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Functional Analysis of the *STRUBBELIG-RECEPTOR FAMILY* in *Arabidopsis thaliana*

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Table of contents

Summary ***3***

Zusammenfassung ***4***

1. Introduction ***5***

1.1 Plant development 5

1.2 Plant receptor-like kinases (RLKs) 6

1.2.1 Diversity and structure of RLK Gene family 6

1.2.2 Function of RLKs 9

1.3 Leucine-rich repeat receptor-like kinases (LRR-RLKs) in plants 10

1.3.1 Structural features of LRR-RLKs 12

1.3.2 Extracellular regions and corresponding ligands 12

1.3.3 Importance of signal transduction pathways and functions in plants ... 14

1.3.4 BRI1 as an example of LRR-RLK pathway 15

1.3.5 The LRR-V/ *STRUBBELIG (SUB)-RECEPTOR-FAMILY (SRF)* 17

1.3.6 Putative functional relevance during plant development 19

1.4 The purpose of this study 19

2. Material and Methods ***20***

2.1 Material 20

2.1.1 Plant material and growth condition 20

2.1.2 Bacteria strains 20

2.1.3 Equipments for microscopy and morphological analysis 21

2.2 Methods 21

2.2.1 Plant work 21

2.3 Molecular analysis 22

2.3.1 DNA-extraction 22

2.3.2 Expression analysis 27

3. Results ***37***

3.1 Expression of the *SRF* Genes 37

3.1.1 *In-situ* Hybridization (ISH) of *SRF1-8* 37

3.1.2 AtGenExpress data of the *SRF*-gene family 48

3.1.3 Promoter GUS analysis of *SRF1*, *SRF4*, *SRF6* and *SRF7* 51

3.2. Characterization of T-DNA insertion lines 57

3.3 Phenotype analysis 65

3.3.1 Root analysis 66

3.3.2 Pollen phenotype 68

4. Discussion ***70***

4.1 Expression analysis 70

4.1.1 *In-situ* Hybridization analysis 70

4.1.2 AtGenExpress data 73

4.1.3 Promoter-GUS studies 74

4.2 Reverse genetic approach by using T-DNA lines 75

4.3 Redundancy aspects in the SRF family? 78

4.4 Phenotypic investigations 79

4.4.1 Root phenotype 80

4.4.2 Pollen development investigations 83

4.5 Conclusion and outlook 84

4.5.1 Other possibilities of getting mutants 84

4.5.2 Potential functions of the *SRF* members 85

References: ***87***

Acknowledgement ***98***

Summary

Receptor-like kinases (RLKs) play a crucial role in a diverse range of signaling processes. Investigations of these genes give evidence to their role in plant growth and development, hormone perception, pathogen resistance and self-incompatibility. The most common extracellular motif is the Leucine-Rich-Repeat (LRR) present in over half of the *Arabidopsis* RLKs. Already well-characterized members of LRR-RLKs are CLAVATA1, ERECTA and BRASSINOSTEROID INSENSITIVE1.

The aim of this subject was to identify the function of eight LRR-V/*STRUBBELIG-RECEPTOR-FAMILY* (*SRF/LRR-V*) genes encoding LRR-RLKs in *Arabidopsis thaliana.*

To explore *SRF* gene function several different T-DNA insertion lines in each gene were identified and characterized. *SRF4* appears to be a positive regulator of leaf size as *srf4* showed smaller vegetative leaves than wild type, while *35S::SRF4* plants exhibits larger leaves. Above ground organs of other *srf* mutants displayed no obvious mutant phenotype under normal growth conditions. Subsequently, several double mutants were constructed, which showed also no alteration to wild type. Additional to the above ground phenotypic analysis, root growth of *srf4* and *srf5* mutants was investigated in the presence of different sucrose concentrations. Roots of *srf4* mutants grown in 1% sucrose exhibit shorter roots. These findings also indicate a possible function of *SRF4* in root size.

Expression patterns of all *SRF* genes were characterized by *in-situ Hybridization*. All *SRF* genes show expression in ovules and anthers during late flower stages. *SRF2, SRF4, SRF5, SRF7, SRF8* and *SUB* also show expression in the inflorescence meristem, young flower primordia and arising young flower organs, like sepals, anthers, gynoecium and ovules. In addition the expression of *SRF1, SRF4, SRF6* and *SRF7* was determined by analysing transgenic plant lines expressing the *GUS* gene under the control of the respective *SRF* promoter sequences. These investigations provide new insights in the functional elucidation of the *Arabidopsis thaliana* genome.

Zusammenfassung

Receptor-like Kinasen (RLKs) nehmen als eine der wichtigsten Gruppen von Rezeptoren in einer Reihe von Signal-Transduktions-Prozessen einen entscheidenden Stellenwert während der Pflanzenentwicklung ein. Des Weiteren sind sie an der Hormonerkennung, der Pathogenresistenz sowie der Selbstinkompatibiltät beteiligt. In über der Hälfte der *Arabidopsis thaliana* RLKs tritt das Leucine-Rich-Repeat (LRR) Motiv in ihrer extrazellulären Rezeptordomäne auf. Herausragende und bereits gut charakterisierte Beispiele sind die LRR-RLKs CLAVATA1, ERECTA und BRASSINOSTEROD INSENSITIVE 1.

Das Ziel dieser Arbeit war die funktionelle Analyse von acht *LRR-V/ STRUBBELIG-RECEPTOR-FAMILY (LRR-V/SRF)* Genen, die LRR-RLKs kodieren.

Für funktionelle Analysen wurden verschiedene T-DNA-Insertions-Linien genauer bestimmt und näher beschrieben. *SRF4* ist möglicherweise ein positiver Regulator der Blattgröße, da *srf4* Mutanten kleinere Blätter aufwiesen, wohingegen *35S::SRF4* Pflanzen entsprechend größere. Andere *srf* Mutanten, die sich unter normalen Wachstumsbedingungen entwickelten, zeigten keinen veränderten Phänotyp. Ferner sind Doppelmutanten der jeweils nah verwandten *SRF* Gene angefertigt worden, die ebenfalls keine phenotypische Veränderung gegenüber dem Wildtyp aufweisten. Zusätzlich zu den oberirdischen Organanalysen wurde das Wurzelwachstum von *srf4*- und *srf5*- Mutanten auf unterschiedlichen Saccharose-Konzentrationen untersucht. Die Wurzeln von *srf4* Mutanten, die auf 1% Sucrose gewachsen sind, zeigten verkürzte Wurzellängen. Dies deutet ebenfalls darauf hin, dass *SRF4* ein positiver Regulator der Wurzelgröße sein könnte.

Das Expressionsmuster aller *SRF* Gene wurde mit Hilfe von *in-situ* Hybridisierung charakterisiert. Alle *SRF* Gene sind in späteren Stadien der Blütenentwicklung in den Ovulen und Antheren exprimiert. *SRF2, SRF4, SRF5, SRF7, SRF8* und *SUB* sind auch im Infloreszenzmeristem, Blütenprimordium und jungen Blütenorganen, wie den Sepalen, Antheren, dem Gynoecium und den Ovulen exprimiert. Außerdem wurde die Expression von *SRF1, SRF4, SRF6* and *SRF7* mittels transgenen Reporterlinien bestimmt. Dabei exprimierten die transgenen Pflanzen das *GUS*-Gen, welches von der jeweiligen Promotersequenz gesteuert wurde. Diese Arbeit stellt damit einen wichtigen Beitrag für die funktionelle Aufklärung des *Arabidopsis* Genoms dar.

1. Introduction

1.1 Plant development

How a single cell becomes a complex organism, is the main question of developmental biology. Cell division, cell growth, cell differentiation and cell death belongs to the essential steps in the development of plants. For that the mechanisms and the factors controlling the behavior of cells towards cell division, growth and death need to be understood.

The flowering plant *Arabidopsis thaliana* represents an important model system to identify genes and determine their function. Advantages of using *Arabidopsis thaliana* are the small size of the plants for an easy handling, the short generation time of six weeks, the possibility to grow well under controlled conditions (on soil or media), high fertility (up to 10 000 seeds per plant) and easy maintenance of mutant lines by self-fertilization (Page *et al.,* 2002). This was the first plant genome, which was fully sequenced. It has one of the smallest genome in plants with about 125 Mb and 25 500 genes (The Arabidopsis Genome Initiative 2000; Campbell 2006). Sequence comparisons and homology analysis between animal genes with already known function and plant genes can be taken into consideration for first indications about the gene structure and possible functions in plants. The question about the developmental processes of each gene in plants still remains to be unveiled.

Plant evolution is clearly distinguishable from that of animals under absolute different modes and patterns of development. Multicellularity evolved independently in plants and animals and under very different constraints from their unicellular ancestors. Before the evolution of these two kingdoms crucial characteristics diverged such as chloroplasts, autotrophic metabolism, the presence of a semirigid cell wall and the absence of mobility found in plant lineages (Leyser *et al.,* 2003).

Differences in plants and animals are also characterized in lacking specific proteins or pathways. At least key elements are partially conserved in plants, animals and fungi, which regulate processes that are basic to unicellular as well as multicellular organisms. For example essential steps in plant and animal steroid biosynthetic pathways or the ubiquitin mediated proteolysis are highly conserved (McCarty *et al.,* 2000; Scott 2000). Genetic analysis of diverse plant signaling pathways revealed novel families of DNA binding proteins that are unique to plants. The plant-specific proteins were discovered almost exclusively in developmental pathways that are unique to plant biology. It suggests that recruitment of new transcription factors has played a significant role in the evolution of novelties in plants and animals ((McCarty *et al.,* 2000).

1.2 Plant receptor-like kinases (RLKs)

1.2.1 Diversity and structure of RLK Gene family

The RLKs in the *Arabidopsis thaliana* genome is composed of a gene familiy with 610 members. They represents one of the largest gene families that comes to 2,5% of *Arabidopsis* protein coding genes. 60% of all kinases in Arabidopsis are presented by this family and constitute nearly all transmembrane kinases in Arabidopsis (Shiu *et al.,* 2001b, 2003). It represents a remarkable number compared to animal or humans with less than 100 *RLK* genes. In addition the alternative splicing of *RLK* transcripts increases the complexity within the plant genome (Stein *et al.,* 1991; Kumar *et al.,* 1994; Giranton *et al.,* 1995; Tobias *et al.,* 1996; Bassett *et al.,* 2000). This significant high number strongly indicates that the plant cells predominantly use RLKs for sensing external signals and regulating gene expression. RLK proteins in plants consist of a predicted signal sequence, an extracellular region, a single transmembrane region and cytoplasmic carboxyterminal serine/threonine kinase domain.

By contrast, animal RLKs contain predominantly ligand-activated receptor tyrosin kinases (RTKs) (Walker 1994; Shiu *et al.,* 2001b, a). According to the kinase domain sequences and the extracellular domain, the RLK family is divided in 44 different RLK subfamilies. The 417 genes with receptor configurations can be classified in more than 21 structural classes by their extracellular domains (Shiu *et al.,* 2001b). Beside the kinase domain the juxtamembrane domain, localized between transmembrane and kinase domain, plays an important role in downstream signaling cascades by providing a docking site for substrates.

24% of the 610 *Arabidopsis thaliana* genes in the RLK/Pelle family analysed do not have a predicted extracellular domain. They constitute the receptor-like cytoplasmatic kinases (RLCKs). The prolin-rich extensin-like receptor kinases (Silva *et al.,* 2002) does not contain a signal sequence (Shiu *et al.,* 2004) but they are inserted into the membrane via the usual ER-translocator protein machinery with some slight modifications (Singer 1990).

The name "receptor-like kinases" is often applied instead of Receptor kinases (RKs) because their corresponding ligands have yet to be identified with some exceptions. The extracellular domain gene structure is more variable than the intracellular domain, consistent with the function of this domain in sensing different signals.

1.2.1.1 Evolution of the family

Plant RLKs are composed of a monophyletic group with respect to kinase domains when compared to the other eukaryotic kinase families, related to animal RLKs. Raf kinases in animals were paraphyletic to the RLK family and are the closest relatives to RLKs among the *Arabidopsis thaliana* sequences analyzed.
It is most likely that kinase domains from the RLK/Pelle family were recruited multiple times by fusion with different extracellular domains to form the subfamily found in *Arabidopsis.*

According to the proposed hypothetical events that occurred in the evolution of the receptor kinase groups an early gene duplication event led to the founding of two lineages that diversified into the RTK and Raf families on one hand and the RLK/Pelle family on the other (Shiu *et al.,* 2001b). This seems to be occurred before the divergence of plants and animals. The soluble Pelle-like and Raf kinases form complexes with cell surface receptors, which are responsible for signal transduction to downstream effectors (Daum *et al.,* 1994; O'Neill *et al.,* 1998). RLK, RTK, RSK, and Raf kinase genes compose a monophyletic group, named the receptor kinase group. The Pelle kinases in animals are characterized as the homologs of the *Arabidopsis th.* RLK family and form the RLK/Pelle family. The large number of the RLK/Pelle genes only in land plants indicates that the extensive expansion of the RLKs occurred after the divergence of plant and animal lineages. It further demonstrates the functional importance to land plant evolution. The monophyletic origin of the RLK/Pelle family implies that the expansion of the family to its present size in *Arabidopsis* was the result of multiple gene-duplication events like tandem duplication and large-scale block duplication. Gene duplications resulting from unequal crossing-over, polyploidization followed by reshuffling of chromosomal regions (Blanc *et al.,* 2000; The Arabidopsis Genome Initiative 2000; Vision *et al.,* 2000).

The subfamilies differed in their chromosomal distribution. For example the DUF26 subfamily is located on chromosome 4. On the other hand 51 genes from LRR X, XI, and XIII subfamilies are spread over all five chromosomes. More then 30% of the *RLK* family members in *Arabidopsis thaliana* are in tandem repeats with 2 to 19 genes. Conclusively tandem duplication events and large-scale duplications of chromosomes are two of the potential mechanisms responsible for the expansion of the *RLK/Pelle* family in *Arabidopsis thaliana*. *RLKs* that are involved in development seem to have rarely been duplicated after Arabidopsis-rice split, in contrary to those implicated in defense/disease resistance.

