoots (Irish V.F. and Sussex I.M., 1990; Huala E. and Sussex I.M. , 1992; Weigel D. et al., 1992). Therefore, it would be interesting to generate the *sep4 ap1 lfy* triple mutant and investigate whether a floral reversion occurs and if flowering time is affected. The role of *SEP4* as a floral repressor fits well with its expression pattern, which differs from all other *SEP*s, as it is expressed from the seedling stage onwards (Supplementary S7, S8) in vegetative and floral organs (Ma H. et al 1991.; Rounsley S.D. et al., 1995; Savidge B. et al., 1995; Ditta G.S. et al., 2004). This suggests, that one of the main roles of *SEP4* is the repression of flowering time genes to avoid floral reversion and ensure, once the plant switches from the vegetative to reproductive phase, flower development can occur. Together with the finding that *SEP4* activates *FUL* and that *FUL* and *AP1* are involved in the transition from inflorescence to floral meristem identity, *SEP4* can be seen as a “hub” between different phases of flower developmen involved in different developmentl phases. Once floral meristems are established, *SEP4*, together with the other *SEP*s is redundantly involved in floral organ development. Based on available data from *SEP3* (Pelaz S. et al., 2000; Pelaz S. et al., 2001a; Kaufmann K. et al., 2009) and the *sepallata* mutant analyses described in this study (section 3.4), *SEP1-3* seem to be more critical for floral organ development than *SEP4,*as each of those 3 genes can specify all floral whorls (section 3.4), whereas *SEP4* acts in ealier steps in plant development and is involved in the switch from the vegetative to reproductive phase.

Taken together, *SEP4* is expressed earlier than the other *SEP*s and acts as a floral repressor, the gene is involved in the establishment of floral meristem identity (as it activates *FUL*) before contributing to the later establishment of floral organs, together with the other *SEP* genes.

4.20 Overlapping and individual targets show redundant and non-redundant functions between SEP3 and SEP4

The ChIP-SEQ, microarray and qPCR experiments have revealed that SEP3 and SEP4 share many target genes in common, with roles in flower development, reproduction and the floral transition. This indicates that the control of flower development involves a degree of redundancy. Nevertheless, these studies have also shown that both genes have individual targets, suggesting additional, non-redundant functions. For example, *FLC* (a floral repressor) (Michaels S.D. and Amasino R.D., 1999), *LUG* (a cadastral gene involved in A and C -class gene boundary development during flower development) (Liu Z. and Meyerowitz E.M., 1995) and *LBD39* (a gene involved in anthocyanin biosynthesis and Nitrogen metabolism) (Rubin G. et al., 2009) were all identified as individual SEP4 targets not targeted by SEP3. At least the latter two were also affected in their expression in a *sep4* mutant background (Fig. 4.12). In the genome-wide binding study of SEP3 (Kaufmann K. et al., 2009), the genes *SCHLAFMUETZE* (*SMZ*) and *SCHNARCHZAPFEN (SNZ*), two floral repressors belonging to the AP2 family of miR172 regulated transcription factors (Aukerman M.J. and Sakai H., 2003; Mathieu J. et al., 2009) were identified as direct targets, but neither was found as a SEP4 target in this study. Another group of TFs that appeared to be uniquely targeted by SEP3 and not SEP4 were members of the *SQUAMOSA PROMOTER BINDING–LIKE* family (SPLs) that are targeted by microRNA156 (miR156) (Cardon G. et al., 1999; Rhoades M.W. et al., 2002). This micro RNA decreases with increasing plant age, resulting in an increase in *SPL*s that promotes juvenile to adult phase transition and flowering through activation of miR172, MADS-Box genes, and *LFY* (Wu G. and Poethig R.S., 2006; Wu G. et al., 2009).

Interestingly, common targets between SEP3 and SEP4 are often associated with processes involved in flower development, reproduction or floral transition, suggesting that these processes are largely redundantly regulated, whereas individual targets of SEP4 are enriched for cellular processes or the regulation of metabolic processes (section 4.5, Table 4.2). This suggests that some of the individual functions of *SEP*4 are not directly involved in flower development.

Another finding in this study was that the identification of TFs as direct targets by ChIP-SEQ approaches does not necessarily show altered expression in the corresponding mutant backgrounds This confirmed findings of previous studies as well; as for example studies in *Drosophila melanogaster* have demonstrated that transcription factor–binding events do not always coincide with changes in transcriptional activity (Li XY. et al., 2008). The genome-wide identification of AP1 targets in *A. thaliana* revealed that only around ~11 % of target genes are affected in their expression (Kaufmann K. et al., 2010b). Similar observations were made for genome-wide target identification for AG with ~ 5 % being affected in their expression (ÓMaoiléidigh D.S. et al., 2013) and similar results were obtained in our studies with less than 10 % of SEP4 target genes appearing as affected transcripts in the *sep4-1* microarrays. Only 89 of the 1211 SEP4 target genes showed a change in expression in the *sep4-1* mutant versus the WT (7.3 %, section 4.10, Fig. 4.9 a, eS). From the 309 genes that were targeted by SEP3 and SEP4, only 28 genes showed a change in expression in the *sep4-1* mutant versus the WT (~ 9 %, section 4.10, Fig. 4.9 b, eS) and from the 902 individual SEP4 targets, 61 were affected in their expression in the *sep4-1* mutant versus the WT (~ 6.2 %, section 4. 10, Fig. 4.9 c, eS).

4.21 *LUG*, a cadastral gene and individual target of SEP4

LUG is a cadastral gene involved in A and C-class gene boundary development during flower development (Liu Z. and Meyerowitz E.M., 1995). It is one example of a gene targeted by SEP4 and affected in expression, being up-regulated in *sep4-1* mutants compared to WT in qPCR experiments (Fig. 4.12). *LUG* acts as a negative regulator of *AG* and *lug* mutants show homeotic transformations of the first whorl with petaloid, stamenoid or carpeloid sepals and the second whorl petals have stamen characteristics or a reduction in the number of petals (especially in apical flowers). Stamen number is reduced, together with a deviation in the number of carpels, that fail to fuse properly (Liu Z. and Meyerowitz E.M., 1995). The homeotic transformations in the first two whorls, as well as a reduction in the number of petals, result from ectopic *AG* expression. LUG forms a putative co-repressor complex with SEUSS (SEU) and recruitment by SEP3 and AP1 to the *AG* cis-regulatory element leads to repression of the chromatin at the *AG* locus, resulting in the repression of the *AG* gene (Sridhar V.V. et al., 2006) It is known that LUG and SEU do not have a binding domain for *AG,* their binding to the *cis*-regulatory element of *AG* depends on the direct physical interaction between SEU, SEP3 and AP1 and it has been proposed that domain specific expression of *AG* in whorls 3 and 4 is a

result of multiple factors that have opposite effects on *AG* expression (Sridhar V.V. et al., 2006). AP1, SEP3 (and possibly all other SEPs) can function as DNA-binding interaction partners with the LUG-SEU complex and add repressor activity in all four whorls. This repressor effect is enhanced in the perianth by the presence of outer-specific repressors, for example *AP2* (Meyerowitz E.M. et al., 1991; Jofuku K.D. et al., 1994; Chen X., 2004) or *BELLRINGER* (Bao X. et al., 2004). Repression in whorls 3 and 4 is weakened by inner whorl specific activators of *AG,* such as the positive autoregulation by the AG/SEP3 complex (Gomez-Mena C. et al., 2005) or the activities of LFY and WUS (Lenhard M. et al., 2001).

As our experiments have shown, SEP4 has an activating function on *AG* and represses *LUG* (Fig. 4.7, Fig. 4.12). One idea is that SEP4 forms a complex with other unknown proteins (Fig. 4.16) to positively regulate *AG* transcription, whereas another complex is established to repress *LUG* (Fig. 4.16). However, the nature of these complexes is unknown and it is certainly an interesting topic for the future to be further explored. It might be also worth testing whether the expression pattern of *LUG* in *sep4* mutants changes using *in situ* experiments. Single mutants of the *sep4* gene were not affected in their carpel morphology and one explanation for this might be that the other *SEP*s can compensate the loss of the *SEP4* gene.

Fig. 4.16

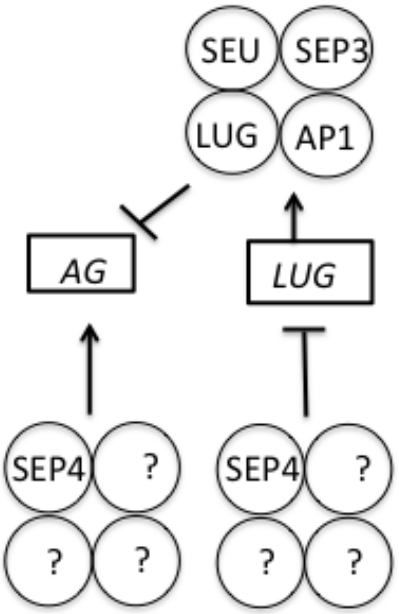


Fig. 4.16: Regulation of *AG* and *LUG* by SEP4

AG is up-regulated whereas *LUG* is down-regulated by SEP4, presumably via complex formations with other currently unidentified proteins to activate *AG* and repress *LUG*. LUG *t*ogether with SEU, SEP3 and AP1 represses *AG* transcription.

4.21 *LBD39*, an example of an individual SEP4 target that is not directly linked to flower development

LBD39 was identified as an individual SEP4 target gene affected in its expression (section 4.7, 4.9, Fig. 4.12). This zinc-finger DNA-binding TF belongs to the plant-specific *Lateral Organ Boundary Domain* (*LBD*) gene family. The *LBD* family contains 43 members, 37 of which are grouped in class 1 and six in class 2. *LBD 3*9 belongs together with *LBD*37 and *LBD38* to class 2 and shows a rather broad expression pattern, with high expression levels in the root, cotyledons, vegetative rosette leaves and the shoot apex (from the vegetative to inflorescence stage) to moderate levels in the flower and young siliques. Within the flower, expression can be found in all floral whorls (expression pattern can be found at *Arabidopsis* eFP Browser, (Lin W.C. et al., 2003; Winter D. et al., 2007). Based on their expression pattern, it has been suggested, that *LOB* genes plays a role in the establishment of boundaries between the meristem and the differentiated lateral organs (Shuai B. et al., 2002). Class 1 members have been implicated in plant development, for example *LBD36* (also known as *ASYMMETRIC LEAVES 2-LIKE 1, ASL1*) is required to repress *KNOX* genes (*BP*, *KNAT2* and *KNAT6*) at the time of leaf initiation alone or in combination with *AS1* (Ori N. et al., 2000; Byrne M.E. et al., 2002). *ASL1* and *AS2* play a role during early stages of flower development as double mutants reveal flower buds that opened precociously and exposed inner organs at early stages (Chalfun-Junior A. et al., 2005). It has been shown that *LBD37, 38* and *39* act as repressors of anthocyanin biosynthesis and nitrogen availability signals and represent a primary component in a signalling pathway that represses anthocyanin production when NO_3^-/N is available. Nitrogen and NO_3^- are the major forms of nitrogen availability to many plants and are macronutrients and signals for plant growth and metabolism (Rubin G. et al., 2009).

This is an interesting result as it links *SEP4* to developmental and metabolic processes that were not described previously. *SEP4* was known to be involved in meristem and flower development (Pelaz S. et al., 2000; Ditta G.S. et al., 2004) , but these results might indicate that it has a broader function than expected, with a more complex role in gene regulation. Further experiments need to be done but it would be interesting to elucidate whether *SEP4* is involved in metabolic processes like Nitrogen uptake. For example, it would be interesting to grow *sep4-1* mutants in the absence of N/NO_3 and investigate whether flowering time, root, flower and seed development differs from WT grown under the same conditions. Another experiment could be to test whether the anthocyanin production is affected in the *sep4* mutant compared to WT. One suggestion is to perform all experiments at two temperature conditions (22 and

27 °C), as the mutant analysis described in chapter 3 revealed, plants are more strongly affected when grown under stressed conditions and if flowering time, root, flower and seed development are affected in the absence of N/NO3, these phenotypes should be easier to detect under elevated temperature conditions. One conclusion from this mutant combinations described in this work is that multiple *SEP* genes enhance the robustness of the plant. Nitrate is a macronutrient and acts as a signal for plant growth. If root development or nutrition uptake in a *sep4* is mutant affected, this could represent one example, that genes originally defined as redundant have individual functions necessary for developmental processes to contribute to the fitness of the plant.

4.22 Antagonistic expression between common targets is another means of transcriptional regulation

One TF can have dual roles, either working as an activator or repressor depending on cellular or promoter contexts. One hypothesis to explain this dual behaviour is a concentration-dependent switch, proposing that a TF is an activator at low concentrations and functions as repressor at high concentrations or the other way round (Papatsenko D. and Levine M.S., 2008). There are three possibilities how a targeted gene can be affected, Firstly, a TF binds a gene, but does not affect its transcription, Secondly, direct binding results in activation of the target, thirdly, direct binding results in repression. Examples for all three fates were seen in this and previous studies (Kaufmann K. et al., 2009; Kaufmann K. et al., 2010b; Yant L. et al., 2010; ÓMaoiléidigh D.S. et al., 2013). When comparing the two closely related TFs SEP3 and SEP4, four ways of transcriptional regulation were seen for their common targets. Firstly, SEP3 and SEP4 target the same gene but have no effect on its transcription (Fig. 4.17 a); secondly, the target is either activated or repressed by both proteins (Fig. 4.17 b). Thirdly the common target is activated or repressed by one of the TFs whereas the other one does not have an effect on the transcriptional regulation of the gene (Fig. 4.17 c). The fourth possibility is that both TFs influence the transcription of the target gene in an antagonistic way, which means that one TF activates the target whereas the other TF represses the target or vice versa (Fig. 4.17 d). Examples in this study are described in section 4.15 (Fig. 4.14). For example *CUC3* is activated by SEP3 and repressed by SEP4, *BGAL7* activated by SEP4 and repressed by SEP3. The fourth possibility is interesting as it suggests a novel way of transcriptional regulation between two closely related TF that were described as redundant.

Fig. 4.17

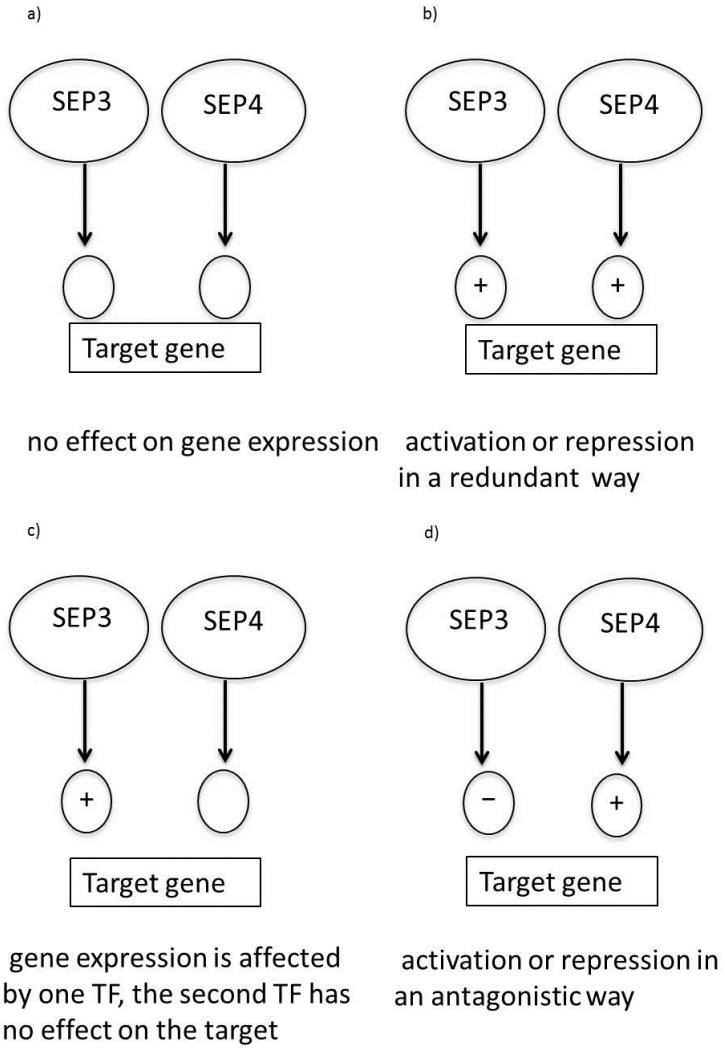


Fig. 4.17: Four different ways of transcriptional regulation for common targets of SEP3 and SEP4

a) common targets are not affected in their expression, b) common targets are affected redundantly, c) common targets are affected by one of the two TFs, d) common targets are affected in an antagonistic way, empty circle = no effect, circle containing a + = activating, circle containing a - = repressing

4.23 *SEP4* and *CUC3* are involved in organ boundary formation

CUC3 together with *CUC1* and *CUC2* belongs to the NAC domain family proteins. The two genes *CUC2* and *CUC3* are mainly required for axillary meristem initiation and boundary specification in various postembryonic organs, including stems, pedicels and leaves, whereas all three *CUC* genes have significant contributions for embryonic shoot meristem and cotyledon boundary formation (Hibara K. et al., 2006). Single mutations in the *cuc3* gene cause cotyledon fusion to a variable degree depending on the allele (Hibara K. et al., 2006). Fusions of floral organs are reported to be very rare, for example, stamen morphology with fused third whorl organs is rarely affected in *cuc3* single mutants. Sepal fusions have not been reported for *cuc3* single, but only for mutant combinations in combination with *cuc1* or *cuc2* (Hibara K. et al., 2006). In addition, the two genes *CUC2* and *CUC3* are involved in leaf morphology as mutants of both genes show serrated leaves (Hasson A. et al., 2011). Axillary shoot formation was also affected in *cuc3* mutants. In WT plants, axillary meristems are formed at the axil of rosette and cauline leaves and form branches, *cuc3* single mutants show disturbed branch formation at a low frequency with a lack of axillary shoots (Hibara K. et al., 2006).

So far, no function has been described for *CUC3* affecting perianth organ number or stamen number. In agreement with this, our analysis of *cuc3-105* mutants also showed no effect on perianth or stamen organ number (Table 4.7).

SEP4 is necessary to establish the floral ground state and and sepal identity as flowers of the *sep1sep2sep3sep4* quadruple mutant are composed of leaf like organs, whereas the *sep1sep2sep3* triple mutant is composed of sepals (Ditta G.S. et al., 2004). Besides the involvement in floral organ formation, *SEP4* has been ascribed a role in maintaining floral meristem identity as shown in a slightly higher frequency of secondary flowers in the axils of first-whorl organs in the *sep1sep2sep3sep4* quadruple mutant compared to the *sep1sep2sep3* triple mutant (Pelaz S. et al., 2000, Ditta G.S. et al., 2004).

Additionally this study has shown *SEP4*'s role in defining the correct number of perianth organs, as shown in the frequent development of extra sepals and petals in *sep4* single mutants (section 3.2, Table 3.4). *CUC3* was identified as a direct repressive target of SEP4 in this work (section 4.10, 4.15, Fig. 4.14). Furthermore previous ChIP-SEQ experiments revealed that *CUC3* is a direct target of SEP3 (Kaufmann K. et al., 2009), and activated by SEP3as shown in expression analysis in this work (section 4.15). Therefore, *CUC3* is one of the common targets that is antagonistically regulated by SEP3 and SEP4; it is down regulated in *sep3-2* and up regulated in *sep4-1* mutants. Based on the idea that the extra perianth organ phenotype

seen in *sep4* single mutants (section 3.2, Table 3.4) and all double mutants carrying a mutant *sep4* allele (*sep1sep4*, *sep2sep4*), with the exception of *sep3sep4* (section 3.3, Table 3.6), is caused by an increased *CUC3* expression in the *sep4* mutant, the *cuc3sep4* double mutant was generated to examine whether the loss of the *CUC3* gene can rescue the extra perianth phenotype (section 3.2, Table 3.4). As seen in section 4.15 (Fig. 4.15, Table 4.7) the opposite effect occurred. Flowers of double mutants produced extra sepals and petals at an even higher frequency than the *sep4-1* single mutant (Table 4.7, *sep4-1* ~30 % vs *cuc3sep4* ~ 70 %) suggesting both genes interact in a pathway involved in specifying organ number. In addition to the increased number of sepals and petals a novel phenotype was discovered, affecting the second whorl. Double mutants showed stamenoid petals that were not described previously either for *sep4* or *cuc3*. Weak mutations of the A class gene *AP2* are reported to show defects in the outer two whorls with sepals replaced by leaf like organs and stamenoid petals (Bowman J.L. et al., 1989). However the involvement of this gene in the development of this phenotype can be excluded as our experiments identified *AP2* as a direct target but its expression did not reveal a change in the *sep4* mutant (section 4.6, Fig.4. 7). Third whorl organs were affected as well, as the double mutant develops on average one stamen less than WT flowers (Table 4.7).

One idea to explain the extra organ phenotype in *sep4* single mutants could be the increased levels of *CUC3*. It is known that *CUC* genes (*CUC1-3*) specify boundary formation and thus promote boundaries. These genes are normally expressed around the auxin maxima that will produce outgrowths (Arnaud N. and Laufs P., 2013). A *sep4* single mutant will have more *CUC3* (Fig. 4.14) and as a result can make more boundaries and therefore more organs. A *sep3* single mutant has decreased levels of *CUC3* (Fig. 4.14), but the effect is invisible or very subtle. To explain the enhanced floral organ phenotype in the *cuc3sep4* double mutant, one could consider the involvement of *CUC2* and *CUC3* genes in cytokinin regulated flower development. It has been shown that *AtIPT4* driven by the *AP1* promoter (*AP1::IPT4)* shows increased cytokinin levels. More interesting is that these transgenic lines show aberrant flower development as e.g. a decreased length in the primary inflorescences and an increase in the total number of flowers and in the number of all floral organs compared to WT (Li et al., 2010). Genome wide expression analyses have revealed increased expression levels for *CUC2* and *CUC3* in transgenic *AtIPT4* lines driven by an *AP1* promoter. This phenotype was rescued in homozygous *cuc2-1 AP1::IPT4* lines that developed floral organs that were indistinguishable from WT and had a normal height of primary inflorescences. Homozygous double mutants for *cuc3-105 AP1::IPT4* showed a recovery in the height of inflorescence and the toal number of flowers, but the floral organ number was still the same as that of the *AP1::AtIPT4* plants, whereas the *cuc2-1 cuc3-105 AP1::AtIPT* triple mutant revealed flowers similar to those of WT

with respect to floral organ number, suggesting that mutations in the *cuc2* and *cuc3* gene can repress the *AP1::IPT4* phenotype. Thus, *CUC2* and *CUC3* mediate the regulation of cytokinin in flower development, whereas *CUC2* seems to have a more important role in developmental responses to cytokinin (Li X.G. et al., 2010). One explanation for the enhanced floral organs phenotype in the c*uc3sep4* double mutant might be that both genes, *CUC3* and *SEP4* repress *CUC1* and/or *CUC2*. A *cuc3* single mutant does not affect the expression of *CUC1* and / or *CUC2* because *SEP4* is still able to repress them. However, in the *cuc3sep4* double mutant, repression of *CUC1* and / or *CUC2 i*s no longer possible resulting in an increased expression of those genes. This suggests that the extra organs in the *cuc3sep4* double mutant come from increased *CUC1* and / or *CUC2* expression levels, whereas the extra organs in *sep4* single mutants come from increased *CUC3* expression levels. It would be interesting to test whether the expression of *CUC1* and *CUC2* is affected in the *cuc3sep4* double mutant.

For the future, it might be interesting to generate the *sep3cuc3* double mutant to test whether the organ number of sepals and petals is decreased or if other floral organs are affected. Based on our studies, *CUC3* was up-regulated by SEP3 and therefore it would be interesting to see if the double mutant is similar to WT or shows a change in the number of floral organs. If we assume that *CUC3* activates or represses unknown downstream genes, this process would be disturbed in the *sep3cuc3* double mutant because *cuc3* is not active and thus no activation or repression of the unknown genes would occur. Therefore it might be that this double mutant could have fewer perianth organs. Also interesting could be the generation of the *sep3sep4cuc3* triple mutant and analyse the floral organ number. *CUC3* is up-regulated by SEP3 and downregulated by SEP4, if both genes are not active *CUC3* expression should be normal and flowers should look like WT. If the *CUC3* gene is not active in this triple mutant, flowers should look like 'typical' *cuc3* single mutants that did not reveal any phenotypes affecting floral organ number or stamen morphology, but revealed changes in the cotyledons. Another interesting cross would be a *cuc3* mutant with a line constitutively expressing the *SEP3* gene or vice versa (35S::*SEP3cuc3*, 35S::*CUC3sep3*) to check whether floral organ development and especially floral organ number is affected. This experiment is less favourable compared to the above-described experiments because lines that constitutively express genes can cause unpredictable effects. Therefore it is difficult to distinguish whether a phenotype in line that constitutively expresses a gene is based on gene function or caused by ectopic expression.