That's why most recent expansion seems to occur in defense/resistance-related genes of the *RLK/Pelle* family. It is possibly the result of strong selection pressure for recognizing pathogens.
By contrast the intracellular domain has been under stronger purifying selection because of functional constraints in transducing signals to downstream components constantly (Shiu *et al.,* 2004). The lineage-specific expansions are limited to specific subfamilies such as legume lectin (L-LEC), leucin-rich repeat subfamily Ia (LRR-Ia), and WALL-ASSOCIATED KINASE (WAK).

1.2.2 Function of RLKs

RLKs support crucial properties for all living systems because of the ability to perceive and process information into the cell and the communication between cells from chemical signals via cell surface receptors. As a consequence developmental pathways in plants are more strongly coupled to light and other external environmental cues than in animals. The abundance of plant RLKs represents a plant-specific adaptation for extracellular signal sensing and propagation.

RLKs can be grouped into two broad categories (Shiu *et al.,* 2001a; Shiu *et al.,* 2004). The first represents RLKs that control plant growth and development, which comprises for example organ shape determination regulated by *Arabidopsis thaliana ERECTA (ER)* gene (Torii *et al.,* 1996), meristem maintenance controlled by *CLV1* (Clark *et al.,* 1997), regulation of cell growth through the phytohormone response via *BRASSINOSTEROID-INSENSITIVE-1 (BRI1)* signaling (Li, J. M. *et al.,* 1997), reproduction procedures of self-incompatibility controlled by *S-LOCUS RECEPTOR KINASES (SRKs)* during pollen/pistil interaction (Stein *et al.,* 1996), control of cell morphogenesis and differentiation by maize *CRINKLY4 (CR4*) (Becraft *et al.,* 1996) and regulation of abscission by *HAESA* (Jinn *et al.,* 2000).

The second group consists of RLKs involved in plant-microbe interactions and defense responses. In this category some RLKs are implicated in bacterial resistance response mediated by the rice resistance gene *Xa21* (Song *et al.,* 1995), in pathogen response regulated by perception of flagellin by *FLAGELLIN INSENSITIVE2 (FLS2)* and in systemin signaling (Scheer *et al.,* 2002). Further they are crucial for interactions with plant symbionts including *NODULATION RECEPTOR KINASE (NORK)/SYMBIOSIS RECEPTOR-LIKE KINASE (SYMRK)* and *HYPER AUTOREGULATION OF NODULATION RECEPTOR 1 (HAR1)* in fungal and/or bacterial symbiosis (Endre *et al.,* 2002) and in early steps of nodulation and Nod factor perception mediated by Lysine motif-containing RLKs (Madsen *et al.,* 2003; Radutoiu *et al.,* 2003).

1.2.2.1 Biochemical properties of plant RLKs

Plant and animal RLKs can be distinguished by the finding that most plant RLKs examined to date show serine/threonine kinase specificity, whereas animal receptors are tyrosine kinases. Two exceptions are POLLEN RECEPTOR-LIKE KINASE1 (PRK1) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK). The Phosphorylation in AtSERK1 occurs intermolecular at serine/threonine and tyrosine residues (Mu *et al.,* 1994; Shah, Vervoort *et al.,* 2001).

1.3 Leucine-rich repeat receptor-like kinases (LRR-RLKs) in plants

Among the transmembrane receptor-like kinases in plants leucin-rich repeat (LRR)-RLKs constitute the largest family with 12 subfamilies based on the kinase domain phylogeny with over ~222 members in *Arabidopsis thaliana* genome (Shiu *et al.,* 2001b; Yin *et al.,* 2002; Torii 2004).

LRR-RLKs control a wide variety of developmental and defense-related processes. Developmental functions include stem cell maintenance and meristem differentiation regulated by CLV1 (Clark *et al.,* 1997), hormone perception by BR1 signal transduction (Li, J. M. *et al.,* 1997; Bishop *et al.,* 2002; Li, Wen *et al.,* 2002; Nam *et al.,* 2002), vascular and leaf patterning controlled by *VASCULAR HIGHWAY1 (VH1)* (Clay *et al.,* 2002), regulation of cell proliferation and organ shape regulated by *ERECTA* (Torii *et al.,* 1996). 1996; Lease et al. 2001), organ abscission controlled by *HEASA* (Jinn *et al.,* 2000), pollen development controlled by *EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS (EMS/EXS)* (Canales *et al.,* 2002; Zhao *et al.,* 2002) or pollen-pistil Interaction controlled by *L. exculentum POLLEN RECEPTOR KINASE* (*LePRK1-3*) (Muschietti *et al.,* 1998; Kim *et al.,* 2002). Plant-microbe interactions, disease resistance and symbiosis are further processes regulated by LRR-RLK. Defense and stress responses are regulated for example by the perception of flagellin by FLS2 (Gómez-Gómez *et al.,* 2000; Gomez-Gomez *et al.,* 2002) or fungal perception regulated by the rice Xa21 (Song *et al.,* 1995). Systemin receptor SR160 plays a role in wounding responses through the perception of BR and systemin in tomato or potatos (Scheer *et al.,* 2002). Further they are crucial for fungal and/or bacterial symbiosis including NORK (Endre *et al.,* 2002), *Lotus japonicus* SYMRK and HAR1 (Krusell *et al.,* 2002; Nishimura *et al.,* 2002).

Beside LRR-RLKs and LRR-RLPs LRR-extensins (LRXs) is a third class of plant extracellular LRR proteins. They are presumed to be cell wall localized and may be critical regulator of cell shape specification, for example LRX1 is required for proper root hair cell morphogenesis and elongation (Baumberger *et al.,* 2001).

1.3.1 Structural features of LRR-RLKs

The LRR-domain forms a protein region of 24 amino acids with conserved leucines where the number of LRRs varies from 1 to 32. The LRR-RLK family is grouped into sub-classes *LRRI-LRR-XIII* based on the structural arrangement of LRRs and the organization of introns in the extracellular domains of the individual LRR-RLKs. The founding is consistent with the pattern found based on the kinase domains within these groups (Shiu *et al.,* 2001b).

1.3.2 Extracellular regions and corresponding ligands

The diverse sequence motifs, which are present in the putative extracellular regions of RLKs, are responsible for interactions with other protein, carbohydrates, or lipids. How receptors transform their extracellular signal into the cell is different when looking at the first step in signaling. Best characterized receptors in animals like transforming growth factor-β (TGF)- β and epidermal growth factor receptors (EGFR) form hetero-oligomers after ligand binding and further hyperphosphorylation, while in the absence of the ligand Receptor Serine/Threonine kinases (RSKs) of the TGF-β family exist as homodimers (Derynck *et al.,* 2003; Dijke *et al.,* 2004). Also EGFRs and other receptor Tyrosine kinases (RTKs) probably exist as monomers in the absence of their ligand (Burgess *et al.,* 2003).

1.3.2.1 The role of the extracellular LRR domain in plants

In plants heterodimerisation has been predicted to occur in the CLAVATA1 (CLV1), ER, ARABIDOPSIS CR4 (ACR4) and BRI1 receptor systems (Li, Wen *et al.,* 2002; Nam *et al.,* 2002; Diévart *et al.,* 2003; Shpak *et al.,* 2003; Cao *et al.,* 2005; Gifford *et al.,* 2005). BRI1 interacts with its coreceptor BRI1-ASSOCIATED KINASE (BAK1) or SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (SERK3) as an heterodimer. Xa21, CLV1 and ER may also function as heterodimers, whereas AtSERK1 forms a homodimer (Shah, Gadella *et al.,* 2001). LRRs have been also identified in plenty of proteins with diverse functions in yeast, flies and humans, where they are involved in protein-protein interactions (Kobe *et al.,* 1994). In plants, proteins with extracellular LRR motifs usually belong to the LRR-RLK family. An additional motif often associated with LRR-RLKs is paired cysteines. At least in the CLV1 signaling system it is implicated in receptor dimerization and for the BRI1 they seem to take over an important function for the signaling (Trotochaud *et al.,* 1999; Shiu *et al.,* 2001b)).

An interesting point to address is the LRR Intercepting Island domain. In some RLKs or RLPs tandem LRRs are intercepted by an island domain. In all known cases so far it is at the identical position between LRRs 4 and 5. These islands could be found in LRR-RLKs genes (e.g. in *BRI1* and *PHYTOSULFOKINE-α RECEPTOR* (*PSKR*) and LRR-RLPs (e.g., *CLV2* and *CLADOSPORIUM FULVUM*-resistance genes (*Cfs*). Researches about the island domains indicate for *BRI1* and *PSKR* that the island is important for ligand binding (Torii 2004). In contrast to *Cf9* and *Cf4* which show 100% identity in this island, but it is not a determinant for ligand recognition, as they interact with different ligands (Thomas *et al.,* 1997).

1.3.3 Importance of signal transduction pathways and functions in plants

LRR receptor kinases may be involved in perception of small molecules as well as peptides. Brassinosteroid (BR) is one example of an organic molecule. The Brassinosteroid receptor (BRI1) is described in greater detail in the section 1.3.3. There is an overwhelming availability for peptide hormones in plants considering the number of *LRR, CR4-type* and *S-domain* receptor kinase genes in *Arabidopsis thaliana.* Regarding the fact that putative peptide ligands found in plants identified so far failed to identify corresponding families of genes encoding peptide hormones in animals, suggests that these peptide signals are highly specialized (McCarty *et al.,* 2000). A number of studies indicate that LRR-RLKs act as dimmers, together with leucin-rich repeat receptor-like proteins (LRR-RLPs) or with receptor-like cytoplasmatic kinases (RLCKs), respectively. This suggests that LRR-RLPs may function as coreceptor with LRR-RLKs. Also LRR-RLPs are involved in similar functions. For example CLV2 and TOO MANY MOUTH (TMM) are involved in developmental processes and Cf9 and Cf4 in defense responses.

In the majority of LRR-RLKs the kinase domain seems required for signal transduction (Clark *et al.,* 1997; Friedrichsen *et al.,* 2000; Endre *et al.,* 2002). There is an overwhelming number of a variety of identified functions in plants, which indicate a high importance during the development of plants. Known functions are already shortly described at the beginning and especially for BRI1 RLK in the next paragraph.

1.3.4 BRI1 as an example of LRR-RLK pathway

The Brassinosteroid (BR) signaling pathway reveals one of the best characterized models for transforming external signals from the cell surface into the cell via LRR-RLKs, aside from the CLV signal transduction pathway and FLS2 process. The Brassinosteroid kinase signaling in plants, provide an insight into the essential control of plant growth, developmental responses and environmental responses such as stem elongation, vascular differentiation, leaf development, seed size, fertility, flowering time, senescence, and resistance to biotic and abiotic stresses (Li, J. M. *et al.,* 1997)Clouse1996, Altmann 1998 or only Wang 2006). *bri 1* mutants show dwarfed height, male sterility and de-etiolation in the dark.
Brassinolid (BL), the active form of BRs acts as the ligand for the LRR-X BRI-receptor in a ligand-dependent steroid hormone signaling. In the absence of Brassinosteroid (BR) BRI1 exist without the BRI1-ASSOCIATED RECEPTOR KINASE (BAK1), also known as *Arabidopsis thaliana* (AtSERK3) and forms an inactive homodimer (Wang *et al.,* 2005).
Upon binding of the ligand further autophosphorylation of BRI1 provides docking sites for known interactors such as the LRR-II RLK BAK1 and Transthyretin (Nam *et al.,* 2004) and following transphosphorylation of BRI1-BAK1 heterodimer. The alternative is that BR-mediated BAK1-BRI1 interactions and following transphosphorylation of BRI1 by BAK1 is necessary for initial BRI1 activation by carboxy-terminal phosphorylation.
But it has been shown that BRI1 and BAK1 may constitute a ligand-independent dimer, due to an association with eachother in the absence of the ligand. Nevertheless BL binding to BRI1 strengthens BRI1-BAK1 receptor dimer formation (Li and Nam 2002; Nam *et al.,* 2002; Russinova *et al.,* 2004).

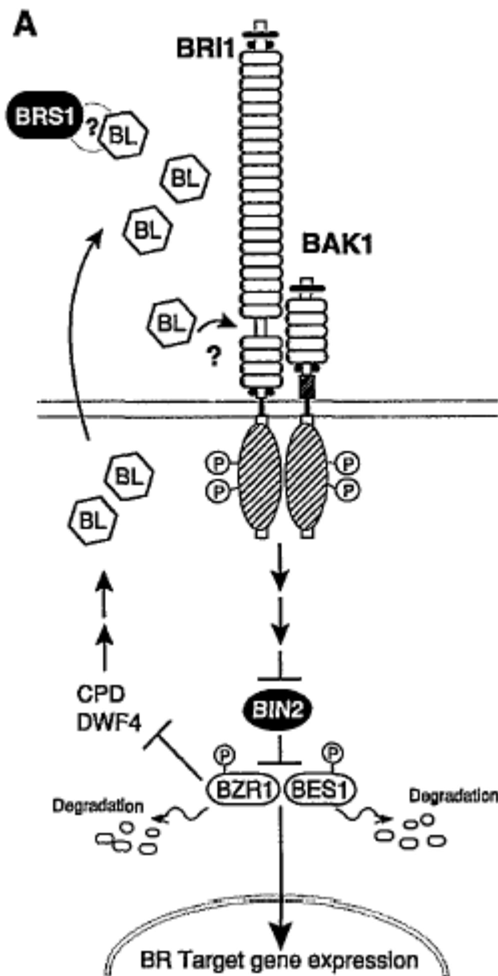


Figure 1-1. Brassinosteriod signaling pathway. The brassinolide (BL) binds to the island domain of BRI1 by itself or via a steroid-protein complex. BRS may modify the hypothetical BL-binding protein. This leads to a transphosphorylation of BRI1-BAK1 receptor heterodimer and the inhibition of BIN2, a cytoplasmic GSK3-like kinase that acts as a negative regulator and stabilization of BZR1 and BES1.
In the absence of BL, BIN2 phosphorylates the downstream components BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1), which triggers the degradation of BZR1 and BES1 via the proteosome pathway. In the presence of BL, stable BZR1 and BES1 translocate to the nucleus and upregulate BR translocate to the nucleus and upregulate BR target gene expression. BZR1 binds to target genes directly in order to turn them off and plays a role in the negative feedback regulation of BL biosynthesis, whereas BES1 acts together with BIM (resembles the animal transcription factor Myc) proteins to bind and to activate the expression of target genes. Schematic from Torii, 2004.