Taken together: this chapter has revealed interesting findings for the *SEP4* gene and its role during development on a molecular level. The identification of direct targets allowed a comparison with its close relative gene *SEP3* and has shown that both genes have many

common targets, especially those involved in flower development and reproduction, suggesting a degree of redundancy for these steps in the life of the plant. SEP4 targets and represses the floral integrators *SOC1*, *FD, SVP* and *AGL24* (section 4.12), overlapping with the tissues it is expressed and suggest a role in preventing floral reversion and ensuring that these genes are down-regulated before floral organ identity genes are activated. These molecular results fit the early flowering phenotype of *sep4* mutants presented in this study for the first time (section 4.14). However, all these floral integrators are common targets between SEP3 and SEP4 and were also shown to be down-regulated by SEP3 (Kaufmann K. et al., 2009), but no effect on flowering time has been described for *sep3* mutants (Lopez-Vernaza M. et al., 2012), except an early flowering phenotype in lines constitutively expressing the *SEP3* gene (Kaufmann K. et al., 2009). Therefore to examine whether SEP4 affects the assembly of higher-order MADS-Boxes protein complexes that regulate flowering time would be certainly interesting for the future. Another possibility might be that SEP4 has a higher affinity to regulate flowering times in contrast to SEP3, which might explain a phenotype affecting flowering time for *sep4* but not for *sep3* mutants. In addition to these common targets, this study also revealed individual targets for SEP3 and SEP4, however many of them were not directly linked to floral or plant reproductive processes. These results suggest non-redundant functions as well and also give an insight into the complexity of GRNs. One example of this study was the identification of direct targets that were not directly associated with floral organ formation as for example the gene *LBD39,* that is reported to have a function in nutrition uptake.

Another interesting finding was that related TFs can have the same target, but regulate them antagonistically.

Some questions remain open and one experiment that needs to be done is a second ChIP-SEQ replicate for g*SEP4*:GFP;*sep1sep4* to verify the targets identified in this study. In addition, further ChIP-SEQ experiments can be performed in different backgrounds, such as g*SEP4*:GFP;*sep2sep4* and g*SEP4*:GFP;*sep3sep4*. After these experiments, it will be possible to obtain an overview of whether SEP4 targets change in different backgrounds. Also interesting would be to compare if the targets that change in a *sep1*, *sep2* or *sep3* mutant background are involved in different developmental processes. This might give an indication about the degree of redundancy, sub- or neo-functionalization of this gene family.

Fig. 4.18

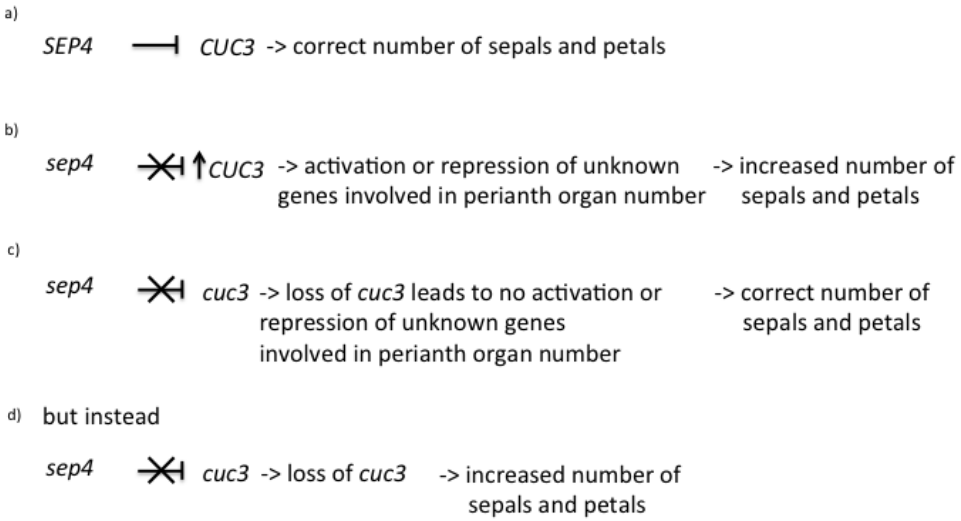


Fig. 4.18: Theory behind the generation of the *cuc3sep4* double mutant.

a) *SEP4* represses *CUC3* and flowers develop the correct number of sepals and petals, b) the loss of the *SEP4* gene results in an increased expression of the *CUC3* gene which might lead to an activation or repression of unknown downstream genes by *CUC3* that are involved in perianth organ number finally resulting in an increase of these organs, c) *cuc3sep4* double mutant flowers should have the correct number of extra perianth organs because *cuc3* cannot act on other unknown downstream genes involved in perianth organs, d) loss of both genes, *sep4* and *cuc3* in the *cuc3sep4* double mutant caused an increased number of perianth organs

Chapter 5

5. General discussion

5.1 Non redundancy in between *A. thaliana SEP*s

Combining genetic and molecular experiments in this and other studies shows that the four *A. thaliana SEP* genes (*SEP1-4*) have redundant as well as non-redundant roles. The single mutant analysis described here revealed individual roles for each *SEP* gene. Perianth organs are affected in *sep3-2* (folded petals) and *sep4* (extra organs), whereas carpel length and morphology is affected in *sep1* and *sep2* mutants respectively (Table 3.5). Double and triple mutant analysis revealed individual as well as common phenotypes, suggesting that the four *SEP*s have individual and combined functions. At a molecular level, this suggests that SEPs activate or repress individual and common targets. The molecular data reported in this study reveal a large degree of redundant as well as non-redundant roles for the two *SEP* genes (*SEP3* and *SEP4*), which is supported by previous expression and protein-protein interaction studies (Fan H.Y. et al., 1997; de Folter S. et al., 2005, Immink R.G. et al., 2009, Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G. et al., 2004). The four *SEP* genes show slightly different expression pattern. *SEP1* and *SEP2* and SEP4 are expressed throughout the floral meristem from stage 2 onwards corresponding to all four floral whorls (sepals, petals, stamens, carpels, Fig. 5.1 a, b, d) (Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G.S. et al., 2004), *SEP3* expression starts slightly later and is expressed in a region corresponding to the inner three whorls (petals, stamens, carpels, Fig. 5.1 c) before the initiation of floral organ primordia (Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G.S. et al., 2004). In this work it has been shown that *SEP4* expression starts much earlier than the other *SEP*s, from the seedling stage onwards in cotyledons and in rosette leaves as confirmed in gene expression experiments (qPCR and semi-quantitative PCR, Supplementary data S7, S8 Fig. 5.1 d) besides its previously reported high expression in cauline leaves (Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G.S. et al., 2004).. All *SEP* genes are expressed in the siliques and seeds (Flanagan C.A.

and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G.S. et al., 2004). This earlier expression pattern of *SEP4* suggests that it is involved in additional developmental processes compared to the other *SEP*s. Protein interaction studies have already shown that SEP proteins have different interaction partners, e.g yeast three hybrid assays revealed complexes for SEP2 and SEP4 that were not found for SEP3 and vice versa (de Folter S. et al., 2005; Immink R.G. et al., 2009). As our study has shown, *sep1* and *sep2* mutants have phenotypes affecting the carpel and yeast studies have shown that SEP1, SEP2 and SEP3 interact with *ARABIDOPSIS BSISTER* (*ABS*) whose proteins are important for gametophyte and seed development in *Arabidopsis.* Two *ABS* genes exist (*ABS-I* and *ABS-II*), which are derived through alternative splicing (de Folter S. et al., 2006). MADS-Box TFs are known to interact with a variety of other classes of DNA binding proteins, called ternary complex formation. The interplay between these different proteins regulates gene expression of target genes (Shore, P. and Sharrocks A.D., 1995) Ternary complexes were found for ABS-I and AGL16-SEP3, PI-SEP3, AGL74-SEP2 and SEP1-SEP2 (Immink R.G. et al., 2009). These results together with the observed phenotype suggest that *SEP1* and *SEP2* have a specialized function in carpel development and do not act fully redundantly. In contrast, SEP4 has not been shown to interact with the ABS proteins and *sep4* mutants do not reveal a phenotype affecting the carpel. Only a few dimerization partners for SEP4 have been previously identified (AGL24-SOC1-SEP4, SEP1-FUL-SEP4, SEP1-SOC1-SEP4, SEP4-AP1-AGL24, FUL-SEP4-SVP) (Immink R.G. et al., 2009). All of these dimerization partners (SEP1, SOC1, AP1, AGL24, FUL and SVP) were identified as direct SEP4 targets in the genome-wide binding study in this work. *FUL*, *SOC1, SVP* and *AGL24* were also affected in their expression in the *sep4* mutant as shown in microarray and qPCR experiments. SEP4 is a repressor of *AGL24, SVP* and *SOC1* (Fig. 4.10) and an activator of *FUL* (Fig. 4.11). Genome-wide binding profile studies of SEP3 identified thousands of binding sites in the genome, targeting floral organ identity genes, hormone-related genes and floral activators (Kaufmann K. et al., 2009).

The extra perianth phenotype of *sep4* single mutants reported here (section 3.2, Tables 3.4, 3.5) also differs from all the other *sep* phenotypes, which mainly affect the morphology or shape of floral organs (*sep3-2*, petal blade, *sep1*, carpel length, *sep2,* carpel morphology), further underlining the non-redundant roles of single *SEP* genes. Once again, all these data show that *SEP4* is the most different from the others *SEP*s, as it has a wide expression pattern in the plant (Fig. 5.1 d) and cannot specify floral organ identity, as shown in the analysis of the triple mutants (section 3.4, Table 3.14, Fig. 3.3, Fig. 3.4). However, it is still unknown how this extra perianth phenotype or the inability to confer floral organ identity can be explained at a molecular level.

Fig. 5.1

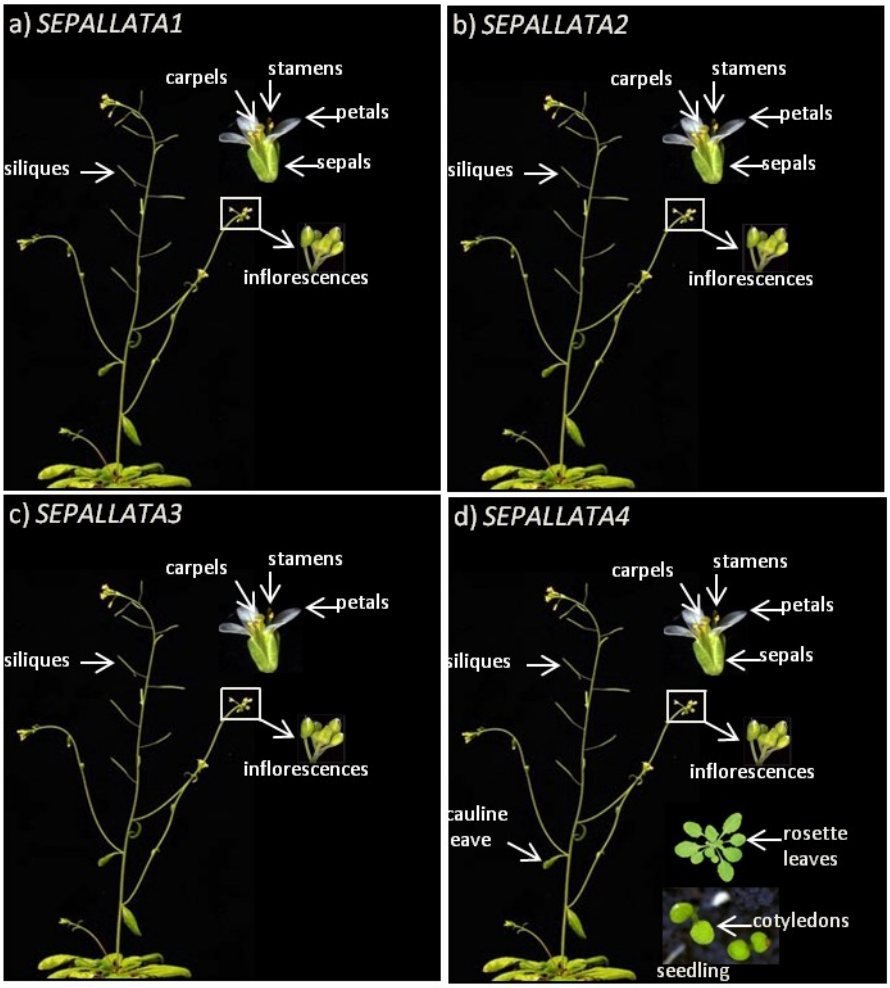


Fig. 5.1: Expression pattern of *SEPALLATA* genes in *A. thaliana.*

a) SEP1 is expressed in the reproductive phase throughout the floral meristems corresponding to all four floral whorls and siliques, [1)] b) *SEP2* is expressed in the reproductive phase throughout the floral meristems corresponding to all four floral whorls and siliques,[1)] c) *SEP3* expression is restricted to the floral meristems corresponding to the inner three whorls and siliques, [1)] d*) SEP4* expression is detected in the vegetative phase from the seedling stage onwards in cotyledons, rosette leaves [2)], and in cauline leaves and in the reproductive phase in floral meristems corresponding to the four floral whorls and siliques [1).]

[1)] Previous expression data published from (Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G.S. et al., 2004)

[2)] Expression in seedlings and rosette leaves shown in this work (S7, S8)

5.2 *SEP* genes enhance the fitness of the plant

As molecular and genetic studies in this and other studies have shown, *AtSEP*s and *SEP*-like genes in other different plant species, such as petunia (Ferrario S. et al., 2003; Vandenbussche M. et al., 2003) and tomato (Ampomah-Dwamena C. et al., 2002; Vrebalov J. et al., 2002) do not act fully redundantly, having experienced diversification. One of the most interesting outcomes of this study is the involvement of the *SEP* genes in the fitness of the plant as the phenotypic characterization has shown (section 3). As soon as single mutants were exposed to a higher temperature, individual phenotypes affecting all floral organs were detectable (section 3. 2) and more severe effects were observed in double or triple mutants leading to infertility (section 3.4, Table 3. 15). The role of *SEP* genes and their effect on the fitness and robustness of the plant to variable environmental conditions was further supported by the observation of an early flowering phenotype for *sep4* single mutants (section 4.14, Fig. 4.13). The decision to flower is important as it has a strong impact on the fitness of the plant. Ideally, plants should start to flower when the environmental conditions are suitable and sufficient to produce fruits and allow seed dispersal. More recently it has been shown that *SEP4* and none of the other *SEP*s redundantly regulates with *SOC1*, *SVP* and *AGL24* inflorescence architecture by repressing the shoot identity gene *TERMINAL FLOWER1* (*TFL1*) (Liu C. et al., 2013). A similar role has been shown in the monocot rice for the *SEP4*, *SVP*, *AGL24* and *SOC1* orthologues, suggesting that this mechanism is conserved between different plant species. (Liu C et al., 2013). Inflorescence architecture plays an important role in the interaction with biotic and abiotic factors, such as pollinators and also affects the fruit set (Evers J.B. et. al. 2011; Iwata T. et al. 2012). After transition from vegetative to reproductive phase, the main inflorescence meristem either acquires floral meristem identity or remains indeterminate, producing lateral meristems on its flanks (Bradley D. et al., 1997, Wang J. and Li J., 2008). *A. thaliana* has a raceme-inflorescence type that has a main SAM growing indefinitely and producing flowers or lateral axes. These lateral axes reiterate the pattern of the main apical meristem (Benloch R. et al., 2007). Recently it has been shown that the inflorescence architecture is strongly affected in the *soc1-2 agl24-3 svp-41 sep4-1* quadruple mutants, revealing a massive inflorescence branching. The main SAM continuously generates secondary and tertiary vegetative shoots, causing infertility as the floral structures were transformed into vegetative structures (Liu C. et al., 2013). This finding shows non-redundant roles between *SEP* genes, as *SEP4* is involved in a pathway controlling inflorescence architecture, whereas the other three *SEPs* do not play a role in this step.

The involvement of *SEP4,* together with the other three flowering time genes *SVP*, *SOC1* and *AGL24* in the repression of *TFL1* in emerging floral meristems correlates with its early expression pattern.

5.3 SEP4 and its function in the GRN

Remarkable progress has been made within the past few years to obtain a better understanding of plant development at the molecular level, based on the advantages of high throughput technologies (Schneeberger K. and Weigel D., 2011; Hamilton J.P. and Buell C.R., 2012). In this study *SEP4* was analysed, employing molecular methods to obtain a better understanding of its role during flower development. This gene is mainly associated with flower development and flower meristem identity (Ditta G.S. et al., 2004). This work has shown that *SEP4* is involved in the regulation in flowering time by repressing flowering time genes (Fig. 4.10, 4.13, 5.2) The purpose of this repression could be to enable the floral organ identity genes together with other co-factors to specify floral whorl development and to maintain the repressed state of flowering genes during the reproductive phase, preventing floral reversion. Floral reversion involves the switch from floral back to vegetative development and is often associated with changing environmental conditions in different plant species (Battey N.H. and Lyndon R.F., 1990). However*, sep4* single mutants are affected in flowering time as they start to flower earlier (section 4.14, Fig. 4.13), but floral reversion has not been observed in these plants. One explanation for this could be redundancy between *SEP4* and other genes that are involved in the prevention of floral reversion, for example *LFY* and *AGL24* (Yu H. et al., 2004). Besides the repression of flowering time genes, SEP4 is involved in maintenance of floral meristem identity, evidenced by its regulatory effect on *FUL*, a MADS-Box gene that is involved in several aspects of flower development including floral induction, as shown by a slight delay in flowering time in *ful* mutants (Ferrándiz C. et al., 2000), meristem identity , cauline leaf morphology and, later on, in carpel and fruit development (Gu Q. et al., 1998; Ferrándiz C. et al., 2000). In addition to processes involved in floral transition and development this gene might have further roles in other processes such as plant growth and Nitogern uptake, as suggested by the targeting of *LBD 39* (section 4.13, Fig. 4.12). *LBD39* and its two homologs (*LBD37*, *LBD38*) are involved in the regulation of plant metabolism by negatively affecting N assimilation and as a consequence the N status and growth of the plant (Rubin G. et al., 2009). Furthermore, the targeting of *CUC3* indicates a role of SEP4 in shoot

meristem formation and cotyledon separation (Hibara K. et al., 2006) and maybe in cytokinin regulated flower development (Li et al., 2010). The enhanced *cuc3sep4* phenotype described in this study with an increased perianth organ number and altered petal morphology (section 4.15, Table 4.7, Fig. 4.15) supports the role of SEP4 in flower development (organ number and morphology).

In summary: *SEP4* affects different phases during the development of the plant (Fig. 5.2) and can be seen as a multifunctional transcription factor. Firstly, *SEP4* represses flowering time genes to avoid floral reversion; secondly, *SEP4* contributes to the establishment of meristem identity and thirdly is involved in the regulation of floral organ identity genes, as seen by positively affecting the transcription of *PI* and *AG* and negatively affecting *LUG* transcription. Thus, *SEP4* can be considered as a "hub" for the transition of different developmental steps in the lifecycle of the plant by its ability to activate or repress genes, depending on the developmental phase. Furthermore, *SEP4* has a particular role in the development of the correct number of sepals and petals (Tables 3.4, 3.5, 3.6), however the molecular evidence explaining this defect is elusive and an interesting topic for future studies.

In addition to processes involved in floral transition and development this gene might have further roles in other processes such as plant metabolism, root development, growth and organ boundary formation as seen in the targeting of *CUC3* and *LBD39*.The combination of genetic and molecular methods has shown interesting findings for the *SEP* family in general and in particularly for the *SEP4* gene. *SEP*s do not act fully redundantly and enhance the robustness of the plant, especially under variable environmental conditions.

Fig. 5.2

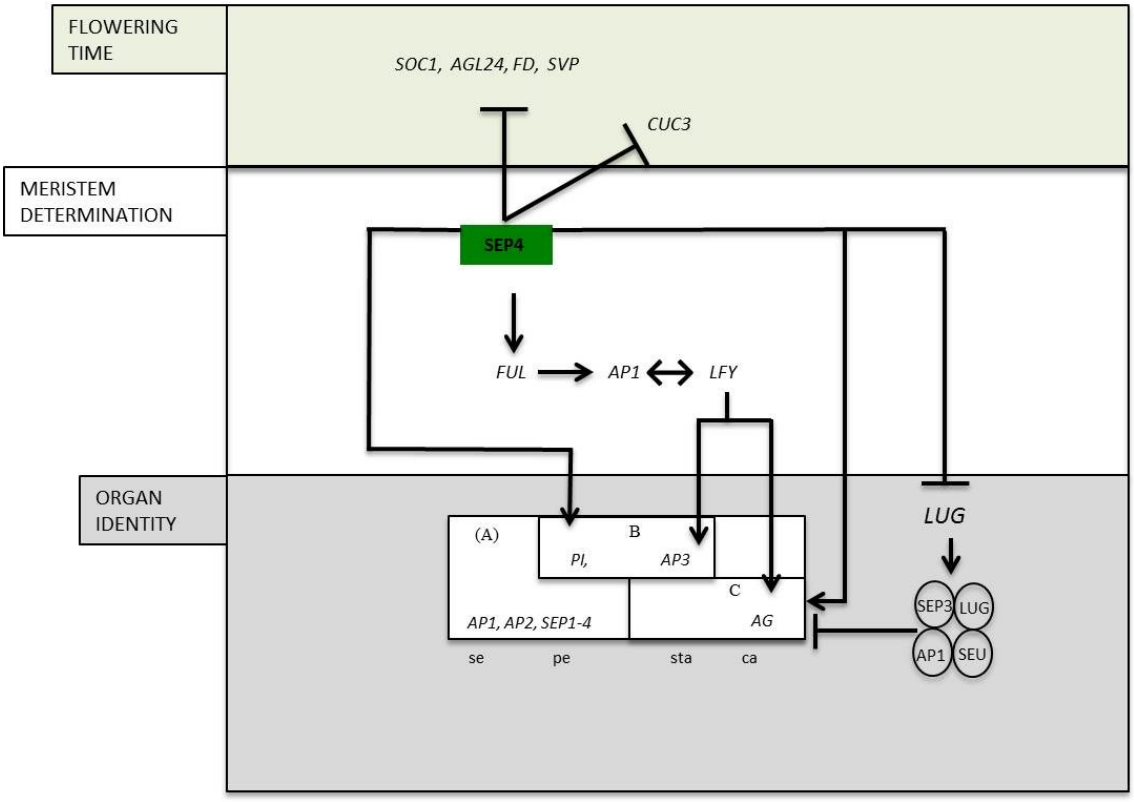


Fig.5. 2 : SEP4, a TF affecting different phase in the life cycle of the plant.

SEP4 represses flowering time genes in the vegetative phases[1)] and activates *FUL*[2)] to contribute to floral meristem establishment. *FUL* together with *AP1* and *LFY* is involved in establishing floral meristems and activating the floral organ identity genes. SEP4 activates the B and C-class genes *PI* and *AG* [3)] and represses the *AG* co-repressor *LUG* [4)]. In addition, the *CUC3* gene is repressed by SEP4 too.[5)]

AG= AGAMOUS, AGL24= AGAMOUS-LIKE24, AP1= APETAL1, AP3= APETALA3, CUC3= CUP SHAPED COTYLEDON 3, FUL= FRUITFUL, LUG= LEUNIG, SOC1= SUPPRESSOR OF CONSTANS1, SVP= SHORT VEGETATIVE PHASE, se= sepal, pe= petal, sta= stamens, ca= carpel

[1)] Shown in this work (Fig. 4.10)

[2)] Shown by Ditta G.S. et al., 2004) in crossing experiments, confirmed on a molecular level in this work (expression of *FUL* in *sep4* mutant Fig. 4.11)

[3)] Shown in this work (Fig. 4.7), [4)]) Shown in this work (Fig. 4.12), [5)] Shown in this work (Fig. 4.18).

5.4 Functional diversification between the four *SEP*s in *A. thaliana*

Repeated rounds of WGD have been reported among plants and particularly for angiosperms (Soltis P. et al., 2009). Immediately after a WGD, all genes exist as duplicates and can follow three fates, gene loss, subfunctionalization and neofunctionalization (Ohno S., 1970). Changes to the duplicated genes can be at the level of gene expression, protein function or both. However it is unlikely that retained duplicated genes act fully redundantly (Ohno S., 1970). The *SEP* genes are present in gymnosperms and occurred via a WGD event around 300 million years ago. The first WGD produced the *SEP3* and *SEP4* genes, whereas *SEP1* and *SEP2* resulted from a more recent WGD (Zahn L. et al., 2005). The four *SEP* genes are all expressed in the flower with slightly different expression patterns, *SEP 1, 2* and *4* are expressed in all four floral whorls, whereas *SEP3* expression is restricted to the inner three whorls expression (Ma H. et al 1991.; Flanagan C.A. and Ma H., 1994; Savidge B. et al., 1995; Mandel M.A. and Yanofsky M.F., 1998; Ditta G.S. et al., 2004), (Fig. 5.1). In addition, *SEP4* has a very broad expression pattern starting from the seedling stage onwards to the flower and differs strongly from the other *SEP* genes (Supplementary data S7, S8, Fig. 5.1 d). The *SEP* genes in *A. thaliana* are described as largely functionally redundant but this study disproved the reported redundancy and has shown non-redundant function for all four single *SEP* genes affecting all floral organs as described in the phenotypic characterization (section 3.2). These findings are supported with the identification of common and individual targets for SEP3 and SEP4 (section 4.6). Based on the data in this study, *SEP3* and *SEP4* acquired novel functions (petal morphology, section 3.2, Table 3.5, flowering time, section 4.14, floral organ number, section 3.2, Table 3.5), what can be interpretated as neofunctionalization. Thus, differences in the expression pattern of *SEP3* and *SEP4* affect the morphological outcome and result in a diversified function in *A. thaliana*.
In contrast, *SEP1* and *SEP2* have the same expression pattern and both affect the carpel. Also these two genes provide an example of diversification and have undergone neofunctionalization, as both genes are necessary for flower development, in particular carpel development but affect the morphology in different ways (carpel length and carpel shape). One explanation for these different morphological outcomes could lie in the protein-protein interaction, as it has been shown that SEP1 and SEP2 have different protein interaction partners (Immink R.G. et al., 2009) or in changes in cis-regulatory elements in the *SEP* genes.

This supports the finding that duplicated E-function genes have been retained in different species and the maintenance of multiple *SEP* genes in the genome after WGD caused

modifications and novel or diversified potein functions (Irish V. F. and Litt A., 2005). *Arabidopsis thaliana* is not an exception in the plant kingdom. Therefore this study provides an example that the functional redundancy as previously reported does not exist and changes in the expression pattern, different interaction partners on the protein level or different targets result in a different morphological outcome. These subtle differences enhance the fitness and robustness of the plant and contribute to their ability to respond to perturbations in their environment.Thus, the maintenance of multiple genes followed by subfunctionalization or neofunctionalization contributes to the ability of an organism to radiate and propagate under various external conditions.

Fig. 5.3

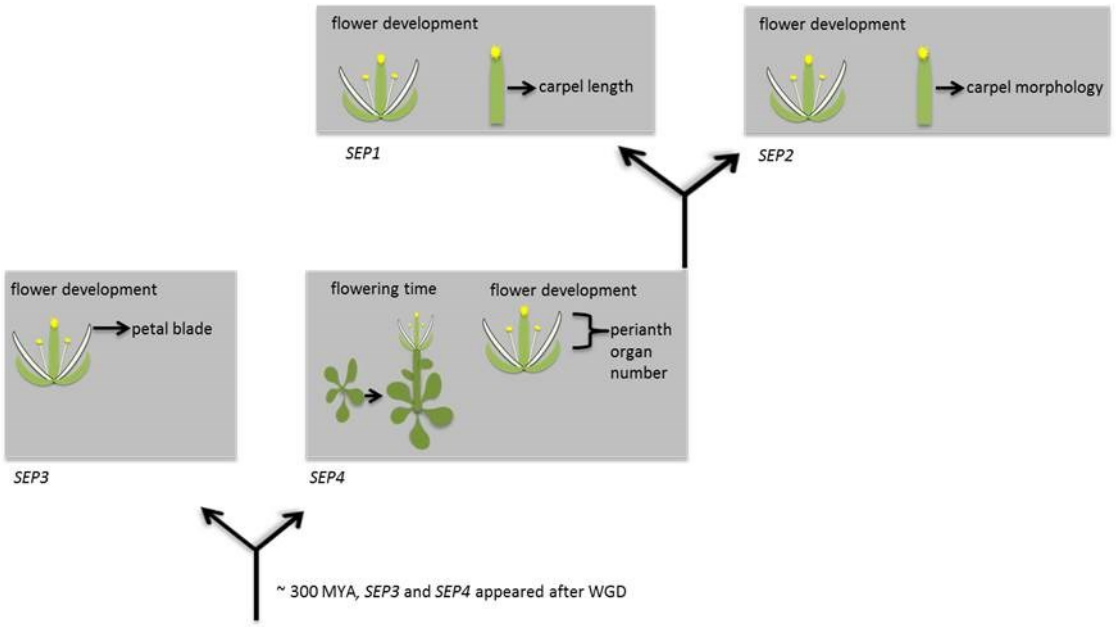


Fig. 5.3: Diversification among *SEP*s in *A. thaliana*

SEP3 and *SEP4* are a result from a WGD around 300 MYA. Both genes are necessary for flower development, but *SEP3* is involved in petal morphology, as *sep3* single mutants are affected in their petal blade, whereas *sep4* single mutants are affected in the number of perianth organs. In addition, *SEP4* is involved in flowering time, as single mutants are earlier flowering, providing an example for a novel function of the gene (neofunctionalization). *SEP1* and *SEP2* resulted from a more recent WGD. Both genes are involved in flower development and play a role particularly in carpel development. *SEP1* is involved in regulating carpel length, as single mutants reveal shorter carpels, whereas *SEP2* is involved in carpel morphology with single mutants showing bent carpels. *SEP1* and *SEP2* have undergone a diversification process as well.

5.5 Future research opportunities

5.51 Biological experiments

This work has shown that the previously described redundantly acting *A. thaliana SEP*s are not fully redundant and that the function of multiple genes enhances the fitness and ability of the plant to propagate. As shown in this study is it a matter of finding the right experimental conditions to assess the biological roles of these genes. Regarding the economic importance of flower and fruit development, it might be worth expanding this research into field studies to obtain more knowledge of how the loss of one or multiple genes affects the plant. One experimental approach could be to grow single and double mutants in the field and measure their seed and fruit production or flowering time. One limiting factor is the availability of lines that can be used outside of the greenhouse due to the presence of resistance genes against antibiotics as for example in SALK-Lines or GABI-KAT Lines used in this study. A promising alternative are Targeting Induced Local Lesions IN Genomes (TILLING) Lines, that carry induced point mutations obtained through chemical mutagenesis (Colbert T. et al., 2001). These lines could be used outside of the greenhouses according to GMO laws for future experiments. At least for *SEPALLATA*2 these lines are available (CS92213, CS94108, CS90103) and could be used for future studies. Another alternative could be to use different ecotypes and exploit the natural variation to dissect the function of individual *SEP* genes and their effect on plant robustness and development. Considering the observations that plants were more affected at higher temperatures, one idea is to compare accessions from areas from cold as for example *Lu-1* (a Swedish accession) to those from hot climates as for example *Kas-2* (an accession from India) . It would be interesting, whether different phenotypes can be seen in those accessions, suggesting that different accessions have different targets or different protein-protein interaction partners, and to unravel whether plants have developed different ways to cope with enhanced environmental conditions.

Another interesting question is whether mutants are more susceptible to plant pathogens and pests compared to WT. More recently *LFY* has been shown to be involved in plant pathogen response, suggesting a function in directing plant resources away from defence responses towards flower and fruit development to maximize reproductive fitness (Winter C.M. et al., 2011). This is an interesting finding, as *LFY* has previously only been associated with developmental processes such as meristem identity and flower development (Weigel D. et al., 1992, Parcy F. et al., 2002). This study has shown that SEP4 binds to genes involved in response to stimulus and abiotic stress (section 4.5, Tables 4.2, 4.3) and thus an interesting experiment

could be to examine the growth of a virulent bacterial strain as for example *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 on cauline and rosette leaves in *sep4* mutants and compare them to WT. This strain serves as a model for understanding plant-bacterial interactions causing economically important diseases and is able to infect *Arabidopsis* and other plants such as tomato or potato. Infections mainly occurs on aerial portions of plants, such as leaves and fruits (Xin X.F. and He S.Y., 2013). *SEP4* is earlier expressed than the other *SEP*s and shows a high expression in cauline leaves (Ma H. et al.;1995, Ditta G.S. et al., 2004), therefore it might be interesting to test whether mutants are more prone to infections and what consequences it has concerning aspects such as flowering time and mass production.

5.52 Molecular experiments

Genome wide target identification for SEP1, SEP2 and SEP4

This study provided the identification of SEP4 targets, in a *sep1sep4* background. Apart from some technical difficulties with the second replicate, making it necessary to repeat this experiment, the basis for further experiments is established. These experiments should be the identification of SEP1, SEP2 and SEP4 targets in complemented single mutant backgrounds (g*SEP1:*GFP;*sep1,* g*SEP2:*GFP;*sep2* and g*SEP4:*GFP;*sep4*). After obtaining these targets the next step should be to identify SEP1, SEP2 and SEP4 targets in different complemented backgrounds. Those experiments could include g*SEP1*:GFP;*sep1sep2,* g*SEP1:*GFP;*sep1sep3* and g*SEP1:*GFP;*sep1sep4* for the *SEP1* gene. Similar experiments can be performed for the *SEP2* gene (g*SEP2:*GFP;*sep2sep4*, g*SEP2:*GFP;*sep2sep3* and g*SEP2:*GFP;*sep1sep2)* and for the *SEP4* gene (g*SEP4*:GFP;*sep1sep4,*second replicate*,* g*SEP1*:GFP;*sep2sep4,* and g*SEP4*:GFP;*sep3sep4).* The targets of SEP3 are available (Kaufmann et al., 2009), therefore it might be interesting to do similar experiments for the *SEP3* gene in different complemented backgrounds (g*SEP3:*GFP;*sep1sep3,* g*SEP3:*GFP;*sep2sep3* and g*SEP3:*GFP;*sep3sep4)* followed by a comparison of how the targets of SEP1, SEP2, SEP3 and SEP4 change in the different backgrounds. For example how do SEP4 targets differ in a complemented g*SEP4:*GFP*;sep1sep4* or *gSEP4:*GFP*;sep2sep4* or g*SEP4*:GFP;*sep3sep4* background differ to those in a complemented g*SEP4:*GFP*;sep4* mutant background? Comparing the SEP1, SEP2, SEP3 and SEP4 targets would shed light in non-redundant and redundant regulation of genes. Targets that appeared in all experiments would suggest a redundancy, whereas targets that only appear in SEP1, SEP2, SEP3 or SEP4 experiments would be individual ones and thus function in a non-redundant way. The identification of targets would firstly give a better insight in the involvement of these genes in flower development and secondly the degree of redundancy in-between these genes at the molecular level. Finally these data could be integrated into GRNs to unravel the

complexity of these networks and obtain a better understanding how flower and fruit development is controlled.

5.6 Summary

*SEP*s do not act fully redundantly and have evolved specific functions affecting all four floral whorls of the flower, evidenced by individual phenotypes for single mutants.

Multiple *SEP*s enhance the robustness and reproduction of the plant, especially under perturbed environmental conditions as seen for all single and mutant combinations. Flowering time, floral organ development, seed as well as fruit development were affected in a variable manner ranging from mild phenotypes in single mutants to sterile triple mutants, a finding especially observed when plants were grown under stressed conditions.

SEP3 and SEP4 have many targets in common, especially those involved in flower development, suggesting a largely redundant regulation of this developmental process. However both genes have individual targets, suggesting non-redundant roles in different developmental stages.

Molecular experiments have shed more light on the complexity of GRNs. Flower development is a network of redundant and individual regulation. *SEP* genes were originally identified as redundantly acting genes necessary for flower and meristem development. This study has shown non-redundant functions for all *SEP* genes, as well as potential additional functions outside of the flower, suggested in the targeting of genes involved in other developmental processes than flowering (e.g. abiotic and biotic stress, metabolic processes).

SEP4 has broad functions besides floral organ development. The identification as a direct repressor of flowering time genes such as *AGL24, FD*, *SOC1* and *SVP* reveals its important role during the vegetative phase of flower development to prevent floral reversion. Another role of the gene is the establishment of meristem identity as shown with its direct activating function on *FUL*, supporting previous studies of Ditta (Ditta G. S. et al., 2004) ascribing *SEP4* a role in determination of meristem identity. Once the plant is in the reproductive phase, *SEP4*, together with the other *SEP*s is involved in floral organ formation.

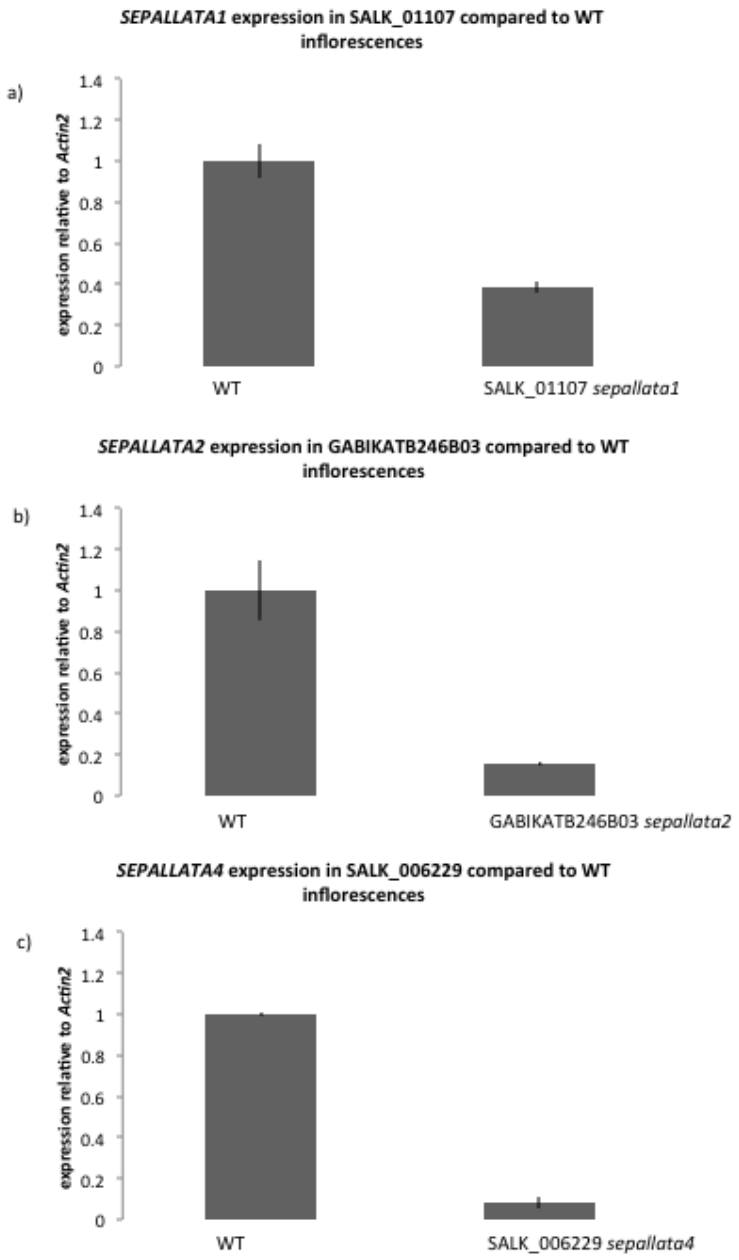
SEP4 can be seen as a multifunctional TF, firstly as a repressor of flowering time genes, secondly as a meristem identity gene that is necessary to establish the ground state of the flower and thirdly as a floral organ identity gene, involved in the specification of al floral

organs. Therefore, the gene can be considered as a “hub” in the lifecycle of the plant integrating different developmental steps by its ability to activate or repress a number of TFs, reflecting its broad expression pattern. However, *SEP4* seems to have a weaker impact on floral organ formation compared to the other *SEPS*, as it is the only one unable to make all four floral organs (sepals, petals, stamens, carpels). In the *sep1sep2sep3* triple mutant, *SEP4* provides the ground state of the flower and gives sepal identity. Whether this is caused by its diverse roles during other, earlier developmental stages (e.g. the expression of the *SEP4* gene could be weaker than the other *SEPs* in floral organ primordia), or by its inability to form complexes with flower specific proteins might be an interesting topic for the future to investigate.

Differences in expression pattern together with the described phenotypes in this study (extra perianth organs, early flowering phenotype in *sep4* single mutants, meristem identity function) and the identification of individual SEP4 targets compared to SEP3, such as *LBD39, LUG, FLC*, provide genetic and molecular evidence that *SEP* genes do not act fully redundantly and have diversified during their evolution.

Supplementary data

S1: Expression analysis in the newly established alleles



S1: **qPCR experiments performed on *SEP* alleles established in this work**.

a) qPCR to test expression of *sep1-2* (SALK_01107C) mutants compared to WT, b) qPCR to test expression of *sep2-2* (GABI KATB246B03) mutants compared to WT, c) qPCR to test expression of *sep4-2* (SALK_006229) mutants compared to WT. All tested lines were significantly down regulated compared to WT with p-values < 0.05, Bars = SEM

S2: ChIP-SEQ targets of SEPALLATA4 from rep.1

Gene Locus	Gene Locus	Gene Locus	Gene Locus	Gene Locus
AT3G60700	AT3G46960	AT4G00550	AT1G49340;	AT4G24530
AT5G18610	AT2G47850	AT5G65880	AT1G51040	AT4G37150
AT5G52960	AT4G02480	AT2G36145	AT2G41620	AT1G02790
AT3G17950	AT4G03530;	AT2G15420	AT2G25430	AT1G10090
AT1G78090	AT1G70010	AT1G49780	AT1G28500	AT3G14710
AT1G78170	AT2G46590	AT5G62000	AT1G27860	AT5G03050
AT1G25570	AT2G12900	AT5G46830	AT3G13250	AT1G44575
AT1G72300	AT5G16750	AT5G28350;	AT4G07800	AT4G14900
AT3G20830	AT4G24450	AT3G61480	AT5G67480	AT1G09575
AT2G42700	AT3G27580	AT2G34470	AT1G32000	AT4G26130
AT5G39390	AT4G37130	AT2G34880	AT1G10430	AT1G31290
AT5G34940	AT1G06470	AT4G32420	AT1G30825	AT3G04520
AT1G09620	AT2G45660	AT5G61520	AT4G19240	AT3G20300
AT2G37740	AT5G10930	AT1G25580	AT3G58070	AT2G14470
AT4G32551	AT2G21640	AT1G52380	AT1G02300	AT5G34960
AT5G15780	AT1G79280	AT3G50900	AT1G09570	AT1G35940
AT5G66560	AT2G42180	AT1G52360	AT3G25910	AT2G14610
AT1G10830	AT4G15260	AT2G33670	AT5G24430	AT4G37410
AT5G56520	AT1G17880	AT3G03680	AT1G13960	AT4G38680
AT4G33810	AT5G07310	AT1G02660	AT3G13730	AT3G26350
AT5G15020	AT4G18390	AT3G22760	AT5G11310	AT2G38920
AT2G01620	AT5G66100	AT1G09230	AT5G66550	AT2G05640
AT2G31930	AT5G44500	AT2G27800;	AT5G60460	AT2G16340
AT1G53830	AT1G16980	AT5G27300	AT3G16920	AT3G60120
AT3G22231	AT3G50560	AT5G03150	AT1G06360	AT4G29370
AT1G13080	AT1G09010	AT4G37550	AT1G06350	AT3G52130
AT1G78995	AT3G63460	AT3G11910	AT3G52700	AT5G51290
AT4G13450	AT4G38740	AT3G20330	AT5G58375	AT2G33470
AT1G74820	AT4G23100	AT4G14530	AT1G50020	AT1G53090
AT2G24330	AT4G19710	AT1G33240	AT1G68875	AT5G57150
AT5G19270	AT5G53160	AT5G47980	AT3G17340	AT4G13480
AT4G05050	AT1G21980	AT3G42660	AT2G02390	AT1G29070
AT2G01310	AT5G08420	AT5G13770	AT2G46560	AT5G10100
AT4G24440	AT3G60720	AT5G40370	AT5G53410	AT5G37790
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AT1G47810	AT2G42490	AT1G12520	AT2G04050	AT2G36450
AT1G75490	AT3G50630	AT1G05900	AT3G18880	AT1G10090
AT3G19570	AT1G60830;	AT3G05620	AT1G63850	AT2G02380
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AT1G28010	AT5G58530	AT5G22270	AT3G50910	AT1G72140
AT2G07290	AT5G44790	AT5G61880	AT4G16790	AT5G13760
AT3G07050	AT2G10970	AT2G25440	AT1G67740	AT4G31900
AT4G34890;	AT5G03590	AT3G57120	AT5G40380	AT3G01860
AT4G34900	AT5G24280	AT3G04510	AT5G49240	AT2G35960
AT1G13260	AT2G03710	AT1G14730	AT3G42930	AT5G45940
AT2G19590	AT3G03910	AT5G06520	AT5G30545	AT3G18450
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AT2G28755	AT2G36080	AT3G02140	AT1G21240	AT1G30400
AT5G59210	AT3G27650	AT5G27470	AT1G51340	AT4G24630
AT5G50720	AT2G04210	AT2G36960	AT3G18890	AT2G02560
AT4G32890	AT1G40105	AT3G50810	AT3G63470	AT3G17330
AT2G36760	AT5G65210	AT4G01910	AT1G68040	AT4G18960
AT3G13480	AT1G32240	AT2G41510	AT3G13740	AT4G38690
AT3G05650	AT3G27410	AT5G65870	AT5G14910	AT5G27330
AT2G04160	AT5G41970	AT5G19050	AT1G12070	AT5G26910
AT4G38200	AT5G03900	AT3G55550	AT1G23340	AT2G23770
AT1G11130	AT3G63310	AT4G11510	AT1G80760	AT3G18090
AT2G39320	AT5G05260	AT4G36380	AT1G36190	AT3G23780
AT5G20690	AT1G07050	AT1G04250	AT3G32230	AT1G76420
AT5G07200	AT1G18400	AT5G58370	AT5G54310	AT2G36970
AT1G53030	AT3G06483	AT2G38820	AT1G50030	AT4G08620
AT1G36640	AT1G69120	AT1G69360	AT4G28703	AT2G24590
AT4G25870	AT4G08780	AT2G45690	AT4G31200	AT4G32375
AT1G14530	AT3G05610	AT2G23660	AT3G26360	AT4G18395
AT1G23380	AT3G25905	AT4G19250	AT5G05560	AT3G55580

AT5G49230	AT3G61850	AT4G19330	AT5G10970	AT3G25630
AT5G44010	AT5G48880	AT1G10870	AT2G31300	AT3G53720
AT5G02710	AT1G70782	AT5G60470	AT2G30910	AT1G45130
AT3G47770	AT1G70780	AT1G31300	AT2G37630	AT4G26650
AT2G23830	AT2G43140	AT3G50890	AT2G13560	AT3G22110
AT5G05450	AT5G46370	AT4G39960	AT1G31120	AT2G47580
AT2G44490	AT1G59930	AT3G58740	AT1G13090	AT5G37310
AT4G04970	AT1G13970	AT3G22121	AT3G44020	AT2G47500
AT4G14650	AT4G16210	AT4G01920	AT3G61100	AT1G79000
AT1G75460	AT1G14770	AT2G44470	AT3G43660	AT1G15130
AT3G06490	AT5G20710	AT2G24390	AT1G76670	AT2G19380
AT1G70040	AT1G32330	AT1G11130	AT2G38910	AT3G44030
AT2G03830	AT3G09280	AT5G14900	AT2G28550	AT2G37460
AT5G27870	AT1G29670	AT4G32980	AT1G51410	AT1G75520
AT5G66080	AT5G15280	AT4G05370	AT5G19280	AT3G50480
AT3G26260	AT4G32430	AT2G23130	AT4G29360	AT5G28970
AT2G14010	AT3G51130	AT2G40810	AT3G23605	AT5G36030
AT1G73300	AT4G25910	AT4G36920	AT4G26730	AT4G19310
AT5G36180	AT1G10522	AT1G72510	AT2G16030	AT2G11480
AT5G17800	AT5G02700	AT2G09970	AT1G05380	AT1G35110
AT3G15030	AT3G28410	AT2G41630	AT5G61510	AT1G05300
AT2G34480	AT5G08240	AT5G44800	AT5G05820	AT3G18870
AT3G46840	AT1G09220	AT3G50620	AT5G42620	AT4G30290
AT3G46850	AT2G16040	AT5G20620	AT3G59350	AT2G47040
AT4G33860	AT2G21185	AT4G37590	AT1G80770	AT2G47030
AT4G33850	AT2G27500	AT1G53080	AT2G23810	AT3G25800
AT1G14120	AT5G52970	AT1G80340	AT2G16020	AT2G11235
AT2G29330	AT3G11900	AT5G62030	AT1G02780	AT1G49500
AT2G40910	AT4G20770	AT4G22240	AT1G80530	AT1G31030
AT3G10140	AT4G14460	AT4G04020	AT3G03890	AT5G18360
AT5G27460	AT2G35950	AT1G18910	AT4G12420	AT1G27880
AT4G22130	AT3G14000	AT5G58900	AT5G59780	AT2G23800
AT1G12500	AT1G60320	AT5G18350	AT2G27460	AT2G11140
AT5G49360	AT2G36560	AT1G68440	AT1G64200	AT1G75500
AT1G65340	AT4G21370	AT2G14100	AT3G44010	AT2G14020
AT1G07380	AT1G26730	AT4G04930	AT3G43980	AT2G27810
AT1G07090	AT5G43900	AT1G61820	AT2G21070	AT3G20630
AT2G21790	AT3G55970	AT4G21160	AT4G35930	AT1G22400
AT4G35240	AT4G09830	AT4G24240	AT4G11140	AT2G44200
AT4G14540	AT5G67490	AT4G13310	AT4G01960	AT5G67500
AT2G23890	AT3G52470	AT4G13290	AT5G51830	AT5G16770
AT4G17410	AT1G18390	AT3G14700	AT3G09920	AT3G20340
AT2G02515	AT2G07280	AT5G46070	AT3G26380	AT4G25880
AT3G53920	AT3G16150	AT3G29792	AT1G76660	AT1G16970
AT1G20440	AT1G64210	AT1G17330	AT2G37750	AT4G11150
AT3G22050	AT5G37260	AT3G62630	AT4G25470	AT5G01380
AT5G20630	AT5G23010	AT5G44230	AT4G33580	AT2G46600
AT3G08680	AT4G33150	AT1G60850	AT4G22250	AT3G18460
AT1G72700	AT4G17070	AT4G19410	AT3G55370	AT1G15660
AT1G15640	AT5G01150	AT5G63370	AT3G04280	AT4G28360
AT2G23640	AT1G80410	AT1G14760	AT5G05460	AT1G52370
AT1G51880	AT1G64620	AT1G11080	AT5G01820	AT5G65800
AT5G11970	AT3G46830	AT3G61530	AT2G04310	AT3G13460