1.3.5 The LRR-V/ *STRUBBELIG (SUB)-RECEPTOR-FAMILY (SRF)*

SUB (Chevalier *et al.,* 2005) and the other eight related genes in Arabidopsis belong to the LRR-V class of RLKs (Shiu *et al.,* 2001b). Three *LEUCINE RICH REPEAT TRANSMEMBRANE PROTEIN KINASES* (*LTKs*) genes from *Zea mays* also belong to this family. *LTK1* was expressed throughout all tissues, whereas *LTK2* and *LTK3* transcripts were only detected in the endosperm. So far no function is known for any *LTK* gene (Li *et al.,* 1998). Additionally twelve genes are identified in *Oryza sativa* (Matsumoto *et al.,* 2001; Goff *et al.,* 2002; Morillo *et al.,* 2006) belonging to this family.

1.3.5.1 *SUB* as the first functionally characterized LRR-V RLK member in *Arabidopsis thaliana*

SUB was found by an EMS mutant screen to look for plants with an ovule defect. The *sub* mutant was isolated by (Schneitz *et al.,* 1997). The *sub* phenotype shows disorganized ovule with irregularities in outer integument, where the ovules are fertile, slightly or severely affected. In addition irregularities in the cell division plane, with a periclinal division in the L2 layer of stage-3 floral meristem were visible. Moreover *sub* plants show reduced plant height together with a twisted stem. Petals were twisted as well at flower stage 13-15. By looking at the cellular level 30-day old *sub* stems revealed a reduced number of epidermal, cortex, and pith cell (Chevalier *et al.,* 2005). Independent research discovered that *SUB/SCRAMBLE (SCM)* is also required for the correct patterning of cells that produce root hairs in the developing root epidermis (Kwak *et al.,* 2005). Taken together, *SUB* is involved in the correct establishment of the cell division plane orientation and controls cell number, cell size and shape.

1.3.5.2 *SRF* gene structure defining a LRR-RLK

The gene structure resembles that of the already described LRR-RLK properties. The putative kinase structure displays an extra-cellular domain (ECD) with the signal peptide, the SUB domain, six leucine rich repeats (LRRs), a transmembrane domain (TM), an intracellular juxtamembrane domain (JM) and an intracellular kinase domain (CD). SUB, SRF1, SRF6, SRF7 contain a proline-rich region in the extracellular domain and SRF3 in the intracellular domain right before the kinase domain.

Two different proteins of SRF1A and SRF1B are translated, due to an alternative splicing process. SRF1B lacks the intracellular kinase domain. Due to sequence homologies in the extracellular domain in the so-called sub domain, SUB and the SRF members becomes an own family.

1.3.5.3 Phylogenetic tree of the *SUB-RECEPTOR-FAMILY* (*SRF*)

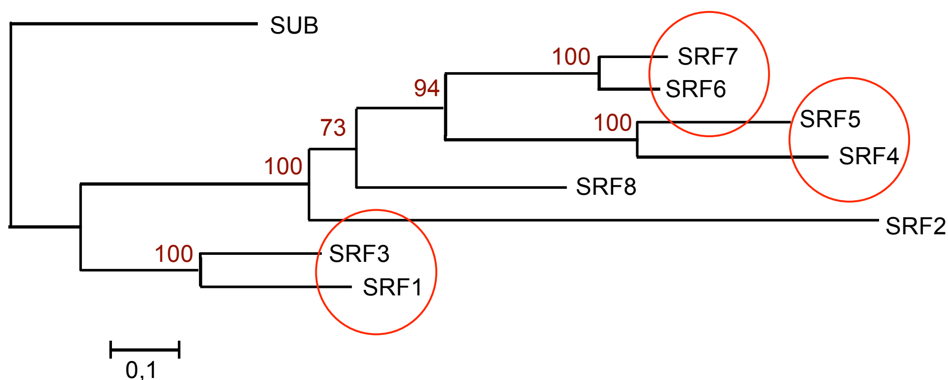


Figure 1-2. Phylogenetic tree of the SRF-gene family. The tree was created with the computer program Tree-Puzzle 5.2 (Maximum likelihood method, JTT-model, 8 gamma-rates). For the analysis the amino acid sequences of the SUB and Kinase domain (~530 aa) of the SRF proteins were used. The branch support values are indicated. The red circles show the pairs of created double mutants.

1.3.6 Putative functional relevance during plant development

SRF4 seems to be involved in correct organ sizing. *Srf4* T-DNA lines showed smaller vegetative leaves (Eyb2007), but also flower sepals and petals are smaller (Banu Eyuboglu and Christine Skornia, personal communication). The roots of *srf4-2* and *srf4-3* are also smaller. SRF4 seems to be involved in a cell expansion process (Banu Eyuboglu and Christine Skornia, personal communication).

1.4 The purpose of this study

The aim of this study was to get information of the general function of this family. To learn more about the expression pattern an *in-situ Hybridization* approach was taken. In this experiment the time dependent and spatial mRNA distribution in flowers could be detected. In a second approach *SRF*-promoter GUS fusions were used. T-DNA insertion lines were used for the functional morphological analysis. The phenotype of the *srf* T-DNA lines grown at different developmental stages under greenhouse condition was evaluated.

2. Material and Methods

2.1 Material

2.1.1 Plant material and growth condition

The seeds were sown on Typ T Einheitserde extra fine (= Topferde) Gebr. Patzer GmbH&Co KG from Sinntal-Jossa, Germany. To avoid a built-up or water dam, Perlit was used on the ground of the pots. For simultaneous growing the pots were stratified four days in a coldroom (4°C). Than the seed pots were transferred to the greenhouse with a plastic cap/hood for app. 6-8 days to have optimal humid condition for germination. The plants were growing under long day conditions (16h/day) with the help of Philips SON-T PLUS 400-W fluorescent bulbs to compensate the missing sunlight. *Arabidopsis thaliana* Heyh. var. Landsberg *erecta* (L*er*), Wassileskija (WS) and Columbia (Col) were used as WT strains. The T-DNA lines were received from different seed resources inclusive SIGnAL (Alonso *et al.,* 2003), the University of Wisconsin Knockout facility (Endre *et al.,* 2002), SAIL (Syngenta Biotechnology, Research Triangle Park, NC, USA) (Sessions *et al.,* 2002), and GABI-KAT (Rosso *et al.,* 2003). All T-DNA lines are listed in detail in Table 3-2a in the Result Chapter.

2.1.2 Bacteria strains

DH5 α E.coli strains were used for cloning the promoter GUS fragments. For plant transformation Agrobacteria GV 3101 strain were used.

2.1.3 Equipments for microscopy and morphological analysis

Images for *in-situ* analysis were taken using a LSM 510 microscope from Zeiss coupled to a Kodak Professional DCS 760 digital camera. GUS-stained whole organ pictures were taken with Olympus SZX12 stereomicroscope with the attendant Color View camera (Soft Imaging System). All pictures were processed using Adobe® version 7.

2.2 Methods

2.2.1 Plant work

2.2.1.1 crosses

Closely related single T-DNA lines were crossed by emasculation and handpolination to get double mutants (see phylogenetic tree of Figure 1-2 in the Introduction Chapter). Also all homozygote insertion lines were cross into L*er* background.

2.2.1.2 Phenotype analysis

To investigate the phenotype of the homozygote T-DNA mutant lines and double mutants not only the aboveground organs were studied also the roots and hypocotyls. Rosette leaves, caulin leaves, stem, flower organs (sepal, petal, stamen and carpel) and siliques were observed under the dissecting microscope. Most of the insertion lines (SAIL) have a BASTA resistance (see Table 3-1). The plant growth can be performed on soil directly without taking sterile conditions and subsequent spraying the 5 days old seedlings with BASTA (AgrEvo Finale ®). The stock solution of 11,33% glufosinate-ammonium was diluted 2000x for spraying over the seedlings, which provides a 300 µM working solution.

For root and hypocotyl analysis surface–sterilized seeds were sown on 1% MS agar plates containing 0,9% agar and generally 1% sucrose (Murashige *et al.,* 1962). Different sucrose (0,3-2%) concentrations were also checked. After Stratification for 4 days the plates were grown vertical arranged either in a cell-culture room at 22 °C with continuous light for 10 days for root growth or placed for 8 h to the light and than wrapped in alufoil and a cardboard box for dark hypocotyls growth for 64 h.For looking of eventual differences in the pollen development, pollen nuclei from different flower stages were stained with DAPI (1 µg/ml dissolved in water), like mentioned in (Johnson *et al.,* 2001). Decolourized 0.1% (w/v) aniline blue was used to stain callose (Regan *et al.,* 1990) for looking at pollen tubes.

2.3 Molecular analysis

General molecular analysis were done like proposed in (Sambrook *et al.,* 2001). Differences are described in the following chapter.

2.3.1 DNA-extraction

DNA extraction from one plant leaf was performed according to the method of (Fulton *et al.,* 1995). One leaf was collected to extract DNA. Plant tissue was grinded with a pistil and liquid nitrogen to receive fine powder. 750 µl microprep buffer was added and incubate at 60°C for 1 h.

2.3.1.1 Identification of the T-DNA

The insertion site was verified by the PCR-based reverse-genetic screens utilizing T-DNA end primers that are directed towards the left and right border repeats in conjunction with 5' and 3' gene-specific primers oriented towards the T-DNA borders. The following sequencing gives information about the exact integration of the T-DNA. Table 2-1a, b shows all primers for T-DNA analysis, which were used. The standard PCR reaction was done with different annealing temperatures according to the primer melting temperature (Tm-4°C).

Table 2-1a. Gene specific primer sequences for analysing the T-DNA locations

Gene (AGI code)	*srf* mutant	T-DNA line code	vector	Location	primer orien-tation	Sequences
At2g20850	*srf 1-2*	Garlic 182A08	pCSA	Intron	for	TGC TTT AGT TGC GGC TAT TAA CAG TCT C
					rev	GAT TCT GGG ATG GTT CCT GTA AAG TTG T
					for	AGA TCG ATG AGA TCT GGG AGA GAC AAC
					rev	GGT ATC TTG CCA GAC AAA AGG TTA TTG
	srf 1-3	Garlic 1297E4	pDAP	Promoter	for	ATC TCA CCG GTG ACG GTA ATG AGT TTA A
					rev	GAG ACT GTT AAT AGC CGC AAC TAA AGC A
	srf 1-7 [a]	Salk 087666	prok2	Exon	for	TGC TTT AGT TGC GGC TAT TAA CAG TCT C
					rev	TGC TAG ACA AAT CTC TAC GCA TAT GTC G
					for	CAG GGA TTT TAG CAA CAA TCA CAT TG
					rev	GGT ATC TTG CCA GAC AAA AGG TTA TTG
At5g06820	*srf 2-1* [a]	Garlic 407 E2	pCSA	Promoter	for	GTA TTG ACC ACA ACA ATG CAA ATG AGC C
					rev	GCA AAA ACA GTT AAG GAC GTT GGA GTC A
	srf 2-2 [a]	Garlic 119 B10	pCSA	Intron	for	CTT TTG GGC AGC CTT GGA A
					rev	GGG TCA GAT TGT TGT AAG CCA TG
	srf 2-3 [a]	Garlic 591 A03	pDAP	Exon	for	TTC CGT TTG GCT TGC CTC C
					rev	CCA AAA GAG CTC GGT AGA TCT CC

(Table 2-1a continued)

At4g03390	*srf 3-1*	Garlic 797F7	pDAP	Promoter	for	TCC ATG TGG GTA ACA ACA ATG TAA GCG A
					rev	GCT AAG GTT CAA TGA ACT TTC GTC GTT G
	srf 3-3	Garlic 244C5	pCSA	Intron	for	ATT ATG ATT CAT TCC TCG AGT GTC GAG C
					rev	ATA TCT CTG AAA GAC GCC CTT ACA TGC T
	srf 3-7 [a]	Salk 029908	prok2	Exon	for	CTC TCA GGA TTG GGT TTT TGG G
					rev	GGA GAT CGA AGG AAT CCA GAT CAG
	srf 3-9	Salk 051048	prok2	Exon	for	AGC TAA TCA GTT CAC CGG AAG CAT CCC
					rev	TTT TCA GAT CGA CCA GGT GGT GGC A
At3g13065	*srf 4-2*	Garlic 230E8	pCSA	Exon	for	GCT GTA CAT TTC TCT ATT CAC ACG TCC A
					rev	CAC AGG TAA GTG AGC GAT TTC AAG TTT C
	srf 4-3	Garlic 253A9	pCSA	Exon	for	AAT GTG TTG GTA TTG CAG AGA TGG CTC T
					rev	TTG ACC ACT TGT TTC CTC CAG TTC TAA C
At1g78980	*srf 5-1* [a]	Salk 010161	prok2	Exon	for	GGT CGT GAA TGG TCA TTC TTG TCC AAA C
					rev	GAA CTT ACA GAT TGG CAA TGT TGG GTG G
	srf 5-2	Gabi 95A05	pAC161	Intron	for	TCC CTA AAT CCA ATC ACA ATG G
					rev	CCA TTG AGC TTG TTT TGT CCA A
At1g53730	*srf 6-2*	Garlic 269D10	pCSA	Promoter	for	GGA AAC CAC AGA GAA GTG AAA GGT TCA
					rev	CTA AAG AGC CAA TCA TAG CCA TTA GCC A

(Table 2-1a continued)

	srf6-4	Salk 062310	prok2	Promoter	for	GGAAACACACAGAGA AGTGAAAGGTTCA
					rev	CTA AAG AGC CAA TCA TAG CCA TTA GCC A
At3g14350	*srf 7-2* [a,b]	Wis mln 21.13	pD991	Intron	for	AGA GAG GTC CCT GAA AAC ACA CAA AAT C
					rev	AAT CTT GGA TCT GCT TCA TGT AAC CGA C
	srf 7-3	Gabi 040G01	pAC161	Intron	for	GAG GAA GCA TTC AGC AAG AAC A
					rev	TCT GGA TCA CTT GGA TTC ATG C
At4g22130	*srf 8-2*	Salk 111750	prok2	5' UTR	for	AGA ACG CGA GTC AA TGT TGC C
					rev	GGT TCT CAG GAA AAC AGA CGG TGT

[a] complex insertion [b] WS (Wassileskija) background

Table 2-1b. Vector-specific primers for analysis of the T-DNA locations

T-DNA line	vector	primer orientation	sequence
Garlic/SAIL lines	pDAP/pCSA	LB	TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C
	pDAP	RB	ATT AGG CAC CCA GGC TTT ACA CTT TAT G
	pCSA	RB	CAA ACT AGG ATA AAT TAT CGC GCG CGG TGT CA
		RB	AAG AAA GGG ATC TTC ACT CGC G
SALK lines	prok2	LB	TGG TTC ACG TAG TGG GCCATC G
	prok2	RB	CGA GAC GAG CAA GAT TGG CC
WIS	pD991	LB	CAT TTT ATA ATA ACG CTG CGG ACA TCT AC
GABI	pAC161	LB	GGG CTA CAC TGA ATT GGT AGC TC
	pAC161	RB	GTG GAT TGA TGT GAT ATC TCC ACT G

2.3.2 Expression analysis

2.3.2.1 RNA isolation

The RNA from the T-DNA lines were isolated with the modified instruction of (Verwoerd *et al.,* 1989). 100 mg grinded plant tissue (sufficient for the following RT-PCR) was mixed with hot phenol containing buffer A (0,1M LiCl, 0,01 M EDTA, 1 % SDS, 0,1 M Tris (pH 9.0) and Chloroform. The upper water phase was than precipitated overnight at 4°C with 8 M LiCl to get the final LiCl concentration of 2 M. 2µg of purified RNA (dissolved in 100µl DEPC water) was used for cDNA amplification. The RNA quality was checked with electrophoresis using denaturating 1,5% agarose gel containing 2,2M formaldehyde.