AT5G50810	AT5G54330	AT4G35900	AT2G23500	AT3G03340
AT5G47040	AT5G55870	AT3G61190	AT3G29695	AT4G36260
AT1G80330	AT3G51120	AT1G18720	AT2G12083	AT5G60930
AT2G30480	AT4G02320	AT2G38250	AT2G32900	AT1G04070
AT3G28930	AT1G76990	AT3G47760	AT1G14720	AT2G26000
AT2G19200	AT4G29780	AT3G47750	AT3G23590	AT5G42900
AT2G12290	AT3G14860	AT1G33800	AT4G13190	AT3G25640
AT4G19700	AT4G18030	AT4G22890	AT5G51820	AT4G38940
AT3G14890	AT1G20620	AT1G35610	AT4G03290	AT1G63710
AT3G57390	AT5G16290	AT3G53270	AT2G47860	AT2G18490
AT1G56100	AT2G04620	AT5G65310	AT1G26240	AT4G38620
AT5G51400	AT3G61540	AT5G58470	AT2G24580	AT2G41090
AT2G33860	AT4G11950	AT1G29660	AT4G24540	AT2G40110
AT4G03520	AT1G12080	AT3G06480	AT3G27420	AT3G54940
AT3G59990	AT2G30130	AT5G43700	AT3G59340	AT5G20700
AT1G28110	AT2G40100	AT4G30030	AT5G03780	AT2G22830
AT1G56380	AT2G28760	AT4G33865	AT4G03820	AT1G36270
AT2G42500	AT3G14010	AT3G01040	AT4G38190	AT1G42510
AT3G58500	AT4G02930	AT4G38750	AT4G17990	AT3G30790
AT2G42830	AT1G78120	AT1G50280	AT4G24730	AT2G13000
AT2G21320	AT1G32010	AT1G61810	AT1G73310	AT5G62880
AT2G03360	AT5G38190	AT4G25490	AT1G32060	AT5G54830
AT4G00160	AT2G41790	AT2G27380	AT2G34790	AT4G22870;
AT4G26640	AT4G11530	AT1G48000	AT5G51410	AT4G22880
AT4G00740	AT4G05470	AT3G23260	AT3G58080	AT2G33490
AT5G11510	AT3G59690	AT2G21310	AT3G11810	AT2G36170
AT1G17890	AT3G03690	AT1G63720	AT2G03330	AT1G45090
AT3G52720	AT5G62000	AT2G44210	AT3G19290	AT2G12100
AT2G16595	AT1G35614	AT2G22300	AT1G47780	AT1G47790
AT2G19580	AT4G35905	AT5G50740	AT1G47786	AT1G67340
AT5G06510	AT2G01290	AT4G16370	AT4G01530	AT5G45950
AT5G08010	AT5G01160	AT5G24660	AT1G12630	AT3G08750
AT1G49490	AT4G13420	AT3G08040	AT2G02520	AT1G15530
AT4G27460	AT2G16090	AT4G19450	AT1G06070	AT3G32400
AT2G32310	AT4G26260	AT5G24870	AT2G36570	AT5G60670
AT2G33480	AT4G16730	AT1G63870	AT1G56360	AT1G26620
AT3G07040	AT1G56140	AT4G25500	AT5G54370	AT2G05320
AT5G66090	AT1G56130	AT1G43040	AT2G30490	AT5G48050
AT2G37920	AT1G56120	AT5G03160	AT1G31270	AT4G05510
AT5G35180	AT5G08430	AT5G03570	AT4G30110	AT5G03560
AT1G44910	AT2G45700	AT4G15280	AT5G64390	AT5G51750
AT5G41410	AT1G66220	AT1G02305	AT1G09070	AT3G59920
AT5G65100	AT5G59090	AT1G36920	AT5G06530	AT2G47570
AT4G37790	AT3G23600	AT2G41100	AT1G17310	AT1G64405
AT4G39410	AT5G39380	AT3G08490	AT4G20270	AT5G23880
AT3G11330	AT5G14400	AT5G48030	AT1G43755	AT5G52950
AT3G07640	AT1G54850	AT5G62040	AT1G43745	AT1G29280
AT3G11700	AT4G30280	AT5G23020	AT3G16940	AT2G21180
AT5G35470	AT1G44510	AT3G26290	AT5G14710	AT4G36910
AT5G21130	AT2G37690	AT3G06530	AT5G13790	AT5G13790
AT1G01480	AT2G05140	AT2G40160	AT1G33817	AT1G33817
AT2G32270	AT4G24550	AT3G44050	AT4G34770	AT4G34770
AT4G00560	AT5G50820	AT2G44820	AT4G38930	AT4G38930

S2: Target list from SEPALLATA4 ChIP-SEQ experiment

All identified targets from the SEPALLATA 4 ChIP-SEQ experiment (g*SEP4*:GFP;*sep1sep4*), rep1 are shown with their gene Locus, FDR > 0.01

S3: 309 common ChIP-SEQ targets between SEPALLATA3 and SEPALLATA4

Gene Locus	Gene Locus	Gene Locus	Gene Locus
AT1G30825	AT1G75450	AT1G75460	AT1G21980
AT5G52960	AT3G14000	AT3G06490	AT2G38060
AT4G35920	AT4G37740	AT1G70040	AT2G45690
AT3G17950	AT2G46600	AT2G40110	AT5G03560
AT1G78170	AT2G37210	AT3G54940	AT1G56590
AT3G04500	AT1G14890	AT5G20700	AT2G36740
AT3G58070	AT5G65800	AT4G25470	AT3G23130
AT1G09570	AT2G47190	AT4G33580	AT5G52950
AT2G41640	AT3G52470	AT5G62880	AT5G58530
AT3G25910	AT4G25450	AT3G15030	AT4G34540
AT3G18080	AT3G16150	AT3G55370	AT2G03710
AT2G42700	AT2G31570	AT2G34480	AT4G04960
AT2G40150	AT1G64620	AT3G46840	AT4G24540
AT2G44500	AT3G25640	AT3G46850	AT2G42690
AT5G11310	AT3G57390	AT5G01820	AT3G27420
AT1G08930	AT1G33270	AT4G15440	AT4G32980
AT5G66550	AT4G29780	AT2G29330	AT4G03820
AT3G16920	AT3G16140	AT2G40910	AT4G38190
AT5G66560	AT1G80420	AT2G04310	AT5G13800
AT2G27690	AT3G14860	AT3G13380	AT2G40810
AT3G52700	AT4G18030	AT1G14720	AT4G36920
AT4G18700	AT2G33860	AT1G18410	AT5G14230
AT1G50020	AT4G37310	AT5G49360	AT1G24280
AT3G17340	AT4G03520	AT1G15530	AT3G55560
AT1G68875	AT5G03790	AT3G53270	AT2G41630
AT3G57880	AT3G26280	AT4G37150	AT1G77280
AT1G30400	AT2G42830	AT1G29660	AT1G28140
AT3G19280	AT4G00740	AT1G07090	AT5G20620
AT1G53830	AT5G62000	AT2G34710	AT1G78480
AT2G36080	AT1G68840	AT2G21790	AT1G53080
AT3G17330	AT3G17930	AT3G49790	AT4G37590
AT1G13080	AT2G19580	AT5G43700	AT5G62030
AT1G78995	AT2G34470	AT4G00540	AT1G72310
AT4G18960	AT5G06510	AT1G44575	AT4G11280
AT1G78990	AT3G14880	AT4G14900	AT2G01990
AT1G76420	AT1G12070	AT2G28305	AT5G58900
AT2G29320	AT4G27460	AT1G09575	AT2G21060
AT3G27410	AT1G49490	AT1G20440	AT3G58080

AT1G75490	AT5G13780	AT4G26130	AT1G33280
AT3G55580	AT3G58620	AT5G20630	AT3G19290
AT1G07050	AT3G17970	AT1G72700	AT5G64770
AT3G25630	AT4G28703	AT3G60120	AT4G01530
AT1G69120	AT1G50030	AT1G50280	AT1G12630
AT1G45130	AT1G56600	AT5G11970	AT4G24240
AT1G13260	AT5G03150	AT4G25490	AT2G21380
AT2G19590	AT3G11910	AT3G02180	AT3G62630
AT3G25905	AT5G10970	AT2G30480	AT2G25900
AT1G47610	AT4G37790	AT2G21310	AT1G09070
AT1G79000	AT3G11330	AT2G12290	AT1G14580
AT2G38810	AT1G33240	AT1G53090	AT2G36750
AT5G48880	AT5G25210	AT5G57150	AT5G06530
AT1G70782	AT3G11700	AT2G16860	AT3G17940
AT1G73840	AT5G64760	AT5G10100	AT5G14710
AT4G27450	AT4G37240	AT2G46590	AT5G49740
AT1G08920	AT1G76670	AT4G16370	AT2G21180
AT4G37070	AT1G20620	AT1G60060	AT5G63370
AT3G45680	AT2G28550	AT4G19450	AT4G36910
AT1G14770	AT3G61540	AT2G37760	AT1G78130
AT5G20710	AT2G36760	AT4G37130	AT3G17970
AT2G45160	AT1G12080	AT2G45660	AT1G01480
AT5G60680	AT2G40100	AT4G25500	AT1G14760
AT2G29090	AT2G25440	AT5G10930	AT4G35900
AT1G49500	AT3G04510	AT4G15260	AT3G61530
AT4G39400	AT5G42620	AT5G07310	AT1G18720
AT1G07040	AT5G05820	AT5G23020	AT2G41080
AT1G31030	AT4G38200	AT3G02140	AT4G24550
AT1G78490	AT1G14730	AT1G58340	AT1G09060
AT1G75500	AT5G07200	AT3G26290	AT2G40160
AT2G27540	AT4G12420	AT3G50560	AT2G41620
AT1G13270	AT1G75750	AT3G55550	AT1G15520
AT1G22400	AT5G62000	AT3G63460	AT2G25430
AT2G21185	AT4G35905	AT4G36380	AT2G44820
AT4G29190	AT1G23380	AT1G04250	AT5G13790
AT5G52970	AT3G53280	AT5G60670	AT4G19440
AT5G57140	AT5G59780	AT1G18930	AT4G34770
AT4G14460	AT5G14700	AT4G19710	AT3G23130
AT2G35950	AT2G21070	AT5G53160	AT5G52950
AT1G78440	AT5G53170	AT2G38820	
AT1G21980	AT2G45690	AT1G56590	
AT2G38060	AT5G03560	AT2G36740	

S3: Gene list of common targets between SEP3 and SEP4 ChIP-SEQ experiments

309 identified common target genes between SEP3 ChIP-SEQ experiments and SEP4 ChIP-SEQ experiment, SEP3 data are available from (Kaufmann K. et al., 2009, FDR > 0.01

S4: GO of SEPALLATA3 targets described in section 4.5

function	GO	description	p-value
biological	0065007	biological regulation	7.9e-39
	0050896	response to stimulus	4.1e-36
	0009791	post-embryonic development	2.7e-32
	0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4.1e-32
	0032501	multicellular organismal process	2.3e-20
	0006464	protein modification process	6.5e-20
	0009725	response to hormone stimulus	1.9e-18
	0032502	developmental process	2e-18
	0048856	anatomical structure development	5.5e-17
	0006629	lipid metabolic process	3.1e-16
	0007242	intracellular signaling cascade	8.6e-12
	0006950	response to stress	7.4e-112
	0048513	organ development	8.4e-11
	0003006	reproductive developmental proces	1.5e-10
	0051179	localization	2.5e-07
	0009733	response to auxin stimulus	2.4e-06
	0009908	flower development	2.6e-06
	0048367	shoot development	3e-06
	0009753	response to jasmonic acid stimulus	8.8e-06

S4: GO of SEPALLATA3 targets described in section 4.5

function	GO	description	p-value
Biological	0009739	response to gibberellin stimulus	1e-05
	0048507	meristem development	0.0002
	0048366	leaf development	0.00032

S4: GO of overrepresented SEPALLATA 3 targets described in section 4.5

(Kaufmann K. et al., 2009) using the agriGO analysis tool (Du Z. et al., 2010)

S5: Up-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep4-1*	Up-reg. in *sep4-1*	Commonly up-reg. in *sep3-2* and *sep4-1*
AT1G74090	AT1G73050	AT4G09650	AT5G18610	AT5G53160	AT3G09050
AT1G23760	AT1G16510	AT3G21690	AT5G52960	AT3G13080;	AT3G58990
AT5G52960	AT5G07550	AT5G60500	AT4G19240	AT1G71330	AT5G52960
AT5G58170	AT1G12090	AT5G60510	AT1G02490	AT5G17100	AT4G25080
AT1G23440	AT1G50310	AT1G72430	ATCG00360	AT5G17110	AT5G07580
AT2G38530	AT1G50320	AT3G26125	AT3G03630	AT1G62560	AT1G77760
AT1G19660	AT2G25810	AT1G21270	AT1G17970	AT2G48050	AT2G29290
AT1G75380	AT5G46110	AT5G52060	AT3G03580	AT2G42110	AT2G25810
AT1G20850	AT3G08560	AT4G36490	AT1G69200	AT5G65080	ATCG00570
AT5G16400	AT2G23900	AT3G52160	AT5G12930	AT1G66040	AT3G10520
AT1G13140	AT4G27435	AT5G02840	AT3G57430	AT1G66050	AT1G28610
AT3G45010	AT4G34470	AT1G75900	AT1G05550	AT5G39550	AT2G01590
AT2G32160	AT4G34210	AT4G22710	AT5G08280	AT4G12030	ATCG00580
AT5G51110	AT1G64900	AT4G22690	AT4G11175	ATCG00380	AT5G57785
AT3G22910	ATCG00280	AT1G11850	AT2G37420	AT2G25310	ATCG00280
AT4G04830	AT4G27450	AT1G51680	AT2G22840	AT5G19120	AT4G27450
AT1G10370	AT5G04180	AT4G29250	AT2G11810	AT3G59040	AT1G68190
AT5G14980	AT5G44030	ATCG00270	AT5G45480	AT3G05130	ATCG00840
AT2G40435	AT3G56010	AT1G12900	AT1G60600	AT5G27440	ATCG01300
AT3G16920	AT1G33811	AT1G24030	AT2G15960	AT5G58120	AT4G13770
AT1G64740	AT2G46210	AT3G21180	AT3G22231	AT3G27690	AT3G15310
AT1G02813	AT3G61630	AT3G46520	AT2G30820	AT2G30140	AT3G14395
AT1G23690	AT1G24400	AT2G30520	AT1G02280	AT1G26530	AT1G62560
AT1G02205	AT5G19140	AT4G19170	AT2G35690	AT3G49750	ATCG00430
AT5G59845	AT5G49270	AT5G59370	AT5G09440	AT1G67830	AT4G12030
AT4G33790	AT2G47050	AT2G05380	AT1G78580	AT5G51600	AT5G14740
AT1G68875	AT5G15950	AT2G42990	AT5G40380	AT5G18670	AT5G06530
AT4G01026	AT5G15948	AT3G14415	AT4G30720	AT4G12390	AT4G23600
AT5G45880	AT1G02050	AT3G14420	AT3G55470	AT3G57520	AT2G36870
AT2G30520	AT3G62230	AT2G46370	AT2G26550	AT5G16180	ATCG00630
AT4G29670	AT2G41180	AT1G67990	AT5G15360	AT2G31400	ATCG00420
AT1G13080	AT5G03040	AT5G61340	AT3G18420	AT1G55370	AT4G25170
AT5G54270	AT1G30350	AT5G02790	AT1G23790	AT5G11930	AT5G46230
AT4G30530	AT3G15280	AT1G20150	AT2G40400	AT3G15140	AT3G62820
AT1G10770	AT1G26820	AT1G23040	AT3G56330	AT1G62045	AT3G43670
AT4G21960	AT1G32540	AT4G18395	AT5G65410	AT4G22280	AT2G42220
AT1G28600	AT2G01520	AT1G74670	AT3G15650	AT3G63510	AT3G03190
AT1G65970	AT3G20530	AT1G75910	AT3G56890	AT3G56650	ATCG00560
AT1G60740	AT2G30550	AT1G69120	AT4G07400	AT5G18840	AT3G59400
AT1G03870	AT3G28960	AT4G28160	AT3G58650	AT2G35030	AT4G22490
AT2G37240	AT5G2735	AT4G17340	AT1G13260	AT5G01910	AT2G45180

S5: Up-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep4-1*	Up-reg. in *sep4-1*	Commonly up-reg. in *sep3-2* and *sep4-1*
AT3G62020	AT2G02990	AT5G39400	AT2G24310	AT5G57785	AT1G70830
AT4G34230	AT1G21065	AT2G26410	AT3G44430	AT4G05520	AT1G68520
AT2G45600	AT1G55570	AT3G60780	AT2G18700	AT3G15310	AT1G54040
AT2G20870	AT3G52600	AT1G22400	AT1G07660	AT4G32340	AT4G17560
AT4G25100	AT2G45670	AT2G22240	AT1G07820	AT2G35820	AT2G30520
AT3G56060	AT2G18280	AT1G75050	AT4G02390	AT3G11520	AT4G25100
AT1G54040	AT2G28800	AT1G66850	AT1G43800	AT1G14580	AT3G27690
AT4G12920	AT2G23840	AT5G15110	AT4G25990	AT5G66960	ATCG00530
ATCG00530	AT2G39710	AT3G11930	AT2G38160	AT3G02450	AT1G52000
AT1G47980	AT5G49360	AT1G02930	AT5G62165	AT1G37130	AT2G05520
AT3G25050	AT4G10955	AT1G02920	AT1G30820	AT1G09415	AT2G01520
ATCG00350	AT4G10960	AT2G25510	AT1G70200	AT1G20370	AT1G32540
AT4G33666	AT3G27210	AT1G66200	AT5G03350	AT3G43670	AT3G51600
AT2G04160	AT1G21140	AT3G20270	AT5G43910	AT3G54090	AT5G48490
AT2G42940	AT4G29340	AT4G19430	AT1G64470	AT5G36910	AT5G57660
AT1G63060	AT5G61430	AT5G07560	AT5G14740	AT1G18330	AT3G46780
AT5G03170	AT5G49070	AT2G45800	AT4G37090	AT4G29060	AT1G02930
AT3G46780	AT3G51600	AT2G02720	AT3G59940	AT4G17560	AT1G02920
AT5G63180	AT3G61890	AT1G21250	AT5G46230	AT1G08065	AT2G25510
AT3G18360	AT3G14210	AT4G00360	AT3G18390	AT1G48180	AT3G01550
AT3G26710	AT3G54130	AT2G07560	AT1G10990	AT5G64380	AT4G16510
AT4G20130	AT1G02790	AT3G03470	AT2G38780	AT5G35740	AT2G22990
AT3G53980	AT4G23680	AT4G05180	ATCG00020	AT1G10540	AT1G78370
AT5G20790	AT3G25165	AT1G75790	AT5G49250	AT5G57660	
AT1G77760	AT3G25810	AT4G13560	AT1G52700	ATCG00520	
AT4G02140	AT4G30500	AT3G52470	AT5G59670	AT5G67270	
AT4G27420	AT2G07040	AT3G55100	AT4G25100	AT4G21270	
AT5G44130	AT4G37900	AT2G28355	AT3G49270	ATCG00650	
AT1G22760	AT1G20120	AT1G28430	AT3G22670	AT1G72670	
AT1G75460	AT3G20300	AT4G36040	AT4G03510	AT1G17220	
ATCG00580	AT3G58990	ATCG00840	AT1G09750	AT5G01050	
AT3G10340	AT5G39880	ATCG01300	AT1G11290	AT5G01040	
AT2G03830	AT1G73220	ATCG00430	AT5G52540	AT1G07610	
AT5G50030	AT1G03390	AT5G23010	AT3G17010	AT1G02450	
AT3G26450	AT4G20420	AT5G53490	AT2G30600	AT1G48840	
AT4G01470	AT3G01700	AT2G02450	AT3G46610	AT1G12250	
AT2G37750	AT4G14440	AT1G07720	AT5G14350	AT3G18080	
AT2G27740	AT2G04460	AT4G34510	AT5G07580	AT3G48100	
AT4G11760	AT2G32890	AT1G35290	AT2G31840	AT5G24240	

S5: Up-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep4-1*	Up-reg. in *sep4-1*	Up-reg. in *sep4-1*
AT5G15310	AT1G69850	AT5G08540	AT1G50240	AT1G68640	AT1G71990
AT4G16490	AT1G28610	AT3G10460	AT5G61850	AT3G48730	AT4G02850
AT1G52880	AT3G28830	AT3G56290	AT5G46790	AT5G58575	AT1G75190
AT5G66400	AT1G55560	AT3G27810	AT5G24490	AT5G67420	AT5G09950
AT3G57690	AT3G45210	AT3G21240	AT4G11330	AT3G09210	AT5G62710
AT3G52820	AT2G46860	AT4G38770	AT4G21200	AT3G12930	AT3G51600
AT5G07540	AT1G23250	AT3G51000	AT3G23670	AT4G31850	AT5G08610
AT1G06260	AT1G58120	AT1G29230	AT4G24110	AT3G19184	AT1G12730
AT3G44300	AT1G58122	AT1G79780	AT4G37240	AT2G30520	AT3G62150
AT3G44310	AT2G20740	AT1G23730	AT1G80050	AT3G26520	AT3G58990
AT3G20865	AT5G22430	AT1G52000	AT5G54510	AT1G62200	AT5G46160
AT3G14395	AT3G28790	AT1G52690	AT4G10420	AT3G55940	AT1G50290
AT5G55970	AT2G16960	AT5G43150	AT1G54040	AT5G65650	AT4G16690
AT3G19930	AT4G20050	AT2G39980	AT4G27710	AT1G76420	AT3G54720
AT4G24380	AT3G10520	AT1G02470	AT2G38380	AT2G41720	AT1G28610
AT1G76470	AT1G24520	AT5G58310	AT2G38390	AT3G60980	AT1G70290
AT1G23080	AT5G28470	AT5G16960	AT3G06730	AT3G57860	AT5G49560
AT3G43120	AT1G11190	AT2G43910	ATCG00530	AT1G67800	AT3G49150
AT1G44170	AT3G05960	AT2G43920	AT2G44920	AT5G23910	AT3G14395
AT5G37940	AT2G25080	AT3G02380	AT1G57680	AT3G62110	AT3G43190
AT5G38000	AT3G56230	AT4G34131	AT3G57920	AT1G74890	AT2G47910
AT1G65445	AT1G18990	AT4G34135	AT3G48500	AT1G01140	AT1G29070
AT1G23350	AT2G03850	AT5G07520	AT1G34355	AT1G68190	AT4G34730
AT5G07530	AT4G25590	AT3G28220	AT3G46780	AT5G20250	AT5G64060
AT1G68520	AT1G62560	AT3G01550	AT2G29180	AT1G01670	AT1G68520
AT1G44446	AT5G64040	AT1G55265	AT3G14330	AT1G12790	AT1G59840
AT3G28810	AT1G60590	AT3G06260	AT1G21500	AT3G20440	AT2G35500
AT3G28820	AT1G44224	AT4G30550	AT5G17160	AT1G14770	AT2G29760
AT3G62170	AT4G25780	AT5G06510	AT5G44010	AT3G15560	AT2G45660
AT5G60020	AT4G12030	AT4G00350	AT1G61370	AT5G27200	AT1G78260
AT1G69530	AT4G36640	AT4G25080	AT1G77760	AT2G36870	AT4G11080
AT2G32090	AT1G74550	AT1G23670	AT5G19750	AT2G37470	AT3G14900
AT2G25760	AT3G57040	AT2G15050	AT5G07360	AT5G60100	ATCG00640
AT1G60010	AT1G56680	AT2G40670	ATCG00580	AT2G25625	AT5G48490
AT3G13390	AT1G11920	AT1G19150	AT5G60610	AT5G55340	AT2G13290
AT5G61605	AT1G26480	AT5G12940	AT5G53550	AT2G45180	AT3G25660
AT3G20450	AT5G16490	AT1G71480	AT1G50460	AT1G10522	AT1G59540
AT4G16980	AT3G11480	AT4G01940	AT5G32460	AT1G70830	AT2G36000
AT5G24690	AT1G24620	AT2G01590	AT5G24160	AT2G16270	AT1G04420
AT5G47635	AT2G42690	AT1G04800	AT2G47940	AT1G29270	AT2G30860
AT2G22970	AT3G27690	AT5G07510	AT2G31160	AT1G11160	AT5G17780
AT5G48490	AT3G03910	AT2G46340	AT2G02130	AT5G13950	AT3G16730
AT3G07820	AT3G62640	AT1G68570	AT1G19050	AT1G18370	AT3G10520