2.3.2.2 cDNA amplification

2 µg of purified RNA and 0.54 µg/µl dT(18)-Primer were denaturate for 5 min at 72 °C. For primer annealing it was incubated 2 min at RT. After chilling on ice 20 U M-MuLV Reverse Transcriptase and buffer components were added and incubate for 1 h at 37 °C. After the synthesis the reaction was stopped at 95 °C for 5 min. To verify the cDNA quality, GAPc (cytosolic glyceraldehyde-3-phosphate dehydrogenase-gene)-primer was taken for a control PCR reaction. Gapc encodes for an widely and equal expressed gene in every tissue of the plants thus a good control (Shih *et al.,* 1991).

2.3.2.3 Reverse Transcriptase PCR (RT-PCR)

The primers used for this experiment are listed in Table 2-1c. They are all situated after the insertion in the gene, which is also illustrated in Figure 3-1.

Table 2-1c. Primer sequence for transcript profiling of the T-DNA lines

Gene (AGI code)	gene name	primer orientation	Sequence
At2g20850	*SRF 1*	for	CCC CTT CTG ATC ATC ATC ACC CA
		rev	ACC CTG CAG CTT CAA AGC AC
At5g06820	*SRF 2*	for	TTA TGA ATT TTC CCA GAC CTG AGA C
		rev	GCT CTT TCT TCT TGC AGA TTT ATC G
At4g03390	*SRF 3*	for	CCA CCT CGA CTG CAC CAT CA
		rev	AGG TCT CCG CAG CCT CTC AA
At3g13065	*SRF 4*	for	GTG GGA GGC CAA AAG CAG AA
		rev	GGT TTT AGC CGC AAT CTC CAG
At1g78980	*SRF 5*	for	CGT CGA AGA CAA CCA GTT TGA AGG ATG G
		rev	CGG TAT CAG TGA AAG ACA TCA CAC GCG A
At1g53730	*SRF 6*	for	TCC AAG AAA TCA GGA ATC GGA GCG GG
		rev	CCA CCA CTG TGG ATT TCT TGA CAG CA
At3g14350	*SRF 7*	for	AGC AAC AAT AAC CTT GGA GGC G
		rev	GGG ATC CAG CCT GTG AAC CGA TTG T
		for	TGG TGA TTC CAG CAA TAG CA
		rev	CAA CAG CAG CTT TCT TTG CA
At4g22130	*SRF8*	for	ACACCTGAGGTGCAGGAGCA GAGGGT
		rev	CGGTGCAGTATCCAGCCAAGG GAACA
At3g04120	*gapC*	for	CAC TTG AAG GGT GGT GCC AAG
		rev	CCT GTT GTC GCC AAC GAA GTC

2.3.2.4 *In-situ* Hybridization

The protocol was modified from the publications of (Grossniklaus *et al.,* 1998), (Long *et al.,* 1998) and (Balasubramanian *et al.,* 2000). A detailed protocol can be found at http://plantdev.bio.wzw.tum.de/methods/inSituProtocol.pdf.

Tissue fixation: The flower tissues of 30 days old plants were directly fixed in scintillation vials containing 50% ethanol, 5% glacial acetic acid and 3.7% formaldehyde with twice vacuum application for 10 min. Four different stages were classified: young flower, containing inflorescent meristem (only IM), flower stage (FS) 1-5, middle flowers (FS 6-11) and old flowers (FS 12 upwards). After 12h incubation the samples were dehydrated through an ethanol series (50%, 60%, 70%, 80%, 90%, 95% Ethanol with 0.1% Eosin Y (Sigma, E-4382), each 45 minutes). Eosin Y was used for better visualisation during sectioning. After clearing with a Histoclear series (Xylolersatz XEM-200, Vogel GmbH + Co., KG, National Diagostics, USA), the tissue was slowly infiltrated with molten Paraplast and embedded in suitable molds with a plastic carrier. The molds must be kept at 4°C before sectioning for at least 3 h and are stable for several months.

Sectioning: The sections of 8 mm thickness were made with a Leica microtom (RM 2145) and transferred to already coated slides (SUPERFROST PLUS, Menzel-Glaeser 041300), which supports the stable binding of the tissues. The slides were baked on a heating plate overnight at 42°C. For *Hybridization* the wax was removed from the slides by clearing them twice with 100% Histoclear for 10 min. The slides were rehydrated through an ethanol series from 100% to 30% followed by an uptake in NaCl 0.85% for 2 min and rinsing them in a 1x phosphate-buffered saline solution (PBS; 0,13 M NaCl, 3 mM NaH_2PO_4, 7 mM Na_2HPO_4). The digestion with 1µg/ml proteinase K (Sigma P-2308) in 100 mM Tris (pH7.5), 50 mM EDTA for 30 min at 37°C was important for penetration of the probe into the tissue. Keeping the slides for 2 min in 2mg/ml Glycine-PBS stopped this reaction.

After 2 min in 1 x PBS the tissue was post fixated 2 min with 4% Paraformaldehyde (Fluka 76240) in 1 x PBS (pH7) at room temperature (RT) to decrease the diffusion and the loss of cellular RNA. Afterwards the tissue was acetylated with 1,3% Triethanolamine (Sigma T1377) and 0,3% acetic anhydride (Sigma A6404) in 250ml dH_2O (pH8) for 2 min. It blocks the positive charged amino acids of proteins in the tissue to avoid background and false binding of the probe. The following dehydration with an increasing ethanol series allows a better penetrance of the probes.

Probe synthesis: For all *SRF* genes already prepared cDNA templates (made by Banu Eyuboglu) in pCR II Topo Vector with the suitable RNA transcription promoter were used for synthesis. The *in vitro* transcription of the Digoxygenin-labeled sense or antisense RNA probes was performed with SP6, T3 or T7 RNA polymerase (Roche), the DIG RNA labelling mix (Roche Diagnostics) including DIG-dUTPs, transcriptase (40 U), 2U of RNAse free DNAse (Roche Diagnostics), RNAsin (RNase inhibitor, 25U/ml Amersham Biosciences) and 20 mM $MgCl_2$. There is a detailed list (Table 2-2a) about the vector, direction of the cDNA integrated in the vector, the enzymes, which linearized 5 mg of vector and the length of the synthesised RNA-fragment. The *INO* control-probe was synthesised from pSK-INO, like described in (Sieber *et al.,* 2004). Fragments, which were longer than 450 bp, were hydrolysed with Hydrolysis Carbonate buffer, followed by Neutralization buffer. After purification and drying, the probe was taken up in 50 µl dH_2O and subsequently diluted 1:10 in Hybridization Buffer (50 % Formamide (Invitrogene 15515026), 10% Dextran sulphate Sodium Salt (Amersham 17-0340-01), 1x Denhardts (Sigma D2532), 0,5 mg/ml tRNA (Roche 109541541001) in 10 mM Tris pH 7.5, 1 mM EDTA, 0,3 M NaCl buffer). This serves also for the stock concentration of the different probe concentrations.

Hybridization: The digoxigenin (DIG)-labelled stock RNA probe, diluted with Hybridization buffer depending on the different probe concentrations was denatured at 80°C for 2 min and transferred to ice. For the *INO* control sample 1-5 µl of probe were taken.

The concentration of the other *SRF*-gene probes varies from 5-30 µl. The total volume for one slide was 120µl. The probe was applied on the dried slide and covered with a coverslip. For Hybridisation it was moved to a humidified box and incubated in an oven at 55°C oven overnight (14-16hours). Afterwards the slides were washed once with 2x SSC (300 mM NaCl, 30 mM Na_3-citrate-$2H_20$), four times 30 min with 0.2x SSC at 55°C once at 37°C and RT for 5 min and finally rinsed in 1xPBS. To prevent unspecific binding the tissue was blocked with 1x Blocking reagent (Roche Diagnostics 1 096 176) in 100 mM Maleic Acid and 150 mM NaCl (pH 7.5) for 45 min and for 30 min the second time at RT. For equilibration the slides were flooded with BSA wash solution (1% BSA, 0.3% Triton-X100, 100 mM Tris HCl pH 7.5, 150 mM NaCl) for 45 min at RT. The Anti-Digoxigenin antibodies coupled with alkaline phosphatase (Roche 1 093 274 150 U) were diluted 1:1250 in BSA wash solution and applied to the slides with a cover slip for 1 hour and 30 min at RT. To wash the antibodies the slides were applied three times 30 min with BSA wash solution at RT. Removing all detergent the slides were equilibrated twice for 15 min with TNM-50 (100 mM Tris-HCl pH 9.5, 500 mM NaCl, 50 mM $MgCl_2$). The stabilized substrate solution Western Blue (Promega S3841) was applied to the slides for the subsequent color detection and overlayed with a coverslip. They were incubated in the dark for 12-16 hours. The reaction was stopped by putting them in 1x TE buffer (10 mM TrisHCl pH 8, 1 mM EDTA pH 8). The slides were mounted in 50 ml medium of Glycerol/TE (50% v/v) to keep them for longer term.

Table 2-2a. *In-situ* probe synthesis with c-DNA (wtCol)

***SRF*-gene in pCR II Topo if not mentioned differently**	**Name and length of the cDNA and size inside the vector in kb**	**full length of gene in kb**	**enzyme for linerizing**	**Promotor for RNA-Poly-merase**	**cDNA orien-tation in the vector**
*SRF*1-sense	*SRF* 1 race pCR	2,6	Xho I	Sp6	forward
*SRF*1-antisense	2,6 kb		BamH I	T7	
*SRF*1-sense	*SRF*1 full length	2,6	Xho I	SP 6	forward
*SRF*1-antisense	2,6 kb		BamH I	T 7	
*SRF*2-5'-sense	*SRF* 25'	2,429	Xba I	SP 6	forward
*SRF*2-5'antisense	1,4 kb		BamH I	T 7	
*SRF*2-5'-sense	*SRF*2 full	2,429	Xba I	SP 6	forward
*SRF*2-5'antisense	2,429 kb		BamH I	T 7	
*SRF*3-5'-sense	*SRF* 35'	3,037	Xba I	SP 6	forward
*SRF*3-5'-antisense	1,4 kb		BamH I	T 7	
*SRF*3-5'-sense	*SRF*3 full	3,037	KpnI	T7	reverse
*SRF*3-5'-antisense	3,037 kb		XhoI	SP6	
*SRF*4-5'-sense	*SRF* 45'	2,64	Kpn I	T 7	reverse
4-5'-antisense	1,1 kb (5'UTR is missing)		Not I	SP 6	

(Table 2-2a continued).

*SRF*4 sense (in pGEM T-easy Vector)[1]	*SRF*4 small	2,64	SpeI	T 7	reverse
*SRF*4 antisense (in pGEM T-easy Vector)[1]	0,629 kb		NcoI	SP 6	
*SRF*5-5'-sense	*SRF* 55'	2,492	Apa I	SP 6	forward
*SRF*5-5'-antisense	1,27 kb		Hind III	T 7	
*SRF*5 sense (in pGEM T-easy Vector)[1]	*SRF*5 small	2,64	NcoI	SP 6	forward
*SRF*5 antisense (in pGEM T-easy Vector)[1]	0,603 kb		SpeI	T7	
*SRF*6-5'-sense	*SRF* 65'	2,473	Kpn I	T 7	reverse
*SRF*6-5'-antisense	1,26 kb		Xho I	SP 6	
*SRF*7-5'-sense	*SRF* 75'	2,449	Hind III	T 7	reverse
*SRF*7-5'-antisense	0,921 kb		Xba I	SP 6	
*SRF*8-5'-sense	*SRF* 85'	2,567	Hind III	T 7	reverse
*SRF*8-5'-antisense	1,82 kb		Xho I	SP 6	
INO cDNA in pBluescript SK+					
INO-sense	INO	0,979	EcoRV	T3	
INO-antisense	0,979 kb		XbaI	T7	
SUB cDNA in pBluescript SK-					
SUB full-sense	SUB full length	2,65	XhoI	T3	
SUB full-antisense	2,65		XbaI	T7	

Table 2-2a. Compilation of the *in-situ* Hybridization constructs. The cDNA and corresponding vector, the orientation of the cDNA inside the vector, the enzymes used for linearization, the RNA Polymerase and the resulting size of the construct are presented. For *SRF*4 and *SRF*5 a small sequence spanning over the transmembrane (TM) sequence from the last LRR until the beginning of the kinase domain and a part from 5'UTR until the end of the TM was used. [1] the fragments for the short fragments of *SRF*4 and *SRF*5 were created with the primer described in Table 2-2b. Figure 2-1 illustrates the insertion in the vector with the respective restriction sides and RNA polymerase promoter region.

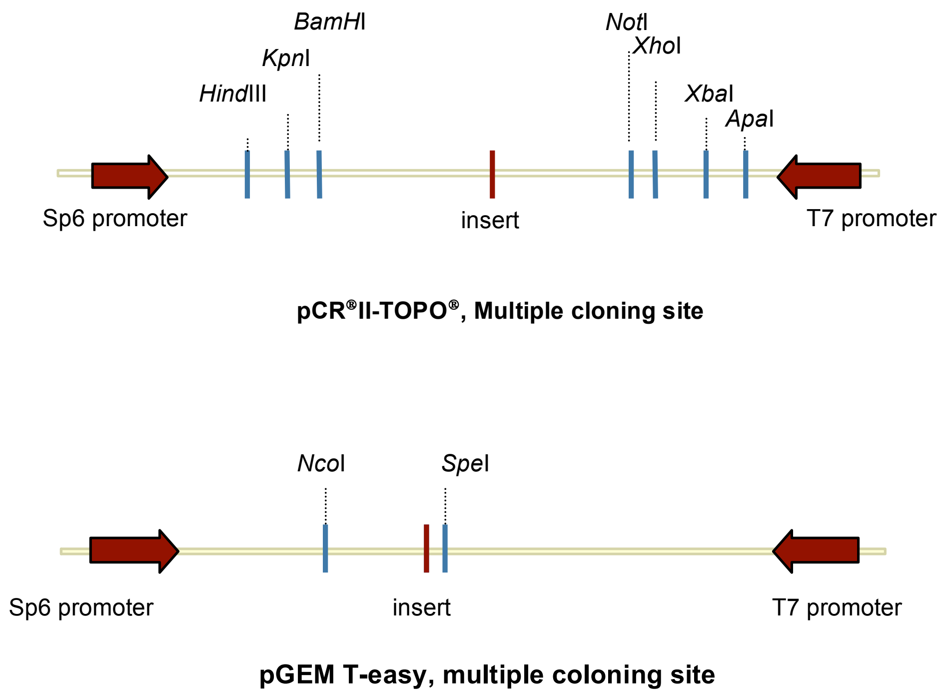


Figure 2-1. cDNA location in the vector used for RNA probe synthesis. Sp6 and T7 are the RNA polymerase promoter.

Table 2-2b. ***In-situ*** **primers used for short constructs**

Gene (AGI code)	gene name	Sequences
At3g13065	*SRF 4*	ACA ATC AAT TTA CTG GTT GGA TCC
		ATA AAC ACG TCC AAT GGT TCC TT
At1g78980	*SRF 5*	GAT GAC TTG AAC GTC GAA GAC AA
		CCC TAG TAG ATT TCC AGG TGA GA

2.3.2.5 Promoter GUS studies

2.3.2.6 Preparation of the Plasmid constructs

The presumed regulatory promoter-sequences were defined as the intergenic sequences between the 5' UTR of each gene sequence and the end of the previous gene in the genome (see Table 2-3 and Figure 2-2 for details). The genomic sequences were PCR amplified with wtDNA from *Arabidopsis th* (Col background) by using 0,05 U Takara LA Taq ™ (Takara Bio Inc.) and 0,005 U PFU Ultra HF (Stratagene) and primers that allowed an introduction of suitable restriction sites (Table 2-3) for cloning the fragment into the pCambia 1305.1 binary vector (www.cambia.org). This resulting plasmid was then transformed to DH5α *E.coli* bacteria strain by electroporation (program: C; V = 2000, C = 0025; hv R = 0201, T= 0005). After confirmation with appropriate enzyme digest, the Agrobacterium strain GV 3101 was transformed with the plasmid like *E.coli* transformation (electroporation program: 1250 V) and then used to transform *Arabidopsis th.* plants with the floral dip method, like described in (Logemann *et al.,* 2006). Silwet L-77 (VAC-IN-STUFF Cat. No. VIS-02, LEHLE SEEDS) was used for easier Agrobacterium integration/infection to the plant (wt Col and L*er* background). The seeds of transformed plants were screened for hygromycin resistance on plates and then transferred to soil. T1 plants were taken for GUS screening. For GUS activity analysis, different stages of flowers (stage1-12 and older) were directly collected with a tweezers in glass vials containing the GUS staining solution (10mM Na-phosphate buffer pH7.0, 10 mM EDTA, 0,1% Triton X-100, 2mM potassium ferrocyanide ($K_4Fe(CN)_6$ * $3H_2O$), 2mM potassium ferricyanide ($K_3Fe(CN)_6$), 100 µg/ml chloramphenicol and 1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolylglucuronide cyclohexylammonium salt, B-7300, Biosynth AG). The samples came twice under vacuum for 10 min and after 10 min slowly release of the vacuum the solution was changed.

They were incubated for at least 24 hours until 2 days at 37 °C and then cleared with 70% Ethanol several times until the chlorophyll is removed (Schoof *et al.,* 2000). A detailed examination of the plant tissue was done under the binocular.

Table 2-3. primer sequences used for GUS constructs (also illustrated in Figure 3-4)

Gene name	Primer name	Amplified size in bp	Primer sequence (from 5' to 3')
*SRF*1 small	*SRF*1GUSAntisenseNcoI	500	CAT GCC ATG GAA AGC AAA ACT TAG CCA AG
	*SRF*1GUSsense BamHI		CGC GGA TCC AAT CTA TAG AGC AAG GCG GAG C
*SRF*1 long	*SRF*1GUSsenselong BamHI	2642	CGC GGA TCC ATC ATG ACC ATA TCC CAC GCT AAC
	*SRF*1GUSAntisense long BglII		GAA GAT CTA CCA TTC CGA ATC CAG TAG ACA CTA AGA G
*SRF*4	*SRF*4 GUS BamHIfor	497	CGC GGA TCC ACA GTC AAG CCC TTG TTT
	*SRF*4GUS NcoIrev		CAT GCC ATG GGA TCC GAC AGT TTT AAG C
*SRF*6	*SRF*6BamHIfor	2251	CGC GGA TCC TGG GCT TCT CAT TTT AAG GG
	*SRF*6NcoIrev		CAT GCC ATG GCC TCT CTC TAG AAC GAT AAG CT
SRF7	*SRF7*BamHIfor	1700	CTT CTT GCT GGC ACA CGC TTG G
	*SRF7*NcoIrev		CAT GCC ATG GTC TTC TTC CTC CTT AAC C

3. Results

3.1 Expression of the *SRF* Genes

One of the initial stages for the functional analysis was to characterize the expression patterns of the *SRF* genes. For this approach *in-situ Hybridization* (ISH) and *β*-glucuronidase (GUS) reporter gene analyses were performed. This can provide an insight into the spatial and temporal appearance of the corresponding mRNA. Our research was focused on the eight members of the *SRF* family, where and when they are expressed under "normal" growth condition in the wild type L*er* *Arabidopsis* flower. Apart from the experimental observations, one can get first impression from AtGenExpress data publicly available in the web (http://weigelworld.org).

3.1.1 *In-situ* Hybridization (ISH) of *SRF1-8*

To describe the expression pattern in the flowers of *Arabidopsis th.*, flower stages (FS) between 1 and 14 (Smyth *et al.,* 1990) were analysed. The experiments for each single gene were carried out in four separated groups of flower stages, namely inflorescence meristem (IM), young flowers (until stage 5), middle-aged flowers (from 5-12) and old flower stage (from stage 12 upwards). All genes and stages were analysed at least three times. Young stages (IM and young flowers) at least five times, because of more details and difficulties of getting appropriate sections. The first investigations were carried out with different stock concentrations of the denoted stock solution (see Material and Methods) ranging from 5-30µl to find the probe concentration with the best signal. The identified probe concentration (10-20 µl of the described stock solution) was used for more investigations. Figure 3-1 shows the fragments of *SRF1-SRF8* that were used for the experiment. *SUB* and *INO* probe were synthesized with the full-length cDNA.

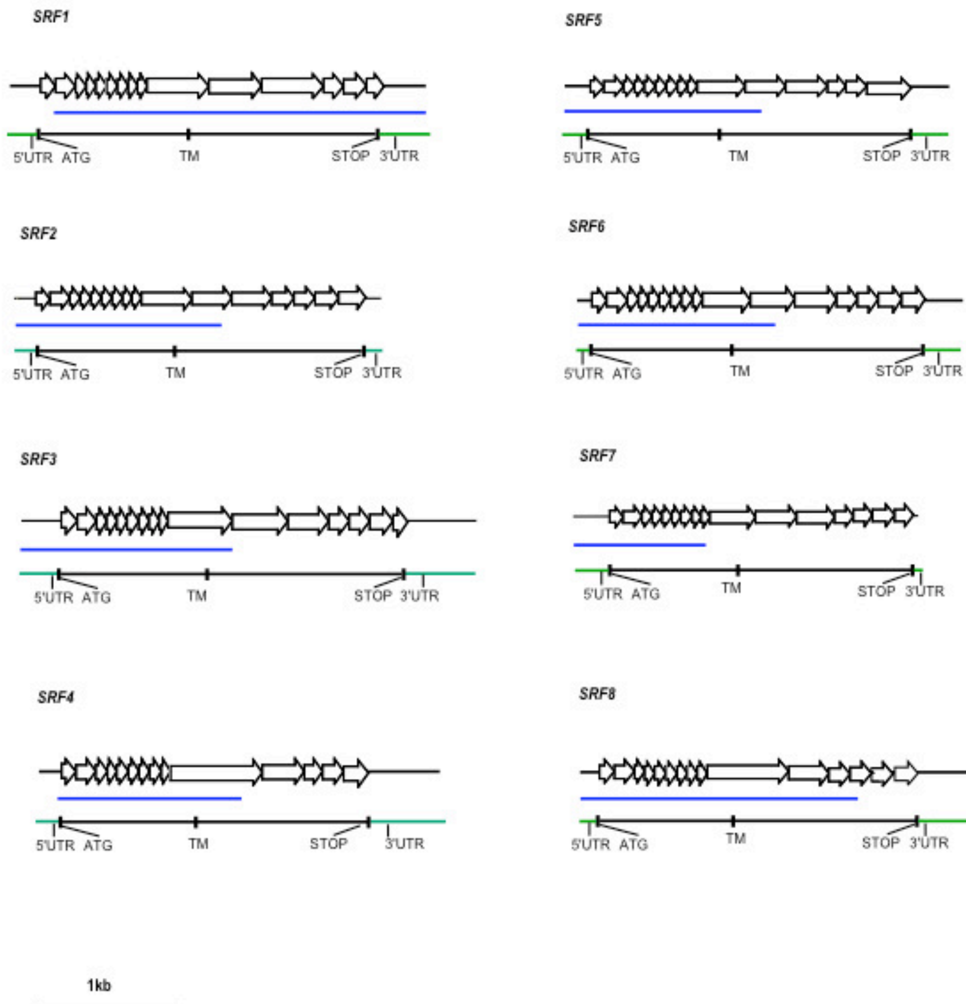


Figure 3-1. Illustration of the cDNA sequences used for *in-situ* hybridization contructs. The arrows indicate the exons of the respective *SRF* gene. The blue line under the gene structure is the sequence length used for the probe synthesis. Abbreviations: UTR- untranslated region; ATG- start codon; TM- transmembrane region.

Transcriptional activity of the eight *SRF* genes was detected in all kind of developing flower organs, for example in the inflorescence meristem, young flower primordia and arising young flower organs, like sepals, anthers, gynoecium and ovules. This is very good exemplified at the picture of *SRF2* (Fig. 3-2 D), *SRF4* (Fig. 3-2 H), *SRF5* (Fig. 3-2 K), *SRF7* (Fig. 3-2 P), *SRF8* (Fig. 3-2 S) and *SUB* (Fig. 3-2 U). A compilation of all *SRF in-situ Hybridization* profiles is shown in Fig. 3-2.

SRF1 shows medium expression in microspores of the anthers, later in the mature pollen and the surrounding tapetum cell layer (around FS 11). Additional *SRF1* is expressed in the ovules. The very low expression in the petals is confined to the edges of the leaves in combination with the vascular tissue.

SRF2 is strongly expressed in the IM and in young flower primordia with the arising sepal primordia and anthers (until FS 5). At FS 5 the expression in the sepals decreased and disappear later. In older flowers around FS 7 and FS 12/13 *SRF2* is expressed in ovules, tapetum, microspores and pollen.

SRF3 as well as *SRF1* show a wide range of weak or medium expression within all flower stages, which was challenging to detect maybe due to a lower expression compared to the other genes. Around FS 11 to 15 a medium ovule expression of *SRF3* could be detected. This is comparable with the AtGenExpress data (see Figure 3-3), where also an average expression in all analysed tissues was detected. By contrast to this experiment quantitative RT-PCR analyses of the ovule receptor kinase called *Solanum chacoense* ovule receptor kinase 5 and 13 *(ScORK5* and *ScORK13),* closely related to *SRF3* showed the strongest expression level in ovary or young fruit tissues (Germain *et al.,* 2005).

SRF4 is expressed in the IM and flower meristem, in young anthers and in the developing gynoecium. Additional *SRF4* displays expression in microspores, pollen, tapetum, petals and ovules (from stage 5 to 15).

SRF5 is expressed in the IM, flower primordia, young flower organs from stage 2 to 7 (gynoecium including ovules, anthers with microspores) and in the later stages it is strongly expressed in the ovules and weak in the pollen.

SRF6 is highly expressed in the anthers and ovules from FS 8 until FS 12. The ScORK28 belongs to the LRR-V or SRF family, where SRF6 and SRF7 are the closest related members of this family (Germain *et al.,* 2007). ScORK28 displays high expression pattern specifically in ovaries.