S5: Up-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep4-1*	Up-reg. in *sep4-1*	Up-reg. in *sep4-1*
AT5G24090	AT5G07420	AT1G35310	AT5G47600	AT2G44910	AT4G23600
AT3G22740	AT2G39210	AT1G74000	ATCG00840	AT2G01590	AT5G67600
AT2G21200	AT5G66690	AT5G02160	ATCG01300	AT2G01830	AT4G25170
AT4G22080	AT5G54570	AT1G14700	AT5G60930	AT5G43530	ATCG00170
AT4G22090	AT5G61640	AT2G42220	ATCG00430	AT3G17360	AT3G19810
AT1G74540	AT5G45340	AT5G07430	AT1G80410	AT2G07680	AT4G15440
AT4G16620	AT5G41900	AT5G17480	AT3G10040	AT5G66470	AT5G65510
ATCG01100	AT3G60020	AT4G27580	AT1G49230	AT2G02710	AT1G08230
AT1G70410	AT2G34860	AT1G30020	AT3G27330	AT1G49870	AT5G15120
AT1G18520	AT2G28630	AT1G20620	AT1G09420	AT5G05510	AT5G45950
AT5G38760	AT4G17690	AT2G19000	AT1G53920	ATCG00420	AT2G30990
AT3G17810	AT5G03240	AT2G19770	AT4G28080	AT2G42220	AT2G30600
AT4G10260	AT1G34310	AT1G71160	AT5G65360	AT1G30520	AT1G70090
AT2G39130	AT2G37090	AT1G32520	AT1G01740	AT1G16070	AT2G20720
AT1G52870	AT3G07850	AT3G16380	AT4G34930	AT3G09580	AT4G26370
AT3G03530	AT3G14040	AT4G28780	AT4G34920	AT4G31730	AT5G65230
AT3G03540	AT4G34850	AT4G25010	AT5G39890	AT2G42610	AT1G51380
AT2G21970	AT3G54900	AT1G51100	AT1G52000	AT1G05900	ATCG00065
AT3G56170	AT5G65380	AT1G48940	AT5G22920	AT3G21300	AT5G02370
AT1G51440	AT3G08940	AT3G51920	AT1G78820	AT1G01060	AT1G10920
AT5G50800	AT5G57660	AT3G03430	AT1G78830	AT5G57180	AT5G56850
AT5G48210	AT4G36780	AT5G53820	AT3G47090	AT1G36280	AT3G21770
AT2G39800	AT3G17060	AT2G28760	AT3G47580	AT4G18440	AT4G05190
AT3G55610	AT1G31330	AT2G30770	AT5G40160	AT1G70070	AT3G62270
AT3G10525	AT3G28180	AT2G39080	AT1G76110	AT1G02730	AT4G37540
AT1G61110	AT1G28375	AT5G22630	AT3G14930	AT1G15180	AT5G55520
AT5G57785	AT3G21700	AT2G27880	AT2G02350	AT5G60270	AT2G33180
AT2G47550	AT1G01900	AT5G28050	AT3G22210	AT4G11530	AT5G24860
AT5G46795	AT5G62920	AT3G15540	AT1G02930	AT1G10470	AT3G03190
AT4G02250	AT5G59290	AT5G15630	AT1G02920	AT2G41990	ATCG00560
AT3G01270	AT5G63060	AT5G61720	AT2G25510	AT3G21040	AT4G10270
AT5G44020	AT3G25170	AT5G13030	AT5G63010	AT2G30740	AT4G22490
AT3G15310	AT4G25900	AT1G23060	AT4G31570	AT2G34510	AT3G59400
AT4G16160	AT5G09550	AT3G62710	AT5G63120	AT1G22370	AT3G24810
AT5G13380	AT4G17560	AT3G15850	AT1G62480	AT3G22550	AT3G57830
AT3G53510	AT1G54860	AT2G29450	ATCG00810	AT1G59990	AT1G79560
AT1G57590	AT1G29200	AT5G47610	AT4G34610	AT2G28560	AT4G24670
AT5G40350	AT3G25760	AT2G21220	AT3G01550	ATCG00570	AT3G04650
AT1G70230	AT3G25770	AT3G43670	AT3G10310	AT4G34400	AT3G04260
AT1G59870	AT3G55250	AT2G40610	AT3G22150	ATCG00180	AT3G05410
AT4G28090	AT1G42970	AT5G52360	AT4G25080	AT4G26170	AT3G62740
AT2G44940	AT4G35010	AT1G52590	AT5G64640	AT3G01480	AT3G32400
AT5G26700	AT2G1673	AT3G47340	AT2G26360	AT2G04530	AT2G29290

S5: Up-regulated transcripts from sep3-2 and *sep4-1* microarrays

Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep3-2*	Up-reg. in *sep4-1*	Up-reg. in *sep4-1*
ATCG00420	AT1G02475	AT5G55930	AT2G03740	AT5G45040	ATG00630
AT2G40610	AT4G23660	AT3G03190	AT3G43860	AT2G40960	AT5G47190
AT5G52360	AT5G50830	ATCG00560	AT1G23610	AT5G03840	AT1G72070
AT1G52590	AT3G05930	AT3G08040	AT1G31580	AT5G58370	AT1G73602
AT4G37980	AT5G01520	AT3G54800	AT1G58430	AT4G11190	AT1G73600
AT3G20220	AT3G23120	AT5G16370	AT4G30270	AT4G16770	AT3G53460
ATCG00570	AT3G23110	AT5G16340	AT3G62820	AT1G63190	AT1G05190
AT5G16920	AT5G38530	AT1G78290	AT5G44400	AT1G71210	AT2G38310
AT3G18660	AT4G38970	AT3G59400	AT5G65840	AT1G64280	AT2G34640
AT5G14780	AT1G52230	AT4G22490	AT5G55450	AT4G10930	AT5G52630
AT1G63710	AT5G44190	AT2G21260	AT5G39730	AT3G21020	AT5G65660
AT1G23600	AT4G19420	AT2G21250	AT5G23270	AT4G18750	AT2G05520
AT2G38080	AT1G15260	AT1G23740	AT4G16190	AT3G62820	AT4G32915
AT4G28395	AT1G69730	AT1G29430	AT3G11430	AT2G04450	AT4G20130
AT1G65450	AT2G17950	AT5G27780	AT5G59320	AT1G13220	AT2G26400
AT1G61563	AT2G03390	AT5G07220	AT1G52680	AT2G20875	AT3G10180
AT1G61566	AT5G53370	AT2G41100	AT1G62940	AT3G25480	AT1G06190
AT2G41090	AT4G30030	AT3G50440	AT1G72910	AT4G24540	AT5G19210
AT5G59040	AT2G21490	AT1G26710	AT1G72930	AT5G11160	AT3G20150
AT1G75920	AT4G22753	AT3G15400	AT5G48930	AT4G37610	AT3G15450
AT4G23600	AT1G29195	AT1G78140	AT2G20570	AT5G06870	AT5G52100
AT5G20700	AT2G27830	ATCG00120	AT2G40475	AT1G33960	AT4G01460
AT4G25170	AT1G51240	AT2G37640	AT1G01630	AT2G15790	AT2G22990
AT5G62750	AT1G51250	AT3G06420	AT5G57345	AT1G03220	AT5G61440
AT5G37810	AT3G18220	AT2G38230	AT5G53190	AT1G03230	AT5G45040
AT5G37820	AT1G06990	AT2G38210	AT1G03130	AT5G55580	AT2G40960
AT3G26860	AT5G15160	AT3G28950	AT1G02205	AT1G78370	AT5G03840
AT1G51805	AT3G01500	AT2G29290	AT3G11980	AT5G08330	
AT5G25460	AT1G59740	AT1G23650	AT1G28230	ATCG00510	
AT4G21380	AT3G10740	AT1G75930	AT4G23900	AT1G13650	
AT3G28750	AT4G01900	AT3G62410	AT3G61160	AT2G41940	
AT1G23240	AT5G57810	AT3G11660	AT4G04610	AT5G28030	
AT3G52810	AT1G12780	AT5G18130	AT4G22240	AT1G13710	
AT1G11755	AT1G11350	AT1G22380	AT4G04020	AT4G13770	
AT3G06100	AT2G45580	AT1G47410	AT1G06650	ATCG00760	
AT4G23670	AT2G44210	AT3G51590	AT2G03200	AT2G18162	
AT1G50640	AT2G33380	AT2G38400	AT3G15510	AT2G18160	
AT3G45290	AT3G51860	AT1G02900	AT2G40890	AT5G40850	
AT1G70940	AT3G56240	AT3G01240	AT2G01670	AT5G50915	
AT4G37760	AT5G24660	AT5G19580	AT4G35060	AT5G06530	
AT5G48140	AT1G80660	AT1G05010	AT4G04930	AT4G35730	

S5: Up-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Up-reg. in *sep3-2*	Up-reg. in *sep3-2*
AT2G43100	AT4G18780
AT1G12110	AT5G27980
AT4G13790	AT5G14940
AT3G29030	AT3G44720
AT5G44480	AT1G28270
AT5G52320	AT1G62990
AT2G41870	AT1G71880
AT5G55620	AT4G24140
ATCG00070	AT2G22990
AT4G04460	AT2G30870
AT4G13770	AT4G34830
AT1G26770	AT2G38940
AT2G41340	AT1G26920
AT3G53420	AT3G47540
AT5G06530	AT2G29940
AT5G11070	AT1G78460
AT1G73630	AT2G28200
AT1G75940	AT5G09520
AT1G03050	AT5G44300
AT4G24640	AT2G05520
ATCG00630	AT5G54690
AT1G75280	
AT1G57750	
AT3G26110	
AT4G22960	
AT3G54700	

S5: Up-regulated transcripts from *sep3-2* and *sep4-1* microarray experiments

All identified up-regulated transcripts in sep3-2 and sep4-1 microarrays compared to WT, as well as common up-regulated transcripts in sep3-2 and sep4-1 microarry experiments,

Fold Change (FD) = greater than 1.5

S6: Down-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Down-reg. in *sep3-2*	Down-reg. in *sep3-2*	Down-reg. in *sep3-2*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Commonly Down-reg. in *sep3-2* and *sep4-1*
AT3G28470	AT5G24420	AT3G24330	AT4G39800	AT1G45145	AT5G04660
AT2G25590	AT1G67865	AT1G71890	AT5G54840	AT1G06250	AT4G10240
AT4G36180	AT5G67100	AT3G52490	AT2G21860	AT3G62180	AT2G34700
AT4G27010	AT1G73620	AT4G26590	AT1G78090	AT3G62040	AT3G29810
AT3G53380	AT3G20520	AT2G18220	AT1G13140	AT1G19640	AT5G21150
AT1G32810	AT5G06670	AT1G07300	AT3G45010	AT5G59310	AT4G18290
AT5G05600	AT5G23260	AT2G34700	AT5G05600	AT5G10170	AT2G25590
AT2G07723	AT5G15720	AT3G57930	AT4G35320	AT3G30720	AT1G17380
AT4G17150	AT1G05450	AT1G56210	AT3G23050	AT1G48100	AT2G38060
AT5G12930	ATMG00210	AT1G15660	AT4G25040	AT5G07550	AT5G05600
AT4G12760	AT2G07725	AT3G29810	AT1G49160	AT2G14170	AT1G02190
AT4G09960	AT1G71691	AT5G08210	AT5G14980	AT4G16740	AT4G09960
AT4G36140	AT5G49180	AT5G47600	AT1G01310	AT4G35190	AT1G51670
AT4G21630	AT3G49690	AT3G45420	AT1G23580	AT1G71340	AT4G36140
AT4G37050	AT1G04645	AT1G02190	AT4G36140	AT1G50310	AT4G21630
AT1G61340	AT3G60970	AT1G03780	AT1G02813	AT3G28007	AT4G37050
AT3G42570	AT3G60160	AT1G25330	AT4G37050	AT3G20520	AT1G73040
AT3G17070	AT3G05800	AT5G60930	AT5G09570	AT1G48330	AT1G61340
AT2G02100	AT1G68290	AT1G73040	AT4G11290	AT1G47610	AT3G42570
AT3G05430	ATCG00690	AT5G42900	AT2G37040	AT4G14080	AT3G17070
AT5G15510	ATCG00660	AT1G06520	AT5G59845	AT1G05450	AT5G09220
ATG01360	AT2G31035	AT3G27330	AT5G56370	AT5G02580	AT5G42900
AT3G48710	AT5G57420	AT3G47460	AT5G66020	AT4G27435	AT1G06520
AT3G20210	AT5G62230	AT2G12480	AT1G77870	AT5G04180	AT5G09990
AT3G07060	ATCG01120	AT3G12670	AT1G68875	AT1G66460	AT2G12480
AT5G12270	AT5G60760	AT3G48900	AT3G17630	AT5G02180	AT1G16705
AT5G61120	AT3G62080	AT5G51810	AT5G45880	AT1G24070	AT5G60140
AT2G40205	AT5G60100	AT1G08260	AT1G01720	AT3G05890	AT5G51810
AT3G08520	AT1G76790	AT2G35310	AT1G28570	AT2G46210	AT2G33850
AT3G11120	AT2G40080	AT3G11000	AT1G10770	AT3G60970	AT3G19550
AT3G56020	AT5G47160	AT4G16270	AT4G30410	AT3G60160	AT1G72520
AT1G30840	AT1G63100	AT3G49270	AT1G16850	AT3G53080	AT5G58400
AT1G75430	AT4G12890	AT2G06200	AT2G43080	AT1G68290	AT4G01730
AT1G22770	AT5G37500	AT2G42830	AT3G25570	AT5G61820	AT2G21100
AT3G55450	AT2G26470	AT4G02700	AT3G25572	AT1G24400	AT3G12000
AT3G20810	AT5G18090	AT3G12000	AT1G65970	AT3G25130	AT4G16870
AT1G10320	AT5G36740	AT2G39850	AT1G60740	AT2G44130	AT3G30720
AT1G76420	AT5G36670	AT2G17620	AT1G76410	AT3G25620	AT1G53885
AT3G30720	AT4G21400	AT1G03830	AT2G13970	AT1G08860	AT1G53903
AT3G50870	AT3G24340	AT1G19485	AT5G65850	AT5G60760	AT1G70270
AT3G48580	AT5G64900	AT1G73960	AT3G08720	AT4G12910	AT5G24420

S6: Down-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Down-reg. in *sep3-2*	Down-reg. in *sep3-2*	Down-reg. in *sep4-2*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Commonly Down-reg. in *sep3-2* and *sep4-1*
AT2G38060	AT5G07810	AT5G11420	AT2G29790	AT1G07880	AT1G67865
AT1G17380	AT2G13540	AT2G47050	AT2G23600	AT2G16910	AT2G34870
AT3G24630	AT1G67120	AT1G63880	AT2G23590	AT1G29140	AT5G53870
AT3G19500	AT3G46770	AT1G79700	AT5G60660	AT3G61220	AT5G51620
AT1G51670	AT2G05120	AT1G02050	AT2G23330	AT5G48900	AT4G32170
ATMG00690	AT5G45780	AT2G26490	AT5G17340	AT2G32430	AT4G38950
AT4G17480	AT1G13050	AT3G62230	AT4G08670	AT1G12070	AT3G20520
AT3G29390	AT2G22960	AT1G67920	AT5G62850	AT5G53870	AT4G16515
AT5G44630	AT1G03410	AT3G52620	AT3G44620	AT1G80760	AT3G53650
AT3G04960	AT5G24470	AT4G12890	AT1G61680	AT4G38950	AT1G15960
AT2G25730	AT5G18000	AT4G08110	AT1G51760	AT1G62710	AT5G15720
AT5G09990	AT5G44440	AT4G02380	AT1G51780	AT1G18280	AT2G21650
AT2G21800	AT3G58530	AT5G37500	AT1G73040	AT2G36020	ATG002100
AT1G16705	AT5G60120	AT1G29395	AT4G01430	AT2G19070	AT2G07725
AT5G57110	AT5G23960	AT1G59640	AT2G26740	AT1G15960	AT1G05450
AT5G24280	AT1G10570	AT4G10440	AT2G26750	AT4G20320	AT2G29660
AT2G33850	AT5G11400	AT3G28960	AT1G06520	AT2G33690	ATG00080
AT5G44750	AT3G10180	AT2G47160	AT4G31330	AT3G25290	ATG00090
AT1G72520	AT1G10540	AT5G14380	AT5G60800	AT1G35490	AT5G49180
AT5G09690	AT3G44050	AT1G75030	AT2G12480	AT1G19960	AT5G09730
AT5G11360	AT3G13682	AT2G24450	AT3G28980	AT1G75300	AT3G60970
AT4G01730	AT1G21090	AT1G16750	AT5G40730	AT2G29660	AT3G60160
AT4G16870	AT5G14070	AT2G19330	AT4G23690	AT1G01600	AT1G72260
AT2G32350		AT1G13150	AT5G51950	AT1G23570	AT1G68290
AT1G53885		AT1G54070	AT4G34230	AT1G69940	AT5G57420
AT1G53903		AT1G69080	AT1G02070	AT5G07410	ATG00660
AT4G27980		AT1G78440	AT5G54470	AT1G14280	AT5G60760
AT2G32280		AT5G62320	AT5G58980	AT1G23520	AT4G22730
AT2G33830		AT1G67870	AT3G13400	AT5G65040	AT4G24450
AT3G21810		AT2G34700	AT3G19390	AT5G62730	AT1G03410
AT4G16515		AT4G01480	AT1G61070	AT5G58390	AT2G22960
AT4G18120		AT3G15900	AT1G12240	AT5G23240	AT1G55020
ATG00080		AT5G25470	AT1G54260	AT1G02850	AT5G18000
ATG00090		AT3G29810	AT1G23130	AT3G57620	AT4G12890
AT5G22980		AT3G28840	AT2G38905	AT1G78960	AT3G58780
AT3G05790		AT1G79800	AT4G14815	AT2G02990	AT5G37500
AT5G07280		AT2G24210	AT3G63330	AT5G23970	AT5G44440
AT5G25950		AT5G52390	AT2G20970	AT3G13220	AT5G23960
AT2G23910		AT2G20700	AT2G15000	AT1G66360	AT2G26470

S6: Down-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Commonly Down-reg. in *sep3-2* and *sep4-1*
AT1G08470	AT1G65370	AT4G19120	AT4G34000	AT1G62540	AT5G11400
AT1G55570	AT4G19460	AT5G39880	AT5G47635	AT3G02480	AT1G71890
AT3G52600	AT5G66080	AT1G73220	AT3G43740	AT5G42860	AT4G12960
AT3G24890	AT5G50030	AT4G32170	AT3G07820	AT1G13990	AT2G23910
AT1G57550	AT2G25350	AT4G20420	AT1G23960	AT1G26480	AT4G21480
AT1G36150	AT4G33040	AT3G01700	AT4G12960	AT2G10970	AT4G26590
AT1G13750	AT4G11760	AT2G32890	AT4G22080	AT1G66540	AT5G22980
AT1G67860	AT5G43620	AT4G04760	AT4G22090	AT3G11480	
AT2G22600	AT1G66500	AT1G02330	AT1G74540	AT5G65350	
AT1G01280	AT5G59770	AT5G59810	AT2G47880	AT4G35480	
AT1G47980	AT5G49260	AT3G28830	ATCG01100	AT2G03710	
AT2G04080	AT1G10070	AT1G55560	AT2G29880	AT1G29050	
AT2G04066	AT5G65020	AT5G59720	AT1G32780	AT3G07010	
AT3G25050	AT3G57690	AT4G22160	AT5G04660	AT5G60360	
AT1G63060	AT4G30960	AT2G46860	AT4G32940	AT5G54095	
AT1G75040	AT5G07540	AT4G31670	AT1G18520	AT3G03910	
AT1G74210	AT5G60140	AT3G20865	AT5G38760	AT3G44610	
AT4G10120	AT1G06260	AT1G61630	AT3G28790	AT4G01730	
AT3G19090	AT3G22840	AT1G11170	AT4G20050	AT3G62640	
AT1G79520	AT5G54940	AT5G45260	AT1G34040	AT1G74020	
AT3G07830	AT1G66350	AT2G18420	AT1G34060	AT1G29440	
AT3G18360	AT5G50630	AT5G44680	AT3G09910	AT5G07420	
AT4G21480	AT5G50520	AT1G76470	AT1G24881	AT5G66690	
AT4G16860	AT3G23030	ATMG00660	AT1G24800	AT2G13680	
AT2G04240	AT1G60710	AT4G22730	AT1G25055	AT1G04310	
AT3G53980	AT5G58400	AT5G49130	AT1G25141	AT4G16870	
AT1G64370	AT5G49360	AT4G18300	AT1G25211	AT5G45340	
AT1G11280	AT2G21100	AT4G24450	AT1G24520	AT5G01200	
AT3G12580	AT3G54000	AT4G15630	AT2G38060	AT1G53885	
AT1G54570	AT4G29340	AT5G07530	AT5G17540	AT1G53903	
AT5G66310	AT2G06050	AT5G44410	AT4G30470	AT1G09930	
AT4G18290	AT5G49070	AT3G22370	AT3G05960	AT2G01610	
AT5G46240	AT3G59480	AT3G28810	AT2G03850	AT2G46640	
AT4G27420	AT2G32460	AT3G28820	AT4G25590	AT4G17690	
AT1G78970	AT1G02790	AT3G62170	AT4G12430	AT4G10260	
AT3G18830	AT3G25165	AT2G37760	AT4G12432	AT4G16590	
AT1G47960	AT1G10160	AT1G55020	AT2G17500	AT5G66590	
AT1G22760	AT3G25810	AT1G69530	AT1G44224	AT4G22730	
AT5G42090	AT5G62360	AT3G13390	AT4G25780	AT5G49130	

S6: Down-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*
AT5G13750	AT4G37900	AT5G61605	AT1G74550	AT4G18300	AT2G31980
AT3G10340	AT1G20120	AT3G20450	AT2G23510	AT4G24450	AT5G15720
AT4G15630	AT2G03850	AT5G66590	AT3G45640	AT1G20450	AT1G73500
AT5G07530	AT4G25590	AT1G52870	AT5G26060	AT5G61260	ATMG00210
AT5G44410	AT4G12430	AT5G22460	AT4G13800	AT2G41330	AT2G07725
AT3G22370	AT4G12432	AT3G56170	AT5G18000	AT3G21180	AT2G02960
AT3G28810	AT2G17500	AT3G21970	AT2G42530	AT4G39180	AT5G49120
AT3G28820	AT1G44224	AT3G01250	AT2G29420	AT1G54050	AT3G43920
AT3G62170	AT4G25780	AT1G09910	AT5G44440	AT3G21670	AT1G13970
AT2G37760	AT1G74550	AT5G46940	AT1G63840	AT2G24280	AT5G49180
AT1G55020	AT2G23510	AT5G43340	AT1G17430	AT3G46520	AT4G14690
AT1G69530	AT1G62540	AT4G28580	AT5G23960	AT5G59370	AT2G37180
AT3G13390	AT3G02480	AT4G01190	AT2G21830	AT5G50335	AT2G37170
AT5G61605	AT5G42860	AT4G16515	AT5G11400	AT2G05380	AT3G28780
AT3G20450	AT1G13990	AT5G50800	AT5G02560	AT2G42540	AT5G20710
AT4G34000	AT1G26480	AT2G19570	AT3G07850	AT2G46370	AT3G21230
AT5G47635	AT2G10970	AT4G01740	AT3G14040	AT1G67990	AT5G47000
AT3G43740	AT1G66540	AT4G14750	AT4G34850	AT4G24000	AT1G24600
AT3G07820	AT3G11480	AT4G17280	AT5G24270	AT1G03420	AT5G57420
AT1G23960	AT5G65350	AT5G48210	AT2G17840	AT1G04430	AT3G17790
AT4G12960	AT4G35480	AT4G26830	AT3G17060	AT1G58160	AT3G23770
AT4G22080	AT2G03710	AT2G28900	AT3G51410	AT1G12400	AT3G28270
AT4G22090	AT1G29050	AT1G61110	AT3G28180	AT5G61340	AT2G22190
AT1G74540	AT3G07010	AT1G02530	AT1G28375	AT5G14760	AT1G80130
AT2G47880	AT5G60360	AT1G02520	AT3G21700	AT2G28470	AT3G27200
ATCG01100	AT5G54095	AT1G72290	AT1G66620	AT1G22710	AT2G47040
AT2G29880	AT3G03910	AT4G02250	AT1G08830	AT2G27180	AT2G47030
AT1G32780	AT3G44610	AT3G01270	AT3G25170	AT1G20150	AT1G68110
AT5G04660	AT4G01730	AT5G57350	AT5G43110	AT4G17483	AT5G51640
AT4G32940	AT3G62640	AT3G29770	AT5G60500	AT3G17860	AT4G01080
AT1G18520	AT1G74020	AT5G13380	AT5G60510	AT1G78680	AT5G55790
AT5G38760	AT1G29440	AT3G29575	AT2G25590	AT4G18395	AT3G58290
AT3G28790	AT5G07420	AT5G47530	AT3G51070	AT5G24420	AT1G32450
AT4G20050	AT5G66690	AT5G40350	AT2G17470	AT1G67865	AT2G23800
AT1G34040	AT2G13680	AT5G53710	AT5G61250	AT2G24190	AT4G13930
AT1G34060	AT1G04310	AT4G28090	AT4G09960	AT1G19890	AT4G13890
AT3G09910	AT4G16870	AT5G26700	AT3G26125	AT3G53960	AT1G80960
AT1G24881	AT5G45340	AT4G27790	AT4G21630	AT1G75910	AT5G23210
AT1G24800	AT5G01200	AT2G22860	AT4G36490	AT3G55310	AT2G11140
AT1G25055	AT1G53885	AT2G21220	AT3G52160	AT3G55290	AT1G48470
AT1G25141	AT1G53903	AT4G25900	AT1G61340	AT1G67750	AT2G26470
AT1G25211	AT1G09930	AT4G20460	AT3G42570	AT1G10980	AT2G26410
AT1G24520	AT2G01610	AT5G09550	AT3G17070	AT4G17340	AT1G59218
AT2G38060	AT2G46640	AT1G03410	AT2G21560	AT2G02140	AT1G58848
AT5G17540	AT4G17690	AT3G14990	AT3G42640	AT3G05610	AT3G58060
AT4G30470	AT4G10260	AT1G54860	AT4G29250	AT2G21890	
AT3G05960	AT4G16590	AT1G77210	AT5G09470	AT2G21730	