SRF7 is expressed in the IM, in the flower primordia, in the arising flower organs, like the gynoecium, sepal and petal primordia and stamen precursors. The expression of the sepals disappeared after stage 5. From stage 6 to 12 expression in the microspores (later pollen) and in the ovules was detectable. The expression in the petals was restricted to the edges of the petals.

SRF8 is strongly expressed in the IM, flower primordia, gynoecium-, anther-, petal- and sepalprimordia. In the following development expression was detectable in ovules, microspores and petals until stage 12.

Pollen expression could not be clearly detected in *SRF6, 7* and *8*. One reason is the high background staining from the pollen itself.

In the later stages of flower development except ovule development, expression of the *SRF* genes decreased or disappeared. Results from *SUB*-ISH experiments showed strong expression in all floral organ primordia and decreasing during flower development (Chevalier *et al.,* 2005) and
Figure 3-2U,V).

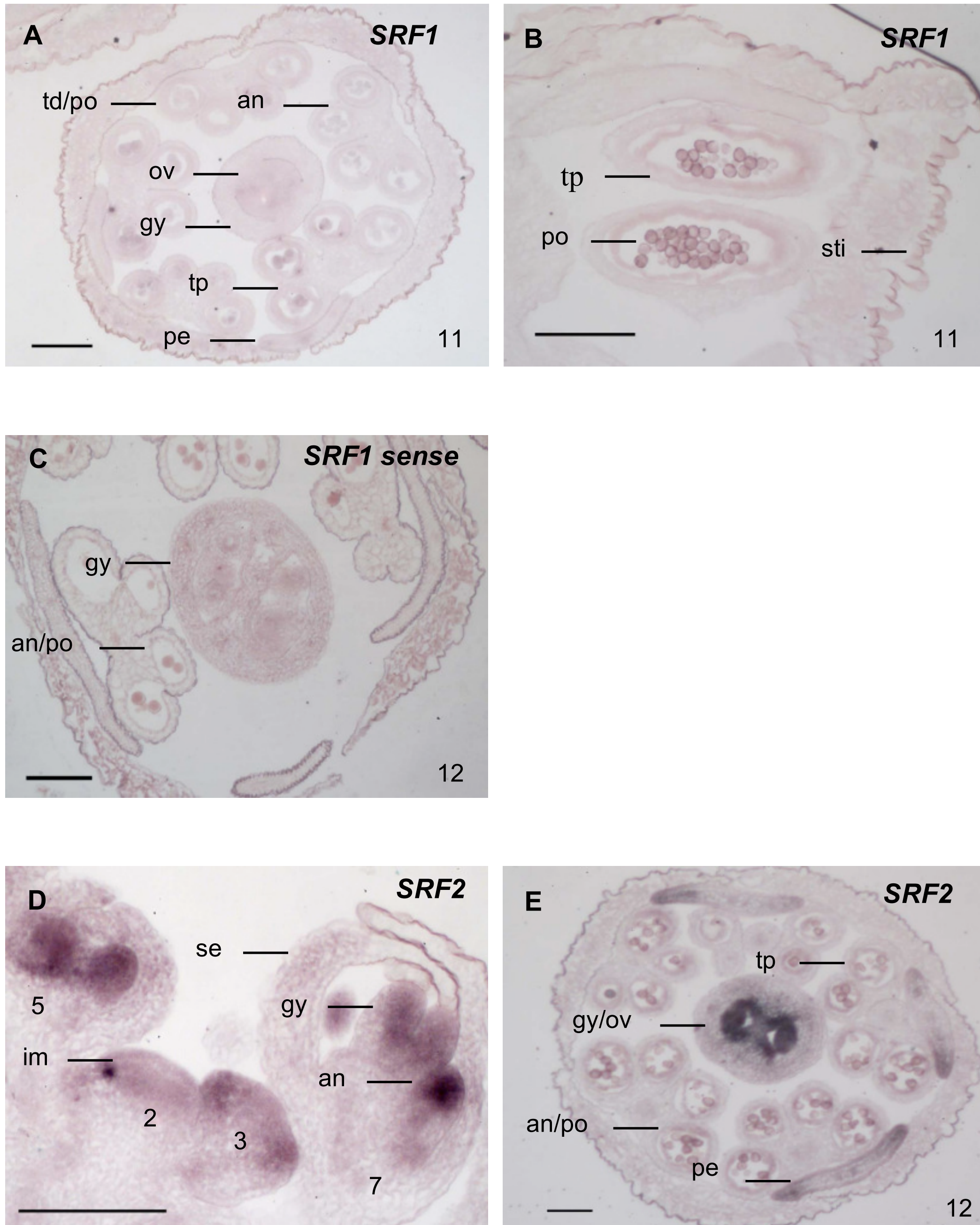
INO was used as a positive control sample, which is mainly expressed on the abaxial side of the ovule primordium and in the outer integument (Balasubramanian *et al.,* 2000) also visible in Figure 3-2W.

As a negative control the sense probe of each gene was used. Normally one expects no signal, but *SRF1sense* probe shows a slight expression in the ovules and pollen (Figure 3-2C). Additional *SRF5sense* probe is lightly visible in the ovules and petals (Figure 3-2M). *SRF7sense* shows even a stronger expression (Figure 3-2R). The reason for this phenomenon is quite unknown (see in the discussion chapter 4). A probable explenation could the existence of antimiRNAs, which are complementary to the *SRF* gene transcript. In *SRF1* six antimiRNA could be identified, which are complementary to the *SRF1* gene transcript.

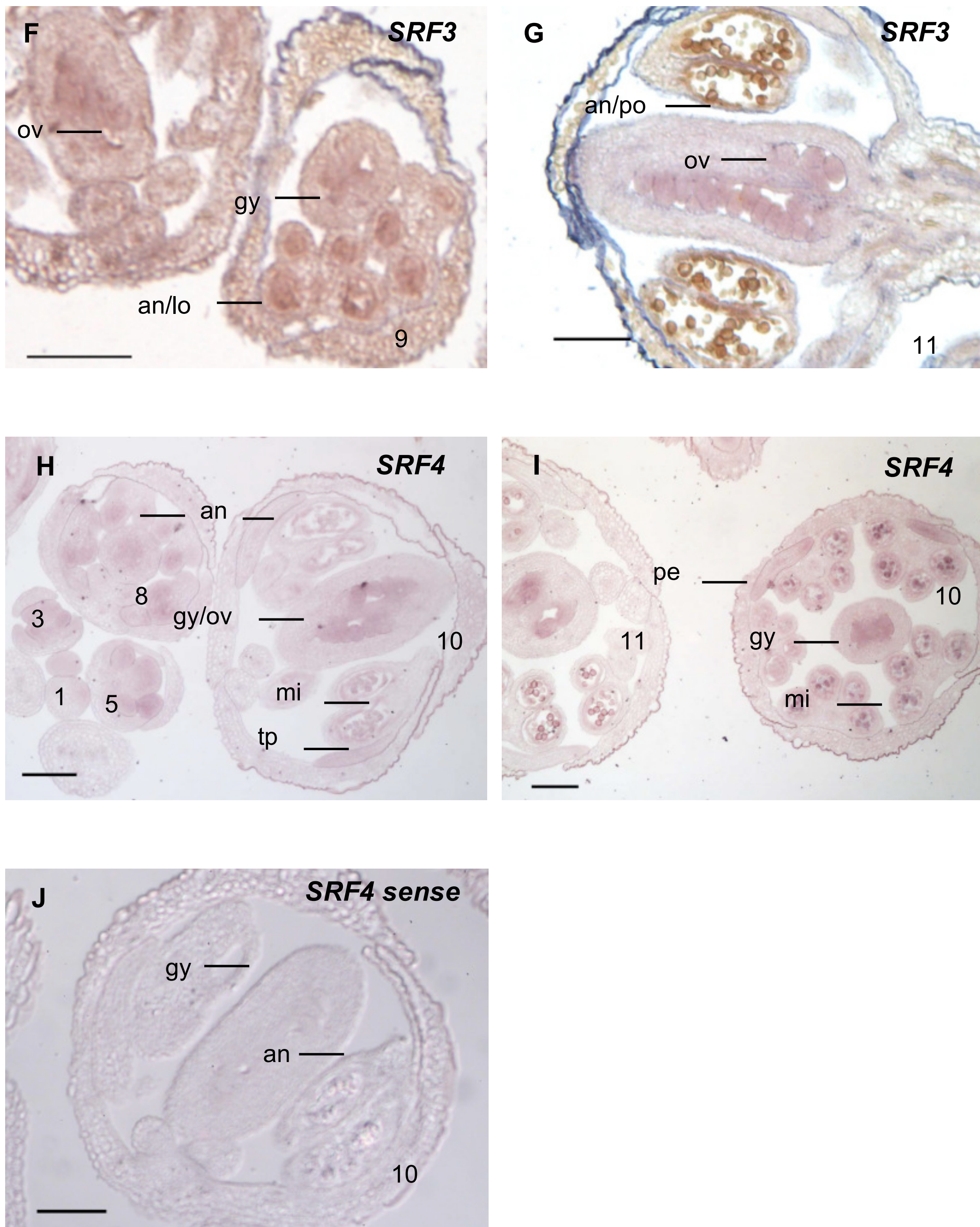
SRF5 has two antimiRNA target sequences matching with the native mRNA. *SRF7* has three antimiRNAs (http://signal.salk.edu/cgi-bin/atta, (Yamada *et al.,* 2003; Adai *et al.,* 2005; Lu *et al.,* 2005; Vaughn *et al.,* 2005; Lu *et al.,* 2006). Curiously other *SRF* members like *SRF2, SRF3, SRF6* and *SRF8* have also analysed antimiRNA, but show no sense expression.

Another point to address is the density and coloration of the ovules and pollen, which can cause a false positive result.

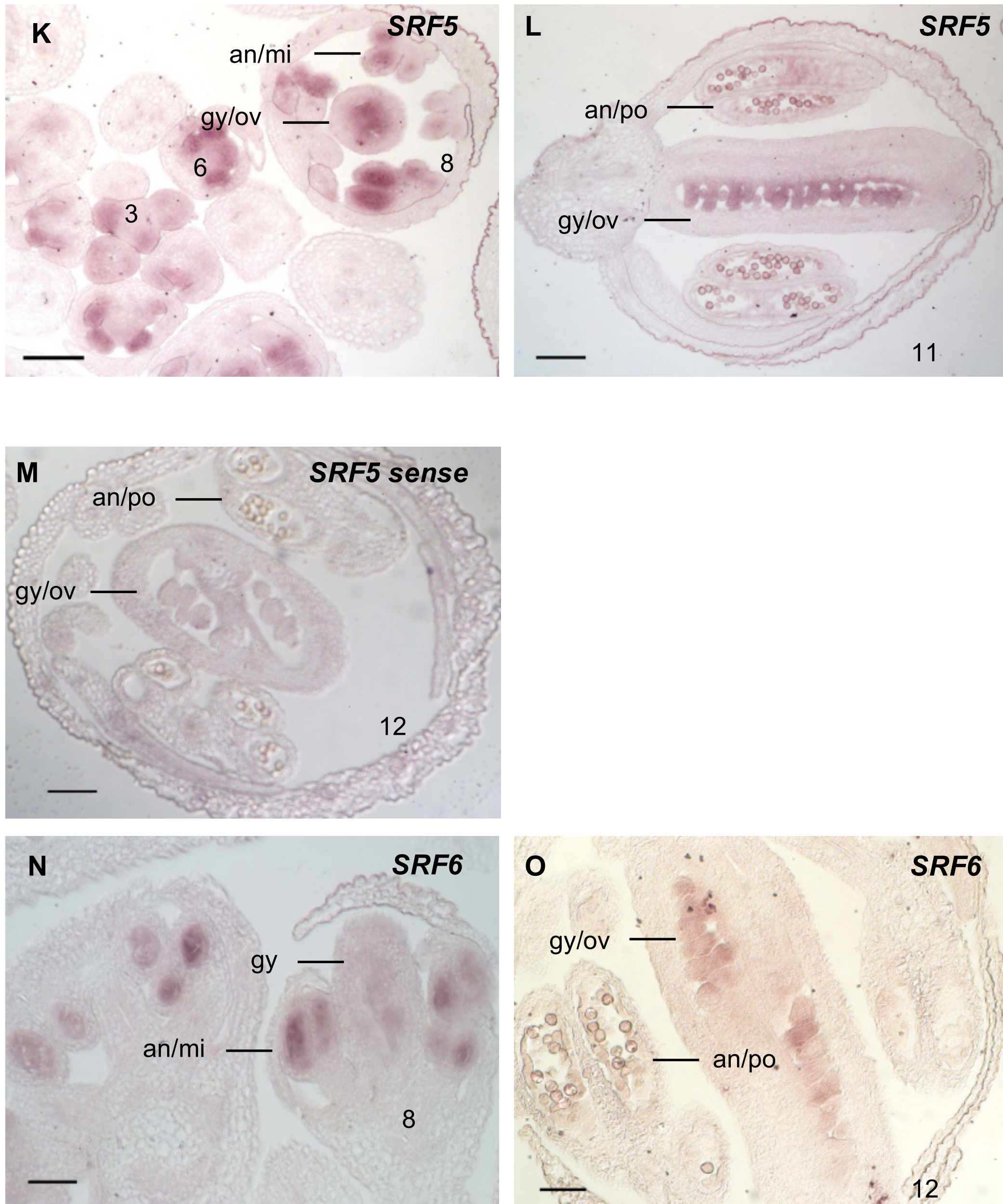
Figure 3-2. *SRF e*xpression profile in *Arabidopsis th.* (ecotype L*er)* floral tissue.