S6: Down-regulated transcripts from *sep3-2* and *sep4-1* microarrays

Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*
AT1G75050	AT3G42960	AT4G14020	AT5G61720	AT3G19550	AT1G80660
AT1G66850	AT3G27810	AT5G07510	AT4G32500	AT5G48140	AT5G20410
AT5G15110	AT5G51810	AT3G14560	AT1G23060	AT3G05930	AT3G61380
AT1G71890	AT4G16710	AT5G56380	AT1G06640	AT3G53350	AT5G65205
AT3G04700	AT3G51000	AT4G35180	AT3G62710	AT1G79450	AT3G54800
AT5G53510	AT4G35110	AT1G35310	AT5G54010	AT1G61800	AT3G50570
AT1G35720	AT5G40780	AT1G74000	AT5G21150	AT5G63760	AT4G13345
AT4G26590	AT3G14595	AT5G09730	AT1G09100	AT5G20230	AT1G11410
AT1G59900	AT4G25760	AT1G19630	AT1G52190	AT5G63580	AT4G25950
AT5G22300	AT1G23730	AT1G72260	AT4G37980	AT4G16146	AT1G67290
AT4G19430	AT1G12880	AT4G37790	AT1G23800	AT5G52340	AT1G61065
AT5G07560	AT5G06760	AT4G35010	AT1G35612	AT5G51030	AT1G02340
AT2G45800	AT1G68610	AT2G16730	AT3G20220	AT1G26610	AT4G10380
AT1G07430	AT5G57880	AT5G05790	AT5G16920	AT1G35910	AT5G56910
AT1G44800	AT5G53120	AT5G05760	AT2G07360	AT2G15535	AT3G58780
AT3G28130	AT3G12000	AT2G40610	AT3G61230	AT1G49620	AT5G53830
AT2G07560	AT5G43150	AT5G52360	AT3G50970	AT2G34870	AT3G26820
AT2G14880	AT1G02470	AT1G52590	AT5G17200	AT5G57240	AT3G26840
AT3G55970	AT5G58310	AT3G18250	AT1G10150	AT3G16340	AT5G07220
AT1G27360	AT1G70720	AT4G24130	AT1G23600	AT4G02540	AT4G13030
AT1G15460	AT1G01250	AT5G07430	AT2G38080	AT5G36150	AT4G20480
AT3G49360	AT5G16960	AT5G17480	AT4G38620	AT1G04670	AT2G41100
AT2G38010	AT2G41010	AT4G27580	AT4G28395	AT1G75880	AT3G50440
AT1G02190	ATCG01090	AT1G30020	AT5G65390	AT3G53650	AT1G26710
ATMG01180	AT5G07520	AT2G19770	AT1G61563	AT2G17710	AT1G64700
AT4G13560	AT5G46690	AT2G19000	AT1G61566	AT4G24480	AT3G15400
AT5G03960	AT3G06260	AT1G71160	AT1G75920	AT1G67360	AT5G42170
AT3G55100	AT1G70270	AT2G36190	AT2G40110	AT1G06990	AT3G26290
AT2G28355	AT5G43780	AT5G22270	AT5G09220	AT2G21650	AT1G72280
AT3G16150	AT5G06510	AT3G07700	AT1G64660	AT1G63420	AT4G10240
AT4G33150	AT1G02260	AT3G03430	AT1G14900	AT3G08900	AT5G65870
AT1G51090	AT2G31230	AT5G53820	AT3G26860	AT5G66460	AT2G25890
AT2G01150	AT3G03080	AT5G16570	AT3G28750	AT1G59740	AT1G06170
AT2G19110	AT5G51620	AT4G39110	AT1G23240	AT3G53990	AT1G71770
AT5G42900	AT1G23670	AT3G15353	AT3G52810	AT3G21720	AT1G23650
AT3G62610	AT1G19180	AT2G02850	AT3G01490	AT4G18550	AT1G75930
AT5G02420	AT2G15050	AT1G52890	AT3G06100	AT5G59120	AT5G05040
AT4G13230	AT5G54400	AT2G01420	AT1G24575	AT2G33380	AT5G05060
AT5G50790	AT2G40670	AT4G10040	AT4G15530	AT5G47550	AT3G11660
AT1G80970	AT5G07010	AT1G73490	AT1G26560	AT5G53050	AT3G28540
AT2G38090	AT4G13630	AT5G59510	AT1G27580	AT4G21903	AT2G41290
AT5G04250	AT1G13280	AT2G27880	AT1G27540	AT4G21910	AT3G51590

S6: Down-regulated transcripts from *sep3-2* and *sep4-1* microarrays

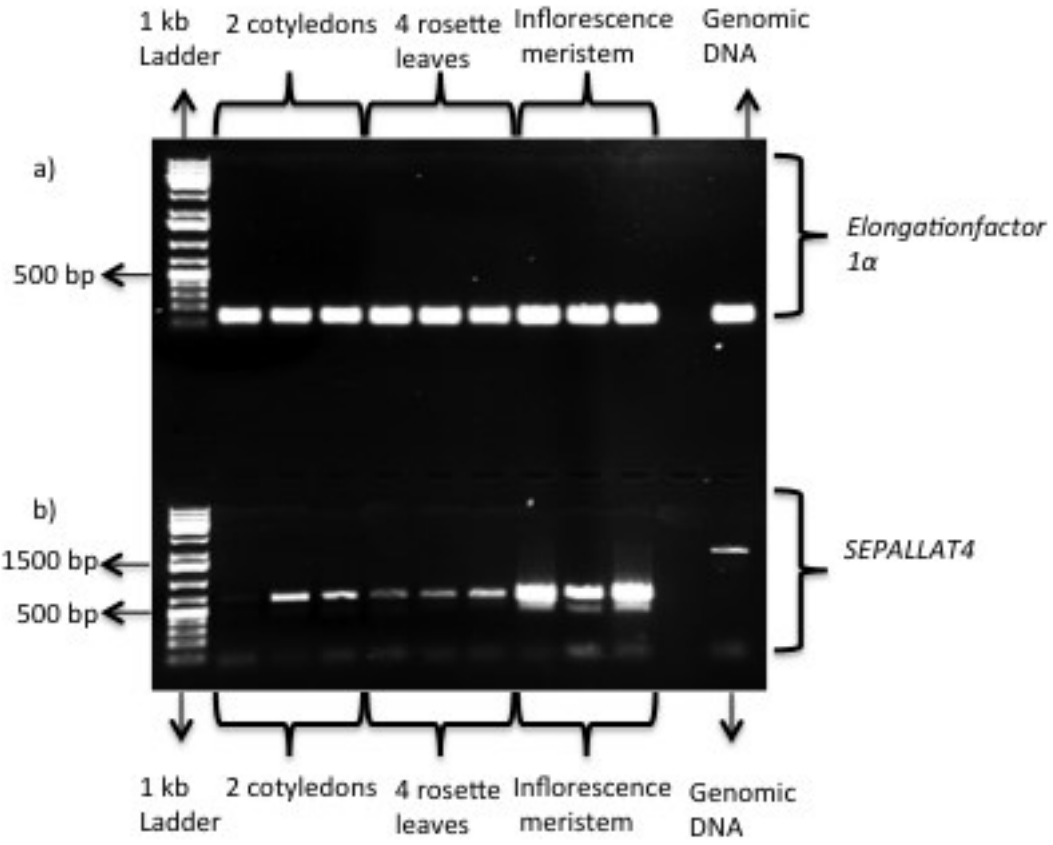
Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*	Down-reg. in *sep4-1*
AT1G17380	AT3G11980	AT5G16240	AT5G27980
AT5G63850	AT1G72510	AT1G56360	AT1G49450
AT3G01240	AT2G09970	ATMG00080	AT2G13570
AT5G22860	AT3G14380	AT1G07340	AT3G13720
AT5G19580	AT1G22010	AT2G20750	AT1G28270
AT1G05010	AT1G67880	AT4G30110	AT4G14780
AT2G03740	AT5G38120	AT3G53420	AT2G23910
AT1G51670	AT3G61160	AT5G58050	AT5G22980
AT4G28250	AT1G58150	AT1G73630	AT2G37770
AT5G47510	AT4G22240	AT1G75940	AT4G15620
AT2G16760	AT4G04020	AT4G24640	
AT3G43860	AT2G03200	AT4G35650	
AT3G43270	AT3G15510	AT1G77450	
AT2G32150	AT2G01670	AT1G75280	
AT1G58430	AT2G07777	AT4G39390	
AT1G52940	AT1G23560	AT5G01870	
AT2G18130	AT4G04930	AT3G48460	
AT3G46120	AT5G16450	AT5G56030	
AT4G30270	AT1G28710	AT2G29940	
AT5G09990	AT1G11700	AT1G78460	
AT5G44400	AT1G62810	AT4G35680	
AT4G03290	AT4G00040	AT5G52310	
AT1G16705	AT4G13790	AT4G04460	
AT1G15360	AT4G01590	AT3G26110	
AT1G72450	AT3G10460	AT2G22960	
AT5G67210	AT4G12930	AT4G13180	
AT3G57680	AT4G19500	AT5G41700	
AT5G59320	AT5G26340	AT1G11545	
AT1G52680	AT5G07990	AT2G28200	
AT4G19840	AT2G33850	AT5G44300	
AT5G35670	AT3G53260	AT1G67560	
AT1G62940	AT1G72520	AT4G00780	
AT4G19230	AT1G66480	AT4G37800	

S6: Down-regulated transcripts from *sep3-2* and *sep4-1* microarray experiments

All identified up-regulated transcripts in *sep3-2* and *sep4-1* microarrays compared to WT, as well as common up-regulated transcripts in *sep3-2* and *sep4-1* microarry experiments, Fold Change (FD) = greater than 1.5

S7: *SEP4* expression analysis at different stages using sqPCR

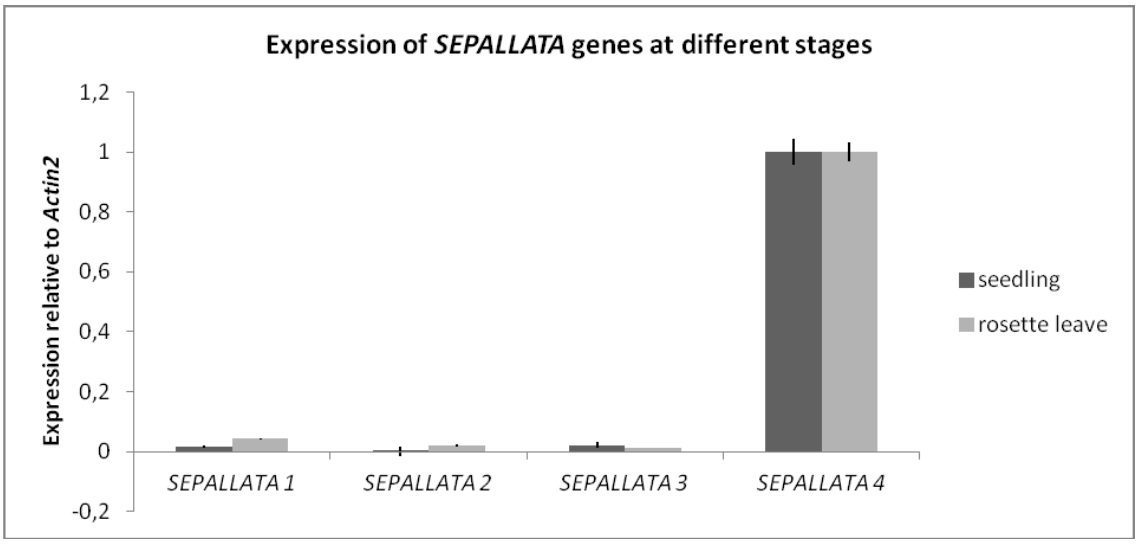
a)



S7: *SEP4* expression in different stages in *A. thaliana* using semi-quantitative PCR.

To test the expression of *SEPALLATA 4* throughout the plant, semi quantitative Real Time PCR with Col WT cDNA was performed on three different developmental stages, once with 2 cotyledons, 4 rosette leaves and Inflorescence meristems at stages (1-12), a genomic DNA sample was loaded to the samples to ensure that the cDNA is not contaminated, *Elongationfactor 1α* was used as a reference gene.

S8: *SEP4* expression analysis at different stages using qPCR



S8: *SEP4* expression in different stages in *A. thaliana* using qPCR .

qPCR experiments with seedling and rosette leaves to test *SEP1-4* expression, *Actin2* was used as a reference genes, qPCR experiments were performed with four biological samples,

Bars = Standard Error of the Mean (SEM)

S9: Gene list common targets and affected in their expression in *sep4-1* microarray

common SEP3 and SEP4 targets and up-reg. in *sep4-1*	common SEP3 and SEP4 targets and down-reg. in *sep4-1*	common SEP3 and SEP4 targets and down-reg. in *sep4-1*	common SEP3 and SEP4 targets and up-reg. in *sep4-1*
Locus	Gene name	Locus	Gene name
AT4G37240	similar to unknown protein	AT5G06510	CCAAT-binding transcription factor
AT4G27450	similar to unknown protein	AT3G16150	L-asparaginase, putative
AT5G52960	similar to unnamed protein product	AT1G12070	Rho GDP-dissociation inhibitor family protein
AT4G37740	*AtGRF2 (GROWTHREGULATING FACTOR 2)*	AT5G20710	*BGAL7* (*beta-galactosidase 7*)
AT4G15440	*HPL1* (*HYDROPEROXIDE LYASE 1)*	AT2G40110	yippee family protein
AT1G76420	*CUC3* (*CUP SHAPED COTYLEDON3*)	AT2G37760	aldo/keto reductase family protein
AT1G13260	*RAV1* (*Related to ABI3/VP1 1*)	AT4G37790	*HAT22* (*homeobox-leucine zipper protein 22*)
AT1G14580	zinc finger (C2H2 type) family protein	AT2G03710	*SEP4* (*SEPALLATA4*)
AT1G14770	zinc ion binding	AT1G47610	transducin family protein / WD-40 repeat family protein
AT5G06530	ABC transporter family protein	AT2G38060	transporter-related
AT4G24540	*AGL24* (*AGAMOUS-LIKE 24)*	AT1G68875	unknown protein
AT5G53160	similar to unknown protein	AT3G26290	*CYP71B26* (cytochrome P450)

common SEP3 and SEP4 targets and up-reg. in *sep4-1*	common SEP3 and SEP4 targets and down-reg. in *sep4-1*	common SEP3 and SEP4 targets and down-reg. in *sep4-1*	common SEP3 and SEP4 targets and up-reg. in *sep4-1*
Locus	Gene name	Locus	Gene name
AT3G18080	glycosyl hydrolase family 1 protein	AT1G78440	*ATGA2OX1* (*GIBBERELLIN 2-OXIDASE 1*)
AT2G45660	*AGL20* (*AGAMOUS-LIKE 20*)	AT5G49360	*BXL1* (*BETA-XYLOSIDASE 1*)

S9: Gene list of affected transcripts in *sep4-1* microarrays and common SEP3 and SEP4 targets,

FDR for ChIP-SEQ = 0.001, FC= >1.5

APPENDICES

S10: List of Oligonucleotides generated and used in this work

Name	Sequence 5'-3'	Description
CA54	GATGCACTCGAAATCAGCCAATTTAGAC	Genotyping *SEP1-1*
CA55	CGTGTATATCTGTGAAGGCTTTGA	Genotyping *SEP1-1*
CA56	AGAGATTGATCGCAAGGCACCAAATGCA	Genotyping *sep1-1*
CA57	GAGCGTCGGTCCCCACACTTCTATAC	Genotyping *sep2-1*
CA58	TTGAAGGCTATTACAGCTATAGCCACTT	Genotyping *SEP2-1*
CA59	AGCAAGTTCGCTGCATCAAGGTGA	Genotyping *SEP2-1*
CA60	AGACAATGTTGGGCTT	Genotyping *sep2-1*
CA182	GCTTAGCATCTCTGCAAAACCAACACAAAGCTA	Genotyping *sep4-1*
CA183	TGTACCTGCAAGTCTTTAGCTGATTGA	Genotyping *sep4-1*
CA184	GATAGAGCGCCACAATAACAAACAATTGCGT	Genotyping *sep4-1*
CA315	ATGGGAAGAGGGAAAGTTGAGC	Genotyping *SEP4-2*
CA316	CATTTCTTGAACGTGCTTGCCATG	Genotyping *SEP4-2*
CA317	GAACGCCAAGTAGATGCATCAC	Genotyping *sep4-2*
CA384	GGACTTGACATTGAAGAGCTTCA	*SVP* RT PCR (Liu et. al)
CA385	TGATCTCACTCATAATCTTGTCAC	*SVP* RT PCR (Liu et. al)
CA386	CAGAAAACGAGAAGCTCTCTG	*SOC1* RT PCR (Liu et. al.)
CA387	TACTCTCTTCATCACCTCTTCC	*SOC1* RT PCR (Liu et. al.)
CA394	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	*FLC* RT PCR
CA395	GGGGACCACTTTGTACAAGAAAGCTGGGTC	*FLC* RT PCR
ATP58	TGGTTCACGTAGTGGGCCATCG	Genotyping *sep4-2,* T-DNA left border
SB1	GTTGGGAAAATCGTACGAGGCTTCACCTAGT	Genotyping *SEP3-2*
SB2	GTCACTTGCCTATTGA	Genotyping *SEP3-2*
SB5	ggg gac aag ttt gta caa aaa agc agg ct TAATGGGTGATTCAATGCAATGTG	g*SEP4* construct for complementation
SB6	ggg gac cac ttt gta caa gaa agc tgg gtc GACCATCCATCCAGGGAAGAATCC	g*SEP4* construct for complementation
SB9	TAGCTCTTCACCTAGCAAATGCC	Sequencing of *SEP4* construct
SB10	GTAGATAGTATAAGATGCCATG	Sequencing of *SEP4* construct
SB13	AAGCATAAGCAGAAAAAAACATATC	Sequencing of *SEP4* construct
SB14	GTCTATTGATCTTGTTCTCTATC	Sequencing of *SEP4* construct
SB24	GCATTCAAGAAAATATAGCGATTAATTCC	Genotyping *SEP1-2*
SB25	GCCTTTGGCGCAGTGTTTTGTTC	Genotyping *SEP1-2*
SB34	GGTGTGAGAAGTCATCATATGG	pPCR *sep1-1*
SB35	CCATTTGCAGAGTTGGATTGC	qPCR *sep1-1*
SB36	CCAAATACCATCAATTCTCCAG	pPCR *sep1-2*
SB37	TGAGCTTTAACACAGCACATGAC	pPCR *sep1-2*
SB39	CACAGTATATGCTTGACCAGCTCTC	Genotyping *SEP2-2*
SB40	TGCATAGACAGGATAAGTACTTCACC	Genotyping *SEP2-2*
SB41	GGGACTTTACTAATGGGGAAGAAG	Genotyping *sep2-2*
SB42	ATAATAACGCTGCGGACATCTACATTTT	Genotyping *sep2-2*, T-DNA left border
Name	Sequence 5'-3'	Description
SB43	CAGGATAAGTATCAAGACTACTTG	*SEP4* sqPCR
SB44	TCCATTAACATTCTGTGATGTTG	*SEP4* sqPCR
SB45	CATCACCATATAGGAGGAGGATG	*SEP2* RT PCR

SB46	Ggctcattctcagggac	*SEP2* RT PCR
SB47	CAGCTACAGAGAGTACTTGAAGCTG	*SEP2* RT PCR
SB48	CCTTGCACCGTCACAGCC	*SEP2* RT PCR
SB49	Ggttgatcactacggtcgtcatcat	*SEP3* RT PCR
SB50	CACTTGGTCCTGCTCCCATTCC	*SEP3* RT PCR
SB51	Gtgccttcaagagaggccttag	*SEP3* RT PCR
SB52	GGAAGAATCAAGCTGTCTCAAGTGAC	*SEP3* RT PCR
SB61	CCAGAACCCAATGTGCCTTCAAG	*SEP3* RT PCR
SB62	GGTCCAAGATCTTCTCCCAACAG	*SEP3* RT PCR
SB63	CTACGGTCGTCATCATCATCAACAAC	*SEP3* RT PCR
SB64	GTAATTATTCACACTTGGTCCTGCTCC	*SEP3* RT PCR
SB66	Gctaaacgtagaaatggtttgc	*SEP2* RT PCR
SB67	CTGACCTTGCACCGTCACAGC	*SEP2* RT PCR
SB68	Gctcaagaaggcttatgagc	*SEP4* RT PCR
SB70	AAACCCAGACGTGACTTGTTTGACG	SEP3 ChIP
SB71	Tgagaatcggacggctttgagg	SEP3 ChIP
SB72	Tttatctgttgggatggaaaga	SOC1 ChIP
SB73	Tctcgtacctatatgcccccact	SOC1 ChIP
SB74	GGAGATCATGAGCAAAAGTAGTAAGCA	AP1 ChIP
SB75	TGGAGATGTAACAAAGGCGAAGATAA	AP1 ChIP
SB76	TCATATATGTCGCATAACGAGGGAGT	AP2 ChIP
SB77	TGCTCATATAAAACAAAACCAAAGAAACA	AP2 ChIP
SB78	TATCTTCTTTTGGCCCAATTTCATTT	FD ChIP
SB79	TTGGTTGCTTAAAAGAATACACAGCA	FD ChIP
SB80	GAACGTTGTGATGTTACTCGGACAAG	AG ChIP
SB81	TCAACAACCCATTAACACATTGGGTA	AG ChIP
SB82	GCCACCATGGTTTCACATCATATC	ARR6 ChIP ref. gene
SB83	CCTTTGCAAGAAGATACTCTGAGC	ARR6 ChIP ref. gene
SB84	GCTATCCACAGGTTAGATAAAGGAG	HSF ChIP ref. gene
SB85	GAGAAAGATTGTGTGAGAATGAAA	HSF ChIP ref. gene
SB86	TGCATGCATTCACAC ATAGTACACAT	*LFY* RT PCR
SB87	TATTATCCGCCGAGCA ATAGACTGTA	*LFY* RT PCR
SB88	GTATGGAGAAGATACTTGAACGC	*AP1* RT PCR
SB89	CAGTTTGTATTGACGTCGGACTC	*AP1* RT PCR
SB90	Cagcatccttacatgctctctcatc	*AP1* RT PCR
SB91	GTTGCAGTTGTAAACGGGTTCAAG	*AP1* RT PCR
SB98	GATAGAGAACAAGATCAATAGACAAGTG	*CAL* RT PCR
SB99	GAGGAGTACTCGAACAATTTGCCC	*CAL* RT PCR
SB100	GGTTGCTCTCATCGTCTTCTC	*FUL* RT PCR
SB101	CTTTGTGAAACGTCTCGGCC	*FUL* RT PCR
SB102	GGGCCAAAACCACCACAAC	*FD* RT PCR
SB103	GAACAGTGACCGTGGTGG	*FD* RT PCR
SB107	CGGCACAGCCGTTGAAAAAG	*AP2* RT PCR
SB108	GAGATTCCCATCTTCCGGTAC	*AP2* RT PCR
SB109	CAAGTCACTTTTTGCAAACGTAG	*AG* RT PCR
SB110	CATAGAGACGACCACGGCTAG	*AG* RT PCR
SB111	CAACAAGCTTCATGAGTATATCAGC	*AP*3RT PCR
Name	Sequence 5’-3’	Description
SB112	CTCATATTGAGTGGCCCAAACATC	*AP3* RT PCR
SB113	GAGGAAAGATCGAGATAAAGAGG	*PI* RT PCR
SB114	CAACTTTTGCATCACAAAGAACTG	*PI* RT PCR

SB115	GGGAAGAAAAAAACTAGAAATCAAGC	*FLC* RT PCR
SB116	GATGCGTCACAGAGAACAGAAAG	*FLC* RT PCR
SB129	GATCTTAACTCTCCTTAGAAAAGTACTAGG	SEP4 ChIP
SB130	GTTACATTATTTTAGTCTTACTTCATCAGG	SEP4 ChIP
SB131	GAGTCTTTGCGCTGACTTTTGC	LUG ChIP
SB132	GTGCAACGATTTTAAAATTGTCATCC	LUG ChIP
SB149	ATAATAACGCTGCGGACATCTACATTTT	Genotyping *cuc3-105*
SB150	ATTGAAACAAAACACATGAAATGG	Genotyping *cuc3-105*
SB151	CTTGCGGTGGAAGATGTGTTAAG	Genotyping *CUC3-105*
SB152	CAACAGACCATAACTCGCTTC	Genotyping *CUC3-105*
SB154	CGGCTAAGCAGTCTAAGCC	*NF-YA10* RT PCR
SB155	CAGAATCCGAGAGATTTGACTC	*NF-YA10* RT PCR
SB156	CGGAGGAAGCCTCTAACTCTAG	*AT5G52960* RT PCR
SB157	GCGAATACAGTGGCTTGTTC	*AT5G52960* RT PCR
SB158	CCATTCAAGACGCAGGTCTC	*BGAL7* RT PCR
SB159	CATGAAACTAGGGTTAACTGTTCTG	*BGAL7* RT PCR
SB160	GCTTAGCAGTTGAAGGAGCC	*AT1G68875* RT PCR
SB161	CTCTACTACCACCAGATCCTCC	*AT1G68875* RT PCR
SB162	CGGATCCACTGGTAAAGATAC	*AT1G12070* RT PCR
SB163	CTTAGAGCCTTCTTTGAGAGTGAAC	*AT1G12070* RT PCR
SB164	GGCGAAGATGGGAGAGAGAGAG	*CUC3* RT PCR
SB165	CTTTGCCGGTAGCTTTCC	*CUC3* RT PCR
SB168	ggg gac aag ttt gta caa aaa agc agg ct CTCGTAATCATGAACTGTGTTTC	35S:: *AT5G52960*
SB169	ggg gac cac ttt gta caa gaa agc tgg gtc TATTCTTTAAGGTCCTCCAAGTATGG	35S:: *AT5G52960*
SB170	ggg gac aag ttt gta caa aaa agc agg ct ATGAAAATGAAACACTTCACTC	35S::*BGAL7*
SB171	ggg gac cac ttt gta caa gaa agc tgg gtcCTAGCACTCCAATTCCAC	35S::*BGAL7*
SB172	ggg gac aag ttt gta caa aaa agc agg ct GAAGCAAAAATGACAGAACTC	35S:: *AT1G68875*
SB173	ggg gac cac ttt gta caa gaa agc tgg gtc AGCTAGCTAGTTAGACTGTGG	35S:: *AT1G68875*
SB174	ggg gac aag ttt gta caa aaa agc agg ct GCAAAGATGGTTTTGAACG	35S::AT1G12070
SB175	ggg gac cac ttt gta caa gaa agc tgg gtc TCACAGCCAATTTTTGCGAATG	35S::AT1G12070
SB178	ggg gac aag ttt gta caa aaa agc agg ct GGAACCGACATAGCTCCCAAC	35S::NF-YA10 (Leyva-González, M. A., et. al., 2012)
SB179	ggg gac cac ttt gta caa gaa agc tgg gtcTCATATATTAAGTTTGCAGCAGC	35S::NF-YA10 (Leyva-González, M. A.,et. al., 2012)
SB180	GAGAAGATAAGGATAAAGAAGATTG	*AGL24* RT PCR
SB181	GTTCATCGGCTTTCTTGAAGATTC	*AGL24* RT PCR
SB182	GCTAAGCATCAGAGAAACCATAG	*FD* RT PCR
SB183	GTGATGAGGATGAAATGGAAGAAAC	*FD* RT PCR
Name	Sequence 5’-3’	Description
SB184	CAACGTGCACAGCAACAGCAGC	*LUG* RT PCR
SB185	GCTGCTGCTGCTGTTGCTGTTGC	*LUG* RT PCR
Act2	GCTCCTCTTAACCCAAAGGC	*Actin2* RT PCR (reference gene)

Act2	ACACCATCACCAGAATCCAGC	*Actin2* RT PCR (reference gene)
attB1	CCACTTTGTACAAGAAAGCTGG	attB1 (sequencing primer p207)
attB1	CAAGTTTGTACAAAAAAGCAGG	attB2 (sequencing primer p207)

Bibliography

Abe M., KobayashiY., Yamamoto S., Daimon Y., Yamaguchi A., Ikeda Y., Ichinoki H., Notaguchi M., Goto K., and Araki T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science. **309,** 1052-1056

Airoldi C.A ., and Davies B. (2012). Gene duplication and the evolution of plant MADS-box transcription factors. J Genet Genomics. **39,** 157–165.

Airoldi C.A., Bergonzi S, and Davies B. (2010). Single amino acid change alters the ability to specify male or female organ identity. Proc Natl Acad Sci U S A. **107,** 18898-18902.

Aleksic J., and Russell S. (2009). ChIPing away at the genome: the new frontier travel guide. Mol Biosyst. **5,** 1421-1428.

Alonso J.M., Stepanova A.N., and Leisse T.J. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science. **301**, 653–657.

Alvarez-Venegas R., Pien S., Sadder M., Witmer X., Grossniklaus U., and Avramova Z. (2003). ATX-1, an Arabidopsis homolog of trithorax, activates flower homeotic genes. . Curr Biol. **13,** 627-637.

Ampomah-Dwamena C., Morris B.A., Sutherland P., Veit B., and Yao J.L. (2002). Down-regulation of TM29, a tomato SEPALLATA homolog, causes parthenocarpic fruit development and floral reversion. Plant Physiol. **130,** 605-617.

Angenent G.C., Franken J., Busscher M., Weiss D., and van Tunen A.J. (1994). Co-suppression of the petunia homeotic gene fbp2 affects the identity of the generative meristem. Plant J. **5,** 33-44.

Aoyama T., and Chua N.H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. . Plant J. **11,** 605-612.

Arnaud N. and Laufs P. (2013). Plant Development: Brassinosteroids Go Out of Bounds. Curr Biol. **23**, R152-R154.

Aukerman M.J., and Sakai H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target gene Plant Cell. **15,** 2730-2741.

Bao X., Franks R. G., Z., L.J., and Liu Z. (2004). Repression of AGAMOUS by BELLRINGER in floral and inflorescence meristems. . Plant Cell. **16,** 1478 -1489.

Bassel G.W., Gaudinier A., Brady S.M. , Hennig L., Rhee S.Y., and De Smet I. (2012). Systems analysis of plant functional, transcriptional, physical interaction, and metabolic networks. Plant Cell. **24,** 3859-3875.

Battey N.H., and Lyndon R.F. (1990). Reversion of flowering. . Botanical Review. **56,** 162-189.

Becker A., and Theissen G. (2003). The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol. **29,** 464-489.

Ben-Naim O., Eshed R., Parnis A., Teper-Bamnolker P., Shalit A., Coupland G., Samach A., and Lifschitz E. (2006). The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. Plant J. **46,** 462-476.

Birchler J.A., Bhadra U., Bhadra M.P., and Auger D.L., (2001)**.** Dosage-dependent gene regulation in multicellular eukaryotes: implications for dosage compensation, aneuploid syndroms and quantitative traits. Dev Biol**. 234,** 275-288**.**

Birchler J.A, Riddle N.C., Auger D.L., and Veitia R.A. (2005). Dosage balance in gene regulation: biological implications. Trends Genet. **21,** 219-226.

Birchler J.A., and Veitia R.A. (2007a). The gene balance hypothesis: from classical genetics to modern genomics. Plant Cell. **19,** 395-402.Biochim Biophys Acta. **1769,** 422-428.

Birchler J.A., Yao H., and Chudalayandi S. (2007b). Biological consequences of dosage dependent gene regulatory systems. Biochim Biophys Acta. **1769,** 422-428.

Blanc G., and Wolfe K. H. (2004). Functional divergence of duplicated genes formed by polyploidy during Arabidopsis. Plant Cell. **16,** 1679–1691.

Blanc G., Hokamp K., and Wolfe H. . (2003). A recent polyploidy superimposed on older large-scale duplications in the Arabidopsis genome. Genome Res. **13,** 137-144.

Blázquez M.A., and Weigel D. (1999). Independent regulation of flowering by phytochrome B and gibberellins in Arabidopsis. Plant Physiol. **120,** 1025-1032.

Blázquez M.A., and Weigel D. (2000). Integration of floral inductive signals in Arabidopsis. Nature. **404,** 889-892.

Bowers J.E., Chapman B.A., Rong J., and Paterson A.H. (2003). Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. Nature. **422,** 433-438.

Bowler C., Benvenuto G., Laflamme P., Molino D., Probst A.V., Tariq M., and Paszkowski J. (2004). Chromatin techniques for plant cells. Plant J. **39,** 776-789.

Bowman J.L., Smyth D.R., and Meyerowitz E.M. (1989). Genes directing flower development in Arabidopsis. Plant Cell. **1,** 37-52.

Bowman J.L., Smyth D.R., and Meyerowitz E.M. (1991). Genetic interactions among floral homeotic genes of Arabidopsis. Development. **112,** 1-20.

Bowman J.L., Alvarez J., Weigel D., Meyerowitz E.M., and Smyth D.R. (1993). Control of flower development in Arabidopsis thaliana by APETALA1 and interacting genes. Development. **119,** 721–743.

Bowman J.L., Sakai H., Jack T., Weigel D., Mayer U., and Meyerowitz E.M. (1992). SUPERMAN, a regulator of floral homeotic genes in Arabidopsis. Development. **114,** 599-615.

Bradley D., Carpenter R., Sommer H., Hartley N., and Coen E. (1993). Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum. Cell. **72,** 85-95.

Brookfield J. (1992). Can genes be truly redundant? Curr Biol. **2,** 553-554.

Byrne M.E., Simorowski J., and Martienssen R.A. (2002). ASYMMETRIC LEAVES1 reveals knox gene redundancy in Arabidopsis. Development. **129,** 1957–1965.

Cardon G., Höhmann S., Klein J., Nettesheim K., Saedler H., and Huijser P. (1999). Molecular characterisation of the Arabidopsis SBP-box genes. Gene. **237,** 91-104.

Castillejo C., Romera-Branchat M., and Pelaz S. (2005). A new role of the Arabidopsis SEPALLATA3 gene revealed by its constitutive expression. Plant J. **43,** 586-596.

Causier B., Schwarz-Sommer Z., and Davies B. (2010). Floral organ identity: 20 years of ABCs. Semin Cell Dev Biol. **21,** 73-79.

Causier B., Castillo R., Zhou J., Ingram R., Xue Y., Schwarz-Sommer Z., and Davies B. (2005). Evolution in action: following function in duplicated floral homeotic genes. Curr Biol. **23,** 1508-1512.

Chalfun-Junior A., Franken J., Mes J.J., Marsch-Martinez N., Pereira A., and Angenent G.C. (2005). ASYMMETRIC LEAVES2-LIKE1 gene, a member of the AS2/LOB family, controls proximal-distal patterning in Arabidopsis petals. Plant Mol Biol. **57,** 559–575.

Chee M., Yang R., Hubbell E., Berno A., Huang X.C., Stern D., Winkler J., Lockhart D.J., Morris M.S., and Fodor S.P. (1996). Accessing genetic information with high-density DNA arrays. Science. **274,** 610-614.

Chen H.W., Bandyopadhyay S., Shasha D.E., and Birnbaum K.D. (2010). Predicting genome-wide redundancy using machine learning. BMC Evol Biol. **10**.

Chen X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. science. **303,** 2022-2025.

Chuang C.F., and Meyerowitz E.M. (2000). Specific and heritable genetic interference by double-stranded RNA in Arabidopsis thaliana. Proc Natl Acad Sci U S A **97,** 4985-4990.

Chuang C.F., Running M.P., Williams R.W., and Meyerowitz E.M. (1999). The PERIANTHIA gene encodes a bZIP protein involved in the determination of floral organ number in Arabidopsis thaliana. Genes Dev. **13,** 334-344.

Clark S. E., Running M. P. , and Meyerowitz E. M. (1995). CLAVATA3 is a regulator of shoot and floral meristem development affecting the same processes as CLAVATA1. Development **121,** 2057-2067.

Clark S.E., Running M.P., and Meyerowitz E.M. (1993). CLAVATA1, a regulator of meristem and flower development in Arabidopsis. Development **119,** 397-418.

Clough S.J., and Bent A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The Plant Journal. **16**., 735-752.

Coen E.S., and Meyerowitz E.M. (1991). The war of the whorls: genetic interactions controlling flower development. Nature. **353,** 31-37.

Colbert T., Till B.J., Tompa R., Reynolds S., Steine M.N., Yeung A.T., McCallum C.M., Comai L., and Henikoff S. (2001). High-throughput screening for induced point mutations. Plant Physiol. **126,** 480-484.

Craigon D.J., James N., Okyere J., Higgins J., Jotham J., and May S. (2004). NASCArrays: a repository for microarray data generated by NASC's transcriptomics service. Nucleic Acids Research **32,** D575-D577.

Crow K.D., and Wagner G.P. (2006). Proceedings of the SMBE Tri-National Young Investigators' Workshop 2005. What is the role of genome duplication in the evolution of complexity and diversity? Mol Biol Evol. **23,** 887-892.

Das P., Ito T., Wellmer F., Vernoux T., Dedieu A., Traas J., and Meyerowitz E.M. (2009). Floral stem cell termination involves the direct regulation of AGAMOUS by PERIANTHIA. Development. **136,** 1605-1611.

Davies B., Egea-Cortines M., de Andrade Silva E., Saedler H., and Sommer H. (1996). Multiple interactions amongst floral homeotic MADS box proteins. EMBO J. **15,** 4330-4343.

Davies B., Motte P., Keck E., Saedler H., Sommer H., and Schwarz-Sommer Z. (1999). PLENA and FARINELLI: redundancy and regulatory interactions between two Antirrhinum MADS-box factors controlling flower development. EMBO J. **18,** 4023–4034.

De Bodt S., Raes J., Florquin K., Rombauts S., Rouzé P., Theissen G., and Van de Peer Y. (2003). Genomewide structural annotation and evolutionary analysis of the type I MADS-box genes in plants. J Mol Evol. **56,** 573-586.

de Folter S., Shchennikova A.V., Franken J., Busscher M., Baskar R., Grossniklaus U., Angenent G.C., and Immink R.G. (2006). A Bsister MADS-box gene involved in ovule and seed development in petunia and Arabidopsis. Plant J. **47,** 934-946.

de Folter S., Immink R.G., Kieffer M., Parenicová L., Henz S.R., Weigel D., Busscher M., Kooiker M., Colombo L., Kater M.M., Davies B., and Angenent G.C. (2005). Comprehensive interaction map of the Arabidopsis MADS Box transcription factors. Plant Cell. **17,** 1424-1433.

DeYoung B.J., Bickle K.L., Schrage K.J., Muskett P., Patel K., and Clark S.E. (2006). The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in Arabidopsis. Plant J. **45,** 1-16.

Dinneny J.R., Yadegari R., Fischer R.L., Yanofsky M.F., and Weigel D. (2004). The role of JAGGED in shaping lateral organs. development. **131,** 1101-1110.

Ditta G.S., Pinyopich A., Robles P., Pelaz S., and Yanofsky M.F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Curr Biol. **14,** 1935-1940.

Du Z., Zhou X., Ling Y., Zhang Z., and Su Z. (2010). agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Res. **38,** W64-70.

Edger P.P., and Pires J.C. (2009). Gene and genome duplications: the impact of dosage-sensitivity on the fate of nuclear genes. Chromos. Res. **17,** 699–717.

Egea-Cortines M., Saedler H., and Sommer H. (1999). Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. EMBO J. **18,** 5370-5379.

Engelbrecht C. C., Schoof H., and Böhm S., (2004), Conservation, diversification and expansion of C2H2 zinc finger proteins in the Arabidopsis thaliana genome. BMC Genomics. **5**, 1-17.

Ermolaeva M.D., Wu M., Eisen J.A., and Salzberg S.L. (2003). The age of the Arabidopsis thaliana genome duplication. Plant Mol Biol. **51,** 859-866.

Fan H.Y., Hu Y., Tudor M., and Ma H. (1997). Specific interactions between the K domains of AG and AGLs, members of the MADS domain family of DNA binding proteins. Plant J. **12,** 999-1010.

Favaro R., Pinyopich A., Battaglia R., Kooiker M., Borghi L., Ditta G., Yanofsky M.F., Kater M.M., and Colombo L. (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. Plant Cell. **15,** 2603–2611.

Fawcett J.A., Maere S., and Van de Peer Y. (2009). Plants with double genomes might have had a better chance to survive the Cretaceous-Tertiary extinction event. Proc Natl Acad Sci U S A. **106,** 5737-5742.

Ferrándiz C., Gu Q., Martienssen R., and Yanofsky M.F. (2000). Redundant regulation of meristem identity and plant architecture by FRUITFULL, APETALA1 and CAULIFLOWER. Development. **127,** 725-734.

Ferrario S., Immink R.G., Shchennikova A., Busscher-Lange J., and Angenent G.C. (2003). The MADS box gene FBP2 is required for SEPALLATA function in petunia. Plant Cell. **15,** 14-25.

Fields S., and Song O.K. (1989). A novel genetic system to detect protein-protein interactions. Nature. **340,** 245-246.

Flanagan C.A., and Ma H. (1994). Spatially and temporally regulated expression of the MADS-box gene AGL2 in wild-type and mutant arabidopsis flowers. Plant Mol Biol. **26,** 581-595.

Friedman W.E. (2009). Evolution pioneers: celebrating Lamarck at 200, Darwin 215. Nature. **461, 688-689.Fusaro A.F., Bocca S.N., Ramos R.L., Barrôco R.M., Magioli C., Jorge V.C., Coutinho T.C., Rangel-Lima C.M., De Rycke R., Inzé D., Engler G., and Sachetto-Martins G.** (2007). AtGRP2, a cold-induced nucleo-cytoplasmic RNA-binding protein, has a role in flower and seed development. Planta. **225,** 1339-1351.

Goethe, J.W.v. (1790). Versuch die Metamorphose der Pflanzen zu erklären. Ettingersche Buchhandlung, Gotha , Germany.

Gomez-Mena C., de Folter, S., Costa M. M., C.., A.G., Sablowski R., and (2005). Transcriptional program controlled by the floral homeotic gene AGAMOUS during early organogenesis. Development. **132,** 429 -438.

Goto K., and Meyerowitz E.M. (1994). Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. Genes Dev. **8,** 1548-1560.

Gramzow L., and Theissen G. (2010a). A hitchhiker's guide to the MADS world of plants. Genome Biol. **11**:214

Gramzow L., Ritz M.S., and Theissen G. (2010b). On the origin of MADS-domain transcription factors. Trends Genet. **26,** 149-153.

Grandi V., Gregis V., and Kater M.M. (2012). Uncovering genetic and molecular interactions among floral meristem identity genes in Arabidopsis thaliana. Plant J. **69,** 881-893.

Guo A., He K., Liu D., Bai S., Gu X., We L., and Luo J. (2005). DATF: a database of Arabidopsis transcription factors. Bioinformatics. **21,** 2568-2569.

Guo H., Lee T.H., Wang X., and Paterson A.H. (2013). Function relaxation followed by diversifying selection after whole-genome duplication in flowering plants. Plant Physiol. **162,** 769-778.

Gustafson-Brown C., Savidge B., and Yanofsky M.F. (1994). Regulation of the Arabidopsis floral homeotic gene APETALA1. Cell. **76,** 131-143.

Hamilton J.P., and Buell C.R. (2012). Advances in plant genome sequencing. Plant J. **70,** 177-190.

Harris E. M. . (1995). Inflorescences and floral ontogeny in Asteraceae: a synthesis of historicaland current concepts. Botanical Reviews **61,** 93-278.

Hartmann U., Höhmann S., Nettesheim K., Wisman E., Saedler H., and Huijser P. (2000). Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. Plant J. **21,** 351-360.

Hasson A., Plessis A., Blein T., Adroher B., Grigg S., Tsiantis M., Boudaoud A., Damerval C., and Laufs P. (2011). Evolution and diverse roles of the CUP-SHAPED COTYLEDON genes in Arabidopsis leaf development. Plant Cell. **23,** 54-68.

He X., and Zhang J. (2005). Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution. Genetics **169,** 1157-1164.

Heijmans K., Morel P., and Vandenbussche M. (2012). MADS-box Genes and Floral Development: the Dark Side. Journal of Experimental Botany. **63,** 5397-5404.

Henschel K., Kofuji R., Hasebe M., Saedler H., Münster T., and Theissen G. (2002). Two ancient classes of MIKC-type MADS-box genes are present in the moss Physcomitrella patens. Mol Biol Evol. **19,** 801-814.

Hepworth S.R., Valverde F., Ravenscroft D., Mouradov A., and Coupland G. (2002). Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. EMBO J. **21,** 4327-4337.

Hibara K., Karim M.R., Takada S., Taoka K., Furutani M., Aida M., and Tasaka M. (2006). Arabidopsis CUP-SHAPED COTYLEDON3 regulates postembryonic shoot meristem and organ boundary formation. Plant Cell. **18,** 2946-2957.

Hiratsu K., Ohta M., Matsui K., and Ohme-Takagi M. (2002). The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flowers. FEBS Letters. **514,** 351-354.

Honma T., and Goto K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature. **25,** 525-529.

Huala E., and Sussex I.M. . (1992). LEAFY interacts with floral homeotic genes to regulate Arabidopsis floral development. . Plant Cell. **4,** 901-913.

Huang H., Tudor M., Weiss C.A., Hu Y., and Ma H. (1995). The Arabidopsis MADS-box gene AGL3 is widely expressed and encodes a sequence-specific DNA-binding protein. Plant Mol Biol. **28,** 549-567.

Hughes M.K., and Hughes A.L. (1993). Evolution of duplicate genes in a tetraploid animal, Xenopus laevis. Mol Biol Evol. **10,** 1360-1369.

Immink R., Pose D., Ferrario S., Ott F., Kaufmann K., Leal Valentim F., De Folter S., Van der Wal F., van Dijk A.D., Schmid M., and Angenent G.C. (2012). Characterisation of SOC1's central role in flowering by the identification of its up- and downstream regulators. Plant Physiology, **162,** 433-449

Immink R.G., Ferrario S., Busscher-Lange J., Kooiker M., Busscher M., and Angenent G.C. (2003). Analysis of the petunia MADS-box transcription factor family. Mol Genet Genomics. **268,** 598-606.

Immink R.G., Tonaco I.A., de Folter S., Shchennikova A., van Dijk A.D., Busscher-Lange J., Borst J.W., and Angenent G.C. (2009). SEPALLATA3: the 'glue' for MADS box transcription factor complex formation. Genome Biol. **10**., R24-R24.16

Initiative, A.G. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature. **408,** 796-815.

Irish V. F., and Litt A. (2005). Flower development and evolution: gene duplication, diversification and redeployment. Curr. Opin. Genet. Dev. **15,** 454–460.

Irish V.F. (1999). Patterning the flower. Dev Biol. **209,** 211-220.

Irish V.F., and Sussex I.M. (1990). Function of the apetala-1 gene during Arabidopsis floral development. Plant Cell. **2,** 741-753.

Irizarry R.A., Hobbs B., Collin F., Beazer-Barclay Y.D., and Antonellis K.J. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. . Biostatistics **4,** 249–264.

Jack T. (2004). Molecular and genetic mechanisms of floral control. Plant Cell. **16,** S1-17.

Jack T., Brockman L.L., and Meyerowitz E.M. (1992). The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. Cell. **68,** 683-697.

Jofuku K.D., den Boer B.G., Van Montagu M., and Okamuro J.K. (1994). Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell. **6,** 1211-1225.

Johnson D.S., Mortazavi A., Myers R.M., and Wold B. (2007). Genome-wide mapping of in vivo protein-DNA interactions. Science **316,** 1497-1502.

Kagaya Y., Ohmiya K., and Hattori T. (1999). RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. Nucleic Acids Res. **27**, 470-8

Kardailsky I, Shukla V.K., Ahn J.H., Dagenais N., Christensen S.K., Nguyen J.T., Chory J., Harrison M.J., and Weigel D. (1999). Activation tagging of the floral inducer FT. Science. **286,** 1962-1965.

Kaufmann K., Melzer R., and Theissen G. (2005). MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. Gene. **347,** 183-198.

Kaufmann K., Muiño J.M., Østerås M., Farinelli L., Krajewski P., and Angenent G.C. (2010a). Chromatin immunoprecipitation (ChIP) of plant transcription factors followed by sequencing (ChIP-SEQ) or hybridization to whole genome arrays (ChIP-CHIP). Nature Methods **5,** 457-472.

Kaufmann K., Muiño J.M., Jauregui R., Airoldi C.A., Smaczniak C., Krajewski P., and Angenent G.C. (2009). Target genes of the MADS transcription factor SEPALLATA3: integration of developmental and hormonal pathways in the Arabidopsis flower. PLoS Biol. **7,** 0854-0875.

Kaufmann K., Wellmer F., Muiño J.M., Ferrier T., Wuest S.E., Kumar V., Serrano-Mislata A., Madueño F., Krajewski P., Meyerowitz E.M., Angenent G.C., and Riechmann J.L. (2010b). Orchestration of floral initiation by APETALA1. Science. **328,** 85-89.

Kayes J.M., and Clark S.E. (1998). CLAVATA2, a regulator of meristem and organ development in Arabidopsis. . Development. **125,** 3843-3851.

Kempin S.A., Savidge B., and Yanofsky M.F. (1995). Molecular basis of the cauliflower phenotype in Arabidopsis. Science. **267,** 522-525.

Kidder B.L., Hu G., and Zhao K. (2011). ChIP-Seq: technical considerations for obtaining high-quality data. Nat Immunol. **12,** 918-922.

Kieffer M., Master V., Waites R., and B. Davies. (2011). TCP14 and TCP15 affect internode length and leaf shape in Arabidopsis. Plant J. **68,** 147–158.

K**leinboelting N, Huep G, Kloetgen A, Viehoever P, and Weisshaar B** (2012) GABI-Kat SimpleSearch: new features of the Arabidopsis thaliana T-DNA mutant database. Nucleic Acids Res. **40**, D1211-1215.

Kofuji R., Sumikawa N, Yamasaki M., Kondo K., Ueda K., Ito M., and Hasebe M. (2003). Evolution and divergence of the MADS-box gene family based on genome-wide expression analyses. Mol Biol Evol. **20,** 1963-1977.

Kotilainen M., Elomaa P., Uimari A., Albert V.A., Yu D., and Teeri T.H. (2000). GRCD1, an AGL2-like MADS box gene, participates in the C function during stamen development in Gerbera hybrida. Plant Cell. **12,** 1893-1902.

Kramer E.M., Dorit R.L., and Irish V.F. (1998). Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. Genetics. **149,** 765-783.

Kramer E.M., Jaramillo M.A., and Di Stilio V.S. (2004). Patterns of gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms. Genetics **166,** 1011-1023.

Krizek B.A., and Meyerowitz E.M. (1996). The Arabidopsis homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function. Development. **122,** 11-22.

Langham R.J., Walsh J., Dunn M., Ko C., Goff S.A., and Freeling M. (2004). Genomic duplication, fractionation and the origin of regulatory novelty. Genetics. **166,** 935-945.

Lee J., and Lee I. (2010). Regulation and function of SOC1, a flowering pathway integrator. J Exp Bot. **61,** 2247-2254.

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

Levy Y.Y., and Dean C. (1998). Control of flowering time. Curr Opin Plant Biol. **1,** 49-54.

Lewis E.B. (1951). Pseudoallelism and gene evolution. Cold Spring Harb Symp Quant Biol. **16,** 159-174.

Li H., Ruan J., and Durbin R. (2008). Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res. **18,** 1851-1858.

Li X.G., Su Y.H., Zhao X.Y., Li W., Gao X.Q., and Zhang X.S. (2010). Cytokinin overproduction-caused alteration of flower development is partially mediated by CUC2 and CUC3 in Arabidopsis. . Gene. **450,** 109-120.

Li XY., MacArthur S., Bourgon R., Nix D, P.D.A., Iyer V.N., Hechmer A., Simirenko L., Stapleton M., Luengo Hendriks C.L., Chu H.C., Ogawa N., Inwood W., Sementchenko V., Beaton A., Weiszmann R., Celniker S.E., Knowles D.W., Gingeras T., Speed T.P., Eisen M.B., and Biggin M.D. (2008). Transcription factors bind thousands of active and inactive regions in the Drosophila blastoderm. PLoS Biol. **6, e27**.

Liljegren S.J., Ditta G.S., Eshed Y., Savidge B., Bowman J.L., and Yanofsky M.F. (2000). SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. Nature. **404,** 766-770.