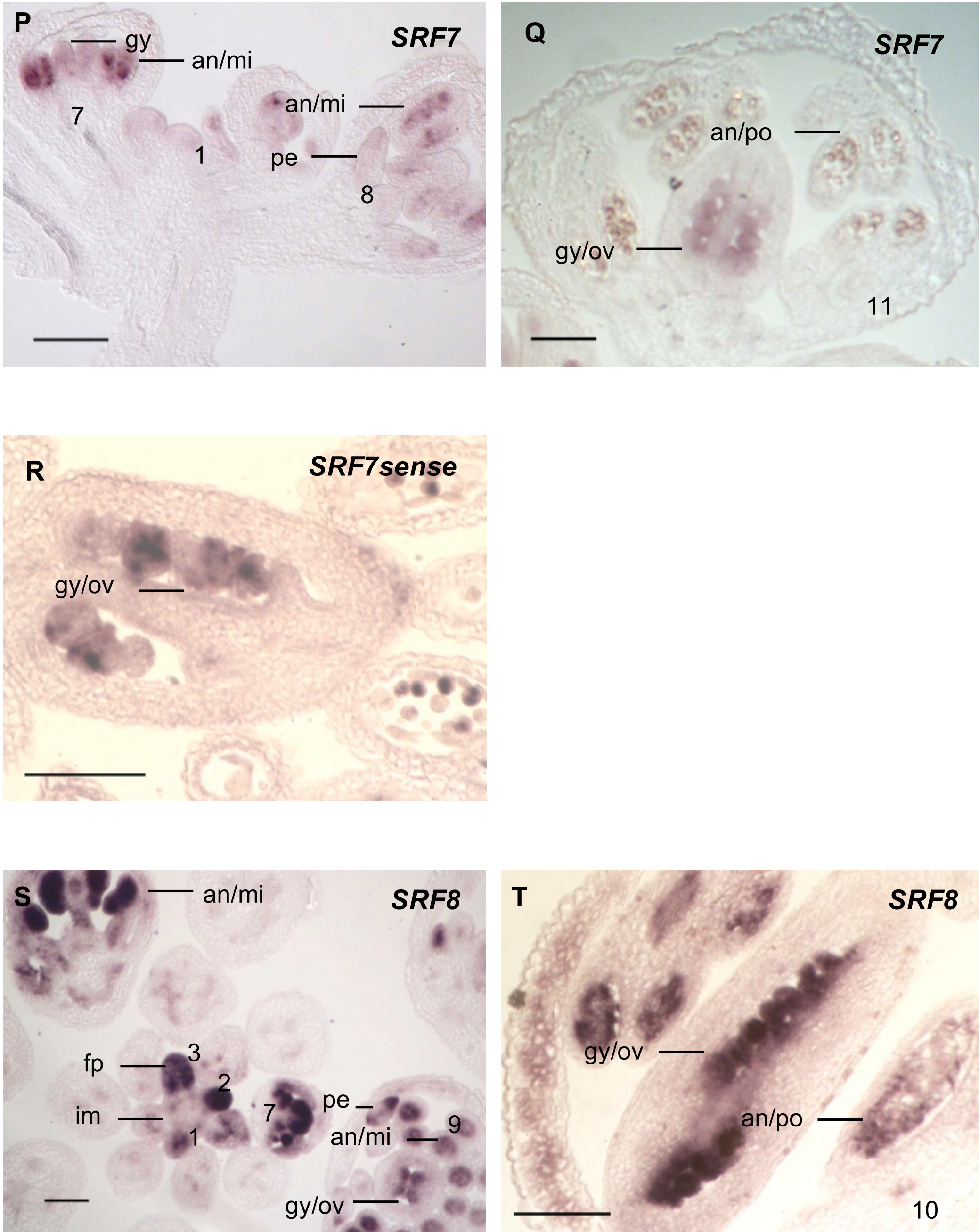
(Figure 3.1 continued).



(Figure 3.1 continued).



(Figure 3.1 continued).



(Figure 3.1 continued).

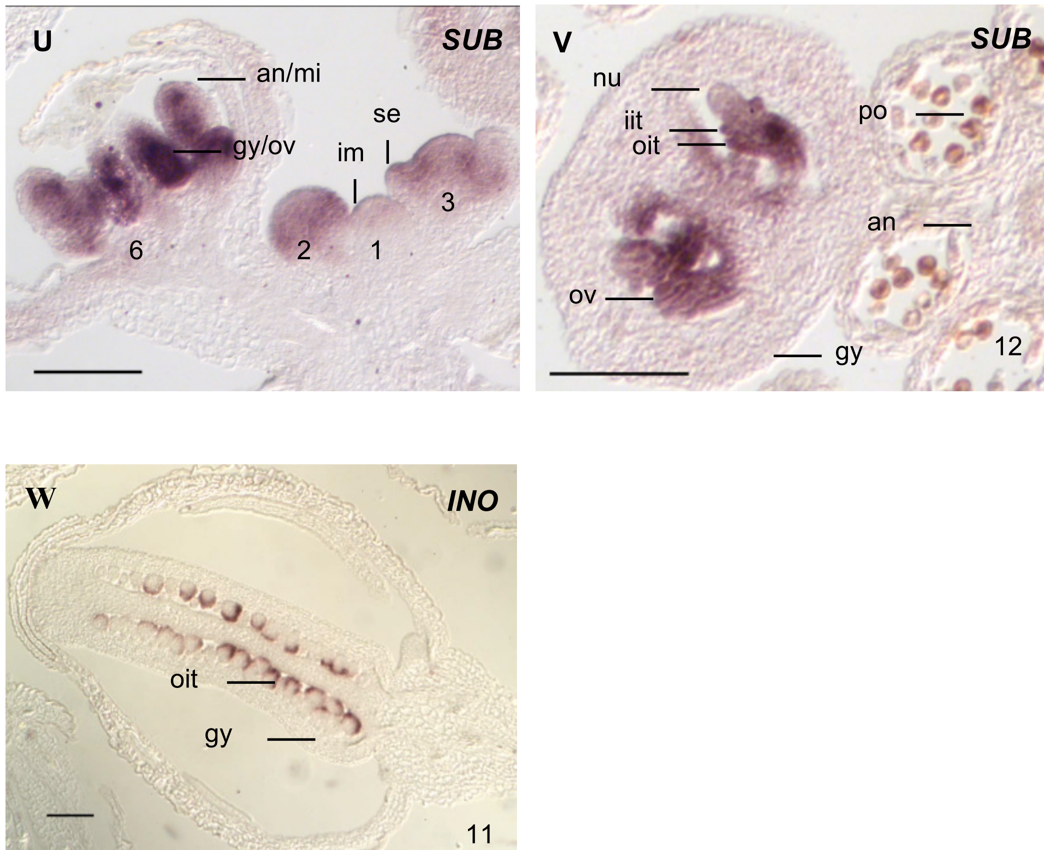


Figure 3-2. *SRF* expression profile in *Arabidopsis th.* (ecotype L*er)* floral tissue. Expression pattern of the *SRF* members*, SUB* and *INO* in wild-type flowers was analyzed with *in-situ Hybridization*. Cross- or longitudinal sections are shown. The antisense and sense probes were labeled with digoxygenin. *INO* served as a positive control. The sense probes of *SRF1, SRF4, SRF5* and *SRF7* were used for the negative control experiment. Cross sections: A, C, E, F, H, I, K, M, S, V. Longitudinal sections: B, D, F, G, H, J, L, N, O, P, Q, R, T, U, V, W**. (A+B) *SRF1*; (C) *SRF1 sense*; (D+E) *SRF2*; (F+G) *SRF3*; (H+I) *SRF4*; (J) *SRF4 sense*; (K+L) *SRF5*; (M) *SRF5 sense*; (N+O) *SRF6*; (P+Q) *SRF7*; (R) *SRF7 sense*; (S+T) *SRF8*; (U+V) *SUB*; (W) *INO.***
Numbers from 1 to 12 refer to flower stage (FS) taken from (Smyth *et al.,* 1990); Abbreviations: an/lo- anther loci, fp- flower primordium, gy- gynoeceum
(consist of two fused carpels), im- inflorescence meristem, mi- microsporocytes, ov- ovule, pe- petal, po- pollen, se- sepal, sti- stigma, td- tetrad, tp- tapetum, oit- outer integument; Scale bar: 90 µm

The expression profile of the *SRF* members is quite similar within the whole family. That could be due to the highly conserved Leucin-rich repeat sequence within the family. Nevertheless, the nucleotide identities between the *SRF* c-DNA sequences used for the probe synthesis were less than 50 %, with two exceptions of 53,1 % between *SRF4* and *SRF5* and between *SUB* and *SRF1* with 51,2% (see table 3-1).

Table 3-1. Nucleotide identities between the *SRF* cDNA sequences of the used *in-situ* probe fragment

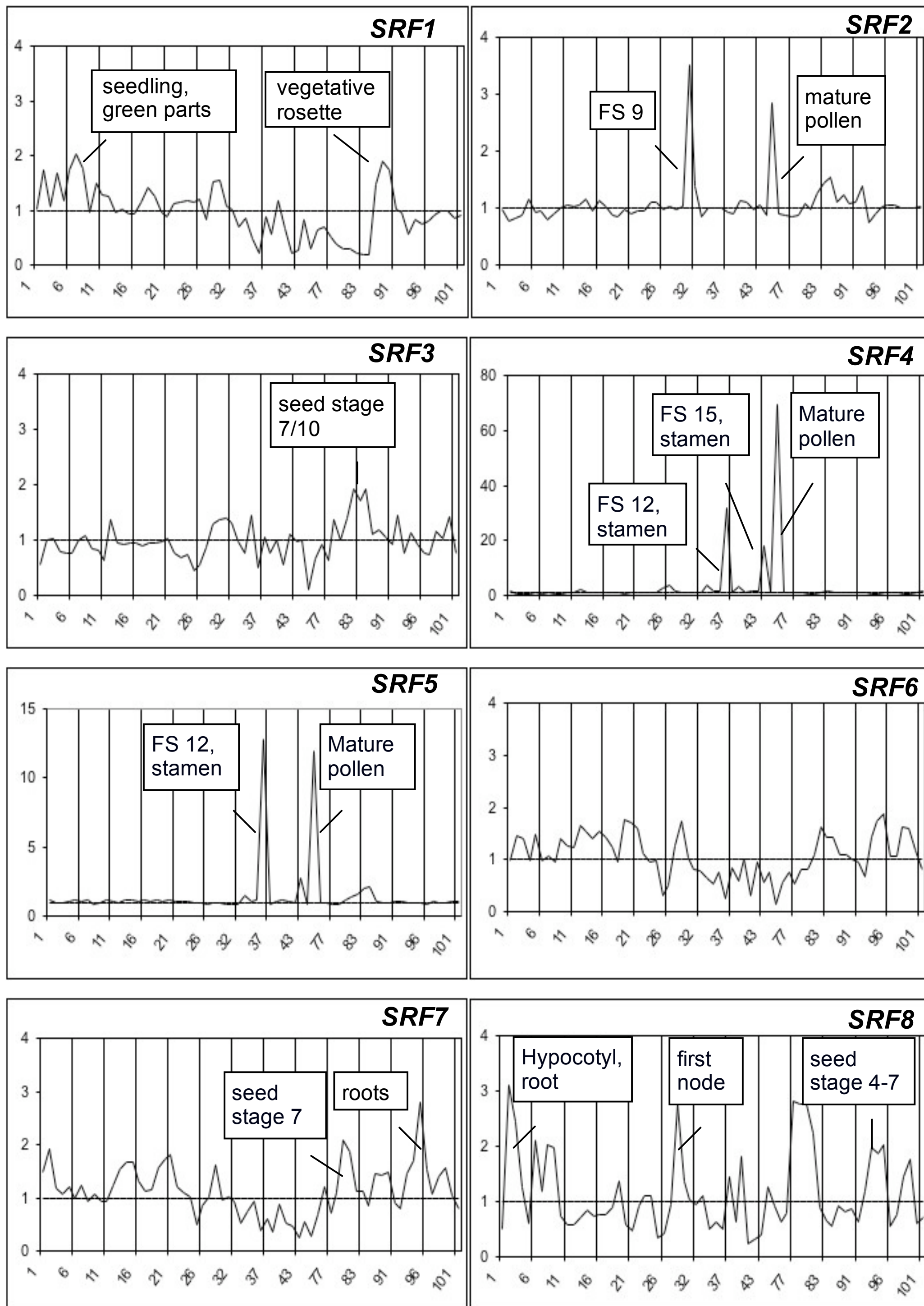
	SRF1	*SRF2*	*SRF3*	*SRF4*	*SRF5*	*SRF6*	*SRF7*	*SRF8*
SUB	51,2	25,1	26,5	22,0	23,4	23,5	15,6	38,5
SRF1		24,8	29,6	20,5	22,6	23,1	16,8	38,0
SRF2			43,4	45,6	44,8	48,4	30,1	38,1
SRF3				39,4	45,2	42,9	33,7	40,9
SRF4					**53,1**	49,1	27,6	33,5
SRF5						48,3	32,3	34,5
SRF6							40,0	36,9
SRF7								21,8
SRF8								

These findings of the ISH experiment shows expression of the *SRF* family transcripts over a wide range of flower development. Especially in ovules and anthers an expression was always visible.

3.1.2 AtGenExpress data of the *SRF*-gene family

The expression pattern of the *SRF*-genes were analyzed with the ATH1 GeneChip dataset developed by Affymetrix and The Institute for Genomic Research (TIGR) by using many developmental stages with sometimes separate organs. These data provide some indications about organ specificity and level of expression. Mainly the intensity of mRNA expression could be evaluated with this kind of experiment. The GeneChip array data (Schmid *et al.,* 2005) are publicly available at the Nottingham Arabidopsis Stock Centre Transcriptomics Service (NASCArrays), the ArrayExpress at the European Bioinformatics Institute (EBI), or the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI). All these data together with the Expressed Sequenced tag database (The Arabidopsis Genome Initiative 2000) about the *SRF* family show that the *SRF* genes are expressed, thus they are likely functional genes. Figure 3-3 illustrates the expression profiles provided and created by (Schmid *et al.,* 2005) with the ATH1 GeneChip dataset. In the *SRF*4 and *SRF*5 profiles there is an obvious high expression in the mature pollen compared to all other tissues. The other members show rather a medium or average expression during the plant development.