Lin W.C., Shuai B., and Springer P.S. (2003). The Arabidopsis LATERAL ORGAN BOUNDARIES-domain gene ASYMMETRIC LEAVES2 functions in the repression of KNOX gene expression and in adaxial-abaxial patterning. Plant Cell. **15,** 2241-2252.

Litt A. (2007). An Evaluation of A-Function: Evidence from the APETALA1 and APETALA2 Gene Lineages. International Journal of Plant Sciences. **168,** 73-91.

Litt A., and IrishV. F. (2003). Duplication and diversification in the APETALA1/FRUITFULL floral homeotic gene lineage: implications for the evolution of floral development. . Genetics. **165,** 821-833.

Liu C, Teo ZW, Bi Y, Song S, Xi W, Yang X, Yin Z, and H., Y. (2013). A conserved genetic pathway determines inflorescence architecture in Arabidopsis and rice. Dev Cell. **25,** 612-622.

Liu C., Zhou J., Bracha-Drori K., Yalovsky S., Ito T., and Yu H. (2007). Specification of Arabidopsis floral meristem identity by repression of flowering time genes. Development. **134,** 1901-1910.

Liu C., T., Thong Z., and Yu H. (2009). Coming into bloom: The specification of floral meristems. . Development. **136,** 3379–3391.

Liu D., Wang D., Qin Z, Z.D., Yin L., Wu L., Colasanti J., Li A., and Mao L. (2013). The SEPALLATA MADS-box protein SLMBP21 forms protein complexes with JOINTLESS and MACROCALYX as a transcription activator for development of the tomato flower abscission zone. Plant J. **77,** 284–296.

Liu Z., and Meyerowitz E.M. (1995). LEUNIG regulates AGAMOUS expression in Arabidopsis flowers. Development. **121,** 975-991.

Livak K.J., and Schmittgen D.T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25,** 402-408.

Lopez-Vernaza M., Yang S., Müller R., Thorpe F., de Leau E., and J.., G. (2012). Antagonistic roles of SEPALLATA3, FT and FLC genes as targets of the polycomb group gene CURLY LEAF. PLoS One. **7**.e30715

Lynch M., and Force A. (2000). The probability of duplicate gene preservation by subfunctionalization. Genetics. **154,** 459-473.

Lynch M., Conery J., and Burger R. (1995). Mutation accumulation and the extinction of small populations . Am Nat. **146,** 489-518.

Lynch V.J., and Wagner G.P. (2008). Resurrecting the role of transcription factor change in developmental evolution. Evolution. **62,** 2131-2154.

Ma H, Yanofsky M.F., and Meyerowitz E.M. (1991). AGL1-AGL6, an Arabidopsis gene family with similarity to floral homeotic and transcription factor genes. Genes Dev. **5,** 484-495.

Ma H., Yanofsky M.F., and Meyerowitz E.M (1995)**.** The Arabidopsis MADS-box gene AGL3 is widely expressed and encodes a sequence-specific DNA-binding protein. Plant Molecular Biology **28,** 549-567.

Malcomber S.T., and Kellogg E.A. (2005). SEPALLATA gene diversification: brave new whorls. Trends Plant Sci. **10,** 427-435.

Mandel M.A., and Yanofsky M.F. (1995). A gene triggering flower formation in Arabidopsis. Nature. **12,** 522-524.

Mandel M.A., and Yanofsky M.F. (1998). The Arabidopsis AGL9 MADS box gene is expressed in young flower primordia. Sex. Plant Reprod. **11,** 22–28.

Mandel M.A., Gustafson-Brown C., Savidge B., and Yanofsky M.F. (1992). Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. Nature. **360,** 273-277.

Mantovani R. (1999). The molecular biology of the CCAAT-binding factor NF-Y. Gene. **239,** 15-27.

Mardis E.R. (2008). Next-generation DNA sequencing methods. Annu Rev Genomics Hum Genet. **9,** 387-402.

Martinez-Castilla L.P.and Alvarez-Buylla E. R.. (2003). Adaptive evolution in the Arabidopsis MADS-box gene family inferred from its complete resolved phylogeny. Proc Natl Acad Sci. **100**, 13407-13412

Masiero S., Colombo L., Grini P.E., Schnittger A., and Kater M.M. (2011). The emerging importance of type I MADS box transcription factors for plant reproduction. Plant Cell. **23,** 865-872.

Mathieu J., Yant LJ, M.F., Küttner F., and Schmid M. (2009). Repression of flowering by the miR172 target SMZ. PLoS Biol. **7**.

Melzer R., and Theissen G. (2009a). Reconstitution of 'floral quartets' in vitro involving class B and class E floral homeotic proteins. Nucleic Acids Res. **8,** 2723–2736.

Melzer R., Verelst W., and Theissen G. (2009b). The class E floral homeotic protein SEPALLATA3 is sufficient to loop DNA in 'floral quartet'-like complexes in vitro. Nucleic Acids Res. **37,** 144-157.

Meyerowitz E.M. (1997). Genetic control of cell patterns in developing plants. Cell. **88,** 299-308.

Meyerowitz E.M., Bowman J.L., Brockman L.L., Drews G.N., Jack T., S.L.E., and Weigel D. (1991). A genetic and molecular model for flower development in Arabidopsis thaliana. Dev Suppl. **1,** 157-167.

Michaels S.D., and Amasino R.D. (1999). LOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell. **11,** 949–956.

Michaels S.D., Ditta G., Gustafson-Brown C., Pelaz S., Yanofsky M.F., and Amasino R.M. (2003). AGL24 acts as a promoter of flowering in Arabidopsis and is positively regulated by vernalization. Plant J. **33,** 867-874.

Mitsuda N., and Ohme-Takagi M. (2009). Functional analysis of transcription factors in Arabidopsis. Plant Cell Physiol **50,** 1232-1248.

Mizukami Y., and Ma H. (1992). Ectopic expression of the floral homeotic gene AGAMOUS in transgenic Arabidopsis plants alters floral organ identity. Cell. **71,** 119-131.

Mizukami Y., and Ma H. (1995). Separation of AG function in floral meristem determinancy from that in reproductive organ identity by expressing antisense AG RNA. Plant Mol. Biol. **28,** 767–784.

Moon J., Suh S.S., Lee H., Choi K.R., Hong C.B., Paek N.C., Kim S.G., and Lee I. (2003). The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant J. **35,** 613-623.

Moore R.C., Grant S.R., and Purugganan M.D. (2005). Molecular population genetics of redundant floral-regulatory genes in Arabidopsis thaliana. Mol Biol Evol. **22,** 91-103.

Muiño J.M., Hoogstraat M., van Ham R.C., and van Dijk A.D. (2011). PRI-CAT: a web-tool for the analysis, storage and visualization of plant ChIP-seq experiments. Nucleic Acids Res. **39,** W524-527.

Nam J., Kim J., Lee S., An G., Ma H., and Nei M. (2004). Type I MADS-box genes have experienced faster birth-and-death evolution than type II MADS-box genes in angiosperms. Proc Natl Acad Sci U S A. **101,** 1910-1915.

Ng M., and Yanofsky M.F. . (2001). Function and evolution of the plant MADS-box gene family. Nat. Rev. Genet. **2,** 186–195.

Nicol J.W., Helt G.A., Blanchard S.G. Jr, Raja A., Loraine A.E.(2009). The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics. **25**, 2730-2731.

Nilsson O., Lee I., Blázquez M.A., and Weigel D. (1998). Flowering-time genes modulate the response to LEAFY activity. Genetics. **150,** 403-410.

Nocker S.V. (2009). Development of the abscission zone. Stewart Postharvest Review **5,** 1-5.

Norman C., Runswick M., Pollock R., and Treisman R. (1988). Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell. **55,** 989-1003.

Ohno S. (1970). Evolution by Gene Duplication. Allen & Unwin; Springer-Verlag

Okamuro J.K., Caster B., Villarroel R., Van Montagu M., and Jofuku K.D. (1997). The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. Proc Natl Acad Sci U S A. **94,** 7076-7081.

ÓMaoiléidigh D.S., Wuest S.E., Rae L., Raganelli A., Ryan P.T., Kwasniewska K., Das P., Lohan A.J., Loftus B., Graciet E., and Wellmer F. (2013). Control of reproductive floral organ identity specification in Arabidopsis by the C function regulator AGAMOUS. Plant Cell. **25,** 2482-2503.

Ori N., Eshed Y., Chuck G., Bowman J.L., and Hake S. (2000). Mechanisms that control knox gene expression in the Arabidopsis shoot. Development. **127,** :5523-5532.

Otto S.P., and Whitton J. Polyploid incidence and evolution. Annu Rev Genet. **34,** 401-437.

Papatsenko D., and Levine M.S. (2008). Dual regulation by the Hunchback gradient in the Drosophila embryo. Proc Natl Acad Sci USA. **105,** 2901–2906.

Papp B., Pál C., and Hurst L.D. (2003). Dosage sensitivity and the evolution of gene families in yeast. Nature. **10,** 194-197.

Parcy F., Bomblies K., and Weigel D. (2002). Interaction of LEAFY, AGAMOUS and TERMINAL FLOWER1 in maintaining floral meristem identity in Arabidopsis. . development. **129,** 2519–2527.

Parenicová L., de Folter S., Kieffer M., Horner D.S., Favalli C., Busscher J., Cook H.E., Ingram R.M., Kater M.M., Davies B., Angenent G.C., and Colombo L. (2003). Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in Arabidopsis: new openings to the MADS world. Plant Cell. **15,** 1538-1551.

Passmore S., Maine G. T., Elble R., Christ C., and Tye B. K. (1988). Saccharomyces cerevisiae protein involved in plasmid maintenance is necessary for mating of MAT alpha cells. . J. Mol. Biol. **204,** 593–606.

Pelaz S., Tapia-López R., Alvarez-Buylla E.R., and Yanofsky M.F. (2001a). Conversion of leaves into petals in Arabidopsis. Curr Biol. **6,** 82-84.

Pelaz S., Ditta G.S., Baumann E., Wisman E., and Yanofsky M.F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. Nature. **405,** 200-203.

Pelaz S., Gustafson-Brown C., Kohalmi S.E., Crosby W.L., and Yanofsky M.F. (2001b). APETALA1 and SEPALLATA3 interact to promote flower development. Plant J. **26,** 385-394.

Picard D. (1994). Regulation of protein function through expression of chimaeric proteins. . Curr Opin Biotechnol **5,** 511-515.

Pickett F.B., and Meeks-Wagner D.R. (1995). Seeing double: appreciating genetic redundancy. Plant Cell. **7,** 1347-1356.

Pinyopich A., Ditta G.S., Savidge B., Liljegren S.J., Baumann E., Wisman E., and Yanofsky M.F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature. **424,** 85-88.

Pnueli L., Hareven D., Broday L., Hurwitz C., and Lifschitz E. (1994). The TM5 MADS Box Gene Mediates Organ Differentiation in the Three Inner Whorls of Tomato Flowers. Plant Cell. **6,** 175-186.

Posé D., Yant L., and SChmitt, M.. (2012). The end of innocence: flowering networks explode in complexity. Curr Opin Plant Biol **15,** 45-50.

Qu W., Zhou Y., Zhang Y., Lu Y., Wang X., Zhao D., Yang Y., and Zhang C. (2012). MFEprimer-2.0: A fast thermodynamics-based program for checking PCR primer specificity. Nucleic Acids Res. **40**, W205-208.

Reynolds E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. **17,** 208.

Rhoades M.W., Reinhart B.J., Lim L.P., Burge C.B., Bartel B., and Bartel D.P. (2002). Prediction of plant microRNA targets. Cell. **110,** 513-520.

Riechmann J.L., Heard J., Martin G., Reuber L., Jiang C., Keddie J., Adam L., Pineda O., Ratcliffe O.J., Samaha R.R., Creelman R., Pilgrim M., Broun P., Zhang J.Z., Ghandehari D., Sherman B.K., and Yu G. (2000). Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. science. **290,** 2105-2110.

Robertson G., Hirst M., Bainbridge M., Bilenky M., Zhao Y., Zeng T., Euskirchen G., Bernier B., Varhol R., Delaney A., Thiessen N., Griffith O.L., He A., Marra M., Snyder M., and

Jones S. (2007). Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. Nat Methods. **4,** 651-657.

Robinson M.D., and Smyth G.K. (2007). Moderated statistical tests for assessing differences in tag abundance. Bioinformatics. **23,** 2881-2887.

Rojo E., Sharma V.K., Kovaleva V., Raikhel N.V., Fletcher J.C., and (2002). CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. . Plant Cell. **14,** 969–977.

Rounsley S.D., Ditta G.S., and Yanofsky M.F. (1995). Diverse roles for MADS box genes in Arabidopsis development. Plant Cell. **7,** 1259-1269.

Rubin G., Tohge T., Matsuda F., Saito K., and Scheible W.R. (2009). Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis. Plant Cell. **21,** 3567-3584.

Ruelens P., de Maagd R.A., Proost S., Theißen G., Geuten K., and Kaufmann K. (2013). FLOWERING LOCUS C in monocots and the tandem origin of angiosperm-specific MADS-box genes. Nat Commun. **4:2280**

Running M.P., and Meyerowitz E.M. (1996). Mutations in the PERIANTHIA gene of Arabidopsis specifically alter floral organ number and initiation pattern. Development. **122,** 1261-1269.

Samach A., Onouchi H., Gold S.E., Ditta G.S., Schwarz-Sommer Z., Yanofsky M.F., and Coupland G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science. **288,** 1613-1616.

Sauerbrunn N. and Schlaich N. L. (2004). *PCC1*: a merging point for pathogen defence and circadian signalling in *Arabidopsis*. Planta**. 218,** 552-561.

Savidge B., Rounsley S.D., and Yanofsky M.F. (1995). Temporal relationship between the transcription of two Arabidopsis MADS box genes and the floral organ identity genes. Plant Cell. **7,** 721-733.

Schena M., Shalon D., Davis R.W., and Brown P.O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. **270,** 467-470.

Schneeberger K., and Weigel D. (2011). Fast-forward genetics enabled by new sequencing technologies. Trends in Plant Sci. **16,** 282-288.

Schwarz-Sommer Z., Huijser P., Nacken W., Saedler H., and Sommer H. (1990). Genetic Control of Flower Development by Homeotic Genes in Antirrhinum majus. Science. **250,** 931-936.

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

Sessions R. A. (1997). Arabidopsis (Brassicaceae) flower development and gynoecium patterning in wild type and Ettin mutants. Am J Bot. **84,** 1179–1191.

Shannon S., and Meeks-Wagner D.R. (1991). A mutation in the Arabidopsis TFL1 gene affects inflorescence meristem development. . Plant Cell. **3,** 877-892.

Shuai B., Reynaga-Peña C.G., and Springer P.S. (2002). The lateral organ boundaries gene defines a novel, plant-specific gene family. Plant Physiol. **129,** 747-761.

Siefers N., Dang K.K., Kumimoto R.W., Bynum W.E.T. ,Tayrose G., and Holt B.F. (2009). Tissue-specific expression patterns of Arabidopsis NF-Y transcription factors suggest potential for extensive combinatorial complexity. Plant Physiol. **149,** 625–641.

Smaczniak C., Immink R.G., Muiño J.M., Blanvillain R., Busscher M., Busscher-Lange J., Q.D., D., Liu S., Westphal A.H., Boeren S., Parcy F., Xu L., Carles C.C., Angenent G.C., and Kaufmann K. (2012). Characterization of MADS-domain transcription factor complexes in Arabidopsis flower development. Proc Natl Acad Sci U S A. **109,** 1560-1565.

Smyth D.R., Bowman J.L., and Meyerowitz E.M. (1990). Early flower development in Arabidopsis. Plant Cell. **2,** 755-767.

Soltis D.E, Albert V.A., Leebens-Mack J., Palmer J.D., Wing R.A., dePamphilis C.W., Ma H., Carlson J.E., Altman N., Kim S., Wall P.K., Zuccolo A., and Soltis P.S. (2008). The Amborella genome: an evolutionary reference for plant biology. Genome Biol. **9**.

Soltis D.E., Ma H., Frohlich M.W., Soltis P.S., Albert V.A., Oppenheimer D.G., Altman N.S., dePamphilis C., and Leebens-Mack J. (2007). The floral genome: an evolutionary history of gene duplication and shifting patterns of gene expression. Treends in Plant Sci. **12,** 358-367.

Soltis P., and Soltis D. (2013). Angiosperm Phylogeny: A Framework for Studies of Genome Evolution. Plant Genome Diversity **2,** 1-11.

Sommer H., Beltrán J.P., Hiuijser P., Pape H., Lönnig W.E., and Saedler H. (1990). A homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. EMBO J. **9,** 605-613.

Sridhar V.V., Surendrarao A., and Liu Z. (2006). APETALA1 and SEPALLATA3 interact with SEUSS to mediate transcription repression during flower development. Development. **133,** 3159–3166.

Strabala T.J., O'donnell P.J., Smit A.M., Ampomah-Dwamena C., Martin E.J., Netzler N., Nieuwenhuizen N., Quinn B.D., Foote H.C., and Hudson K.R. (2006). Gain-of-function phenotypes of many CLAVATA3/ESR genes, including four new family members, correlate with tandem variations in the conserved CLAVATA3/ESR domain. Plant Physiol. **140,** 1331-1344.

Tautz D. (2000). A genetic uncertainty problem. . Trends Genet. **16,** 475-477.

Taylor J.S., and Raes J. (2004). Duplication and divergence: the evolution of new genes and old ideas. Annu Rev Genet. **38,** 615-643.

Teeri T.H., Uimari A., Kotilainen M., Laitinen R., Help H., Elomaa P., and Albert V.A. (2006). Reproductive meristem fates in Gerbera. J Exp Bot. **57,** 3445-3455.

Theissen G. (2001). Development of floral organ identity: stories from the MADS house. Curr Opin Plant Biol. **4,** 75-85.

Theissen G., and Saedler H. (2001). Plant biology. Floral quartets. Nature. **48,** 351-358.

Theissen G., Becker A., Di Rosa A., Kanno A., Kim J.T., Münster T., Winter K.U., and Saedler H. (2000). A short history of MADS-box genes in plants. Plant Mol Biol. **42,** 115-149.

Thomas J.H. (1993). Thinking about genetic redundancy. Trends in Genetics. **9,** 395-399.

Tooke F., Ordidge M., Chiurugwi T., and Battey N. (2005). Mechanisms and function of flower and inflorescence reversion. J. Exp. Bot. **56,** 2587-2599.

Trevaskis B., Tadege M., Hemming M.N., Peacock W.J., Dennis E.S., and Sheldon C. (2007). Short vegetative phase-like MADS-box genes inhibit floral meristem identity in barley. Plant Physiol. **143,** 225-235.

Uimari A., Kotilainen M., Elomaa P., Yu D., Albert V.A., and Teeri T.H. (2004). Integration of reproductive meristem fates by a SEPALLATA-like MADS-box gene. Proc Natl Acad Sci U S A. **101,** 15817-15822.

Urbanus S.L., de Folter S., Shchennikova A.V., Kaufmann K., Immink R.G., and Angenent G.C. (2009). In planta localisation patterns of MADS domain proteins during floral development in Arabidopsis thaliana. BMC Plant Biol **9,** 5.

Vajda V., Raine J.I., and Hollis C.J. (2000). Indication of global deforestation at the Cretaceous-Tertiary boundary by New Zealand fern spike. Science. **294,** 1700-1702.

Vandenbussche M., Zethof J., Souer E., Koes R., Tornielli G.B., Pezzotti M., Ferrario S., Angenent G.C., and Gerats T. (2003). Toward the analysis of the petunia MADS box gene family by reverse and forward transposon insertion mutagenesis approaches: B, C, and D floral organ identity functions require SEPALLATA-like MADS box genes in petunia. Plant Cell. **15,** 2680-2693.

Veita R.A., Bottani S., and Birchler J.A., (2008). Cellular reactions to gene dosage imbalance: genomic, transcriptomic and proteomic effects. Trends Genet. **24**, 33-35.

Vrebalov J., Ruezinsky D., Padmanabhan V., White R., Medrano D., Drake R., Schuch W., and Giovannoni J. (2002). A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science. **12,** 343-346.

Vroemen C.W., Mordhorst A.P., Albrecht C., Kwaaitaal M.A., and de Vries S.C. (2003). The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. Plant Cell. **15,** 1563-1577.

Wagner A. (1998). The fate of duplicated genes: loss or new function? Bioessays. **20,** 785-788.

Weigel D., and Meyerowitz E.M. (1993). Activation of floral homeotic genes in Arabidopsis. Science. **261,** 1723-1726.

Weigel D., Alvarez J., Smyth D.R., Yanofsky M.F., and Meyerowitz E.M. (1992). LEAFY controls floral meristem identity in Arabidopsis. Cell. **69,** 843-859.

Wellmer F., Alves-Ferreira M., Dubois A., Riechmann J.L., and Meyerowitz E.M. (2006). Genome-wide analysis of gene expression during early Arabidopsis flower development. PLoS Genet. **7, e117**.

Wenkel S., Turck F., Singer K., Gissot L., Le Gourrierec J., Samach A., and Coupland G. (2006). CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of Arabidopsis. . Plant Cell. **18,** 2971-2984.

Western T.L., George W., and Haughn G.W. (1999). BELL1 and AGAMOUS genes promote ovule identity in Arabidopsis thaliana. Plant J. **18,** 329-336.

Whitacre J.M. (2012). Biological robustness: paradigms, mechanisms, and systems principles. Front Genet. **3: 67**.

Winter C.M., Austin R.S., Blanvillain-Baufumé S., Reback M.A., Monniaux M., Wu M.F., Sang Y., Yamaguchi A., Yamaguchi N., Parker J.E., Parcy F., Jensen S.T., Li H., and Wagner D. (2011). LEAFY target genes reveal floral regulatory logic, cis motifs, and a link to biotic stimulus response. Dev Cell. **20,** 430-443.

Winter D., Veinegar B., Hardeep N., Ammar R., Wilson G. V., and Povart N.J. (2007). An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets PLoS One. **2, e718**.

Wu G., and Poethig R.S. (2006). Temporal regulation of shoot development in Arabidopsis thaliana by miR156 and its target SPL3. Development **133,** 3539-3547.

Wu G., Park M.Y., Conway S.R., Wang J.W., Weigel D., and Poethig R.S. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell. **138,** 750-759.

Würschum T., Gross-Hardt R., and Laux T. . (2006). APETALA2 regulates the stem cell niche in the Arabidopsis shoot meristem. . Plant Cell. **18,** 295-307.

Xin X.F., and He S.Y. (2013). Pseudomonas syringae pv. tomato DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. Annu Rev Phytopathol. **51,** 473-498.

Xu L., Xu Y., Dong A., Sun Y., Pi L., Xu Y., and Huang H. (2003). Novel as1 and as2 defects in leaf adaxial-abaxial polarity reveal the requirement for ASYMMETRIC LEAVES1 and 2 and ERECTA functions in specifying leaf adaxial identity. development. **130,** 4097-4107.

Xu X. M., Rose A., Muthuswamy S., Jeong S.Y., Venkatakrishnan S., Zhao Q., and Meier I.

(2007). NUCLEAR PORE ANCHOR, the Arabidopsis homolog of Tpr/Mlp1/Mlp2/megator, is involved in mRNA export and SUMO homeostasis and affects diverse aspects of plant development. Plant Cell. **19,** 537-548.

Yanhui C., Xiaoyuan Y., Kun H., Meihua L., Jigang L., Zhaofeng G., Zhiqiang L., Yunfei Z., Xiaoxiao W., Xiaoming Q., Yunping S., Li Z., Xiaohui D., Jingchu L., Xing-Wang D., Zhangliang C., Hongya G., Li-Jia Q. (2006)**.** The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol. **60,** 107-24.

Yang Y., Fanning L., and Jack T. (2003). The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. Plant J. **33,** 47–59.

Yanofsky M.F. (1995). FLORAL MERISTEMS TO FLORAL ORGANS: Genes Controlling Early Events in Arabidopsis Flower Development. Annu. Rev. Plant Physiol. Plant Mol. Bioi. **46,** 167-188.

Yanofsky M.F., Ma H., Bowman J.L., Drews G.N., Feldmann K.A., and Meyerowitz E.M. (1990). The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. Nature. **346,** 35-39.

Yant L., Mathieu J., Dinh T.T., Ott F., Lanz C., Wollmann H., Chen X., and Schmid M. (2010). Orchestration of the floral transition and floral development in Arabidopsis by the bifunctional transcription factor APETALA2. Plant Cell. **22,** 2156-2170.

Yu H., Ito T., Wellmer F., and Meyerowitz E.M. (2004). Repression of AGAMOUS-LIKE 24 is a crucial step in promoting flower development. Nat Genet. **36,** 157-161.

Zahn L.M., Kong H., Leebens-Mack J.H., Kim S., Soltis P.S., Landherr L.L., Soltis D.E., Depamphilis C.W., and Ma H. (2005). The evolution of the SEPALLATA subfamily of MADS-box genes: a preangiosperm origin with multiple duplications throughout angiosperm history. Genetics. **169,** 2209-2223.

Zhao L., Kim Y., Dinh T.T., and Chen X. (2007). miR172 regulates stem cell fate and defines the inner boundary of APETALA3 and PISTILLATA expression domain in Arabidopsis floral meristems. Plant J. **51,** 840-849.

Zhou D., Xin Z., Yi L., Zhenhai Z., and Zhen S. (2010). agriGO: a GO analysis toolkit for the agricultural community. Nucl. Acids Res. **38**, W64-W70.

Zhu Q.H., and Helliwell C.A. (2011). Regulation of flowering time and floral patterning by miR172. J Exp Bot. **62,** 487-495.