Fig. 3-3. AtGenExpress profiles of the *SRF* family



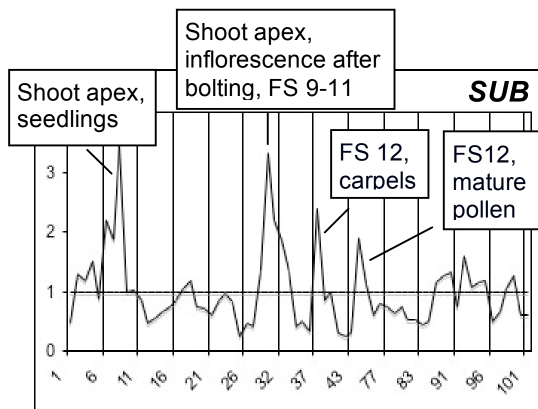


Figure 3-3. Expression profiles of the *SRF* family. These profiles were created and provided by (Schmid *et al.,* 2005) by using AtGenExpress data. The mean-average was mean-normalized to 1, like described in (Schmid *et al.,* 2005). Individual expression profiles are represented by the diagram curve. The y-value shows the average expression. The maximum y-value differs from graph to graph. The different RNA samples are derived from different developmental stages of the plant tissue and are presented by the x-value. **x-values: 1-9**: 7 to 17 days old cotyledons, hypocotyls, roots, SAMs, young leaves and seedlings; **10-21**: 10 to 17 days old rosette leaves including separate leaf parts; **22-25**: 21-23 old total plants and 35 day old senescing leaves; **26-29**: 21 days, cauline leaves, stem, 2nd internode, first**; 31-45**: 21 days old flowers (stage 9-15) with single organs and whole flowers; **73**: 5 weeks old mature pollen; **75-84**: 6-7 weeks old siliques with and without siliques; **87-90**: one to three weeks old rosette leaves; 91-93: leaf, flower and root; **94 and 95**: 8 days old roots; **96 and 97**: 8 days old seedlings (green part); **98-101**: each 21 days old roots and seedlings (green part).

3.1.3 Promoter GUS analysis of *SRF1*, *SRF4*, *SRF6* and *SRF7*

An other method to analyse the expression of genes is the fusion of the putative promoter sequence to the reporter gene *uidA* encoding *β*-glucuronidase (GUS) (Jefferson *et al.,* 1987). The most likely promoter regions for *SRF1, SRF4, SRF6* and *SRF7* were fused to the *GUS* reporter gene and the resulting construct was than transformed to Arabidopsis *Col* and L*er* wild type background. For construct details see Figure 3-4 and Table 2-3 (Material and Methods). The whole tissue can be detect easily and directly *in-vivo* without the need of sections. To assess the GUS expression profiles of *SRF* gene inflorescences (flower stage 1 to 15) of 30 T1 transgenic lines from each *SRF1-short (s), SRF1-long (l)::GUS, SRF4::GUS, SRF6::GUS* and *SRF7::GUS* reporter line were stained (see Figure 3-5). Around 70% of the analysed inflorescences display GUS staining from weak to strong (not all data shown).

For *SRF1* two constructs with differing sizes were made and analyzed to see eventually differences in the expression pattern. The short sequence seems to contain sufficient regulatory information to reproduce the endogenous expression of *SRF1.* Shorter promoter sequences must be tested to get information about the real regulatory promoter sequence. *SRF1*-long (l) and *SRF1*-short (s) shows expression in the filaments, in the connectives of the stamina and the style of the gynoecium at FS 13 to 15. In sepals the expression was visible from FS 6 until FS 12 with a presumed increase. One transgenic line shows strong expression in the ovules (Figure 3-5C). No expression was found in the petals and papilla cells on the stigma of the ovary around FS 12-15.

SRF4 shows a very specific expression in the developing microsporocytes and mature pollen of the anthers from stage 6 to 15. A very weak signal was visible in the petal. Recent analysis showed stronger expression in the sepals, petals, filaments and in the gynoecium (Christine Skornia, personal communication).

SRF6 shows expressions similar to *SRF1* and *SRF7* expression pattern; like that it is expressed in the sepals, in the filaments and connectives of the stamina and in the style. Often a spotted expression profile in the sepals and filaments could be observed. No staining was visible in the young flower primordia, in the petals and anthers (including pollen) and in the papillar cells at stage 10 to 15.

SRF7 becomes active from stage 6 until FS 15, starting with the expression in the sepals and gynoecium. Around stage 10, which is characterized by the fast-growing petals reaching the top of the short stamens until stage 15 *SRF7* was expressed in the sepals, in the filaments, in the anthers and in the style of the gynoecium. Like already mentioned before, from stage 10 to 15 the expression is equal to the expression pattern of *SRF1* and *SRF6.*

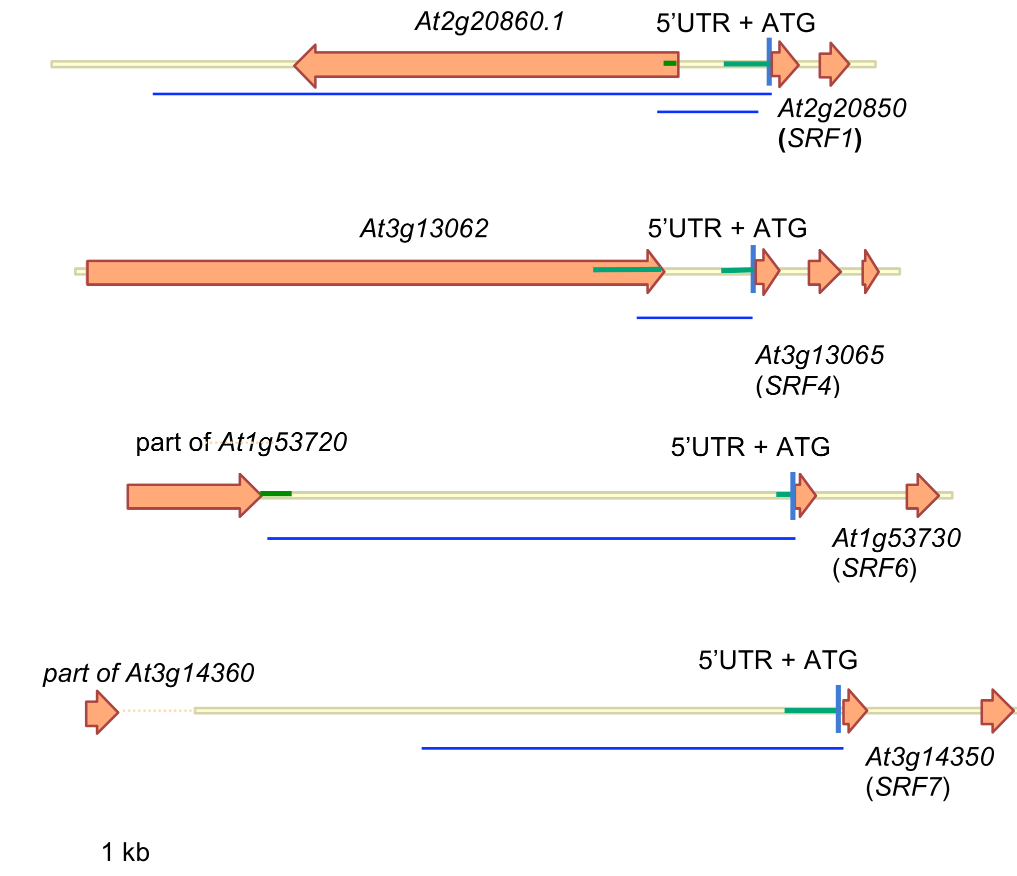
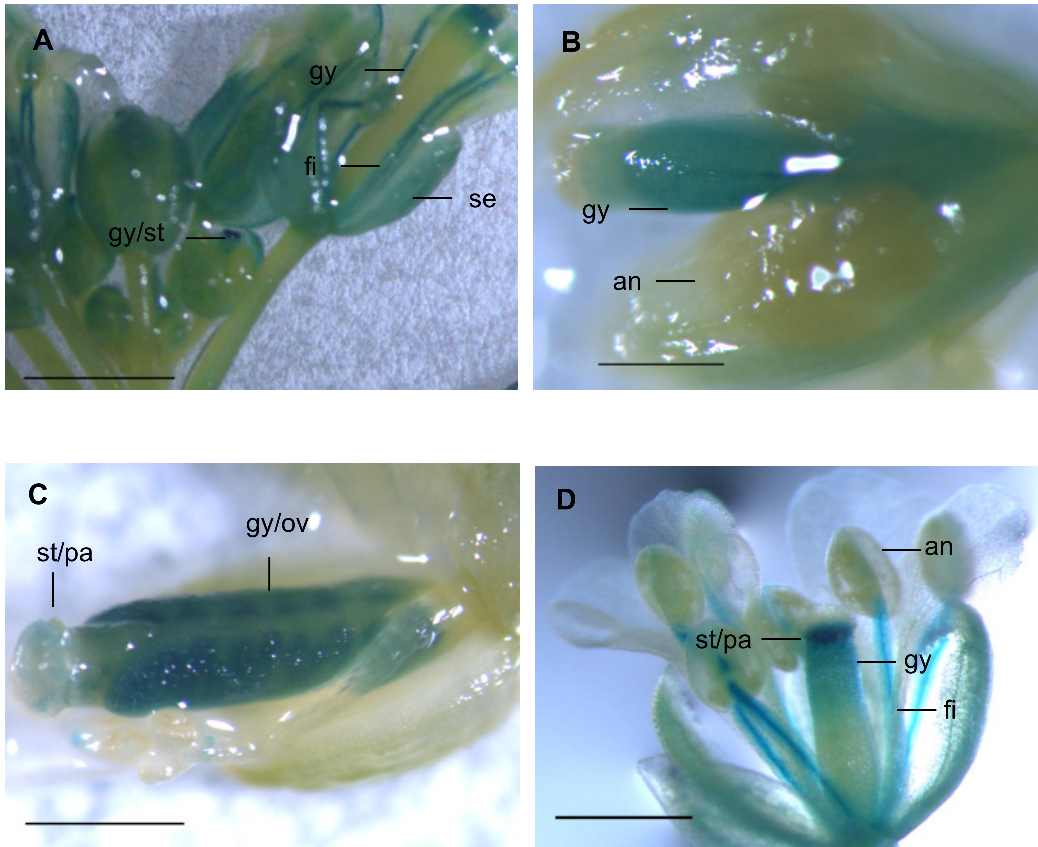


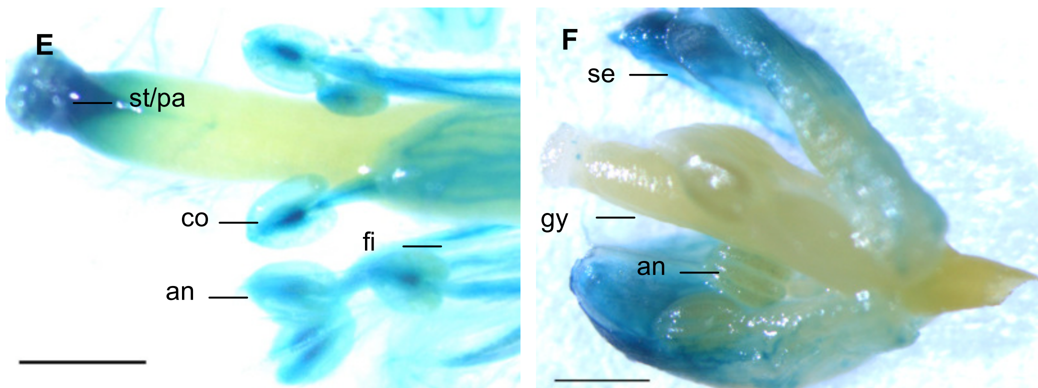
Figure 3-4. Illustration of the used presumed promoter fragments (blue line) cloned into pCambia 1305.1 Vector for GUS analysis. Orange arrows show the first exons of the *SRF* genes and genes before, respectively. The yellow line indicates the intergenic part. The dashed lines are only indications of a much longer part. The green line represents the 5' and 3' UTR of the *SRF* genes and neighboring genes, respectively. The blue vertical stroke shows the start codon of the *SRF* gene.

Figure 3-5. *SRF::GUS* Expression of selected lines in *Arabidopsis th.* flowers

SRF1::GUSs

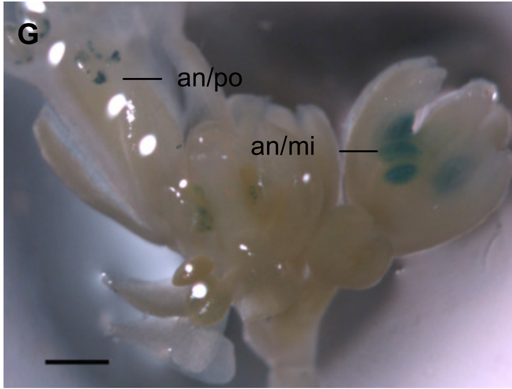


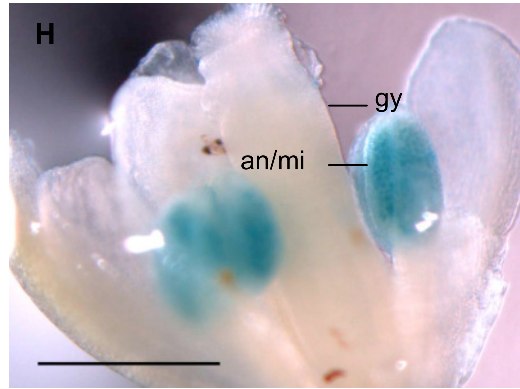
SRF1::GUSl

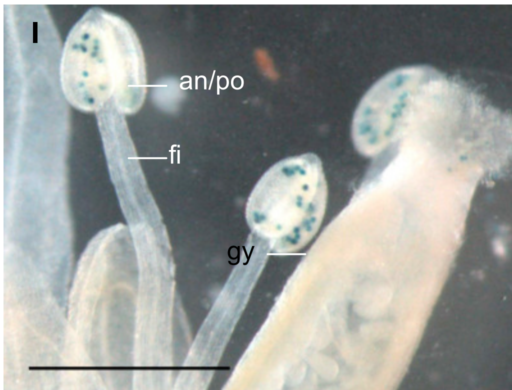


(Figure 3-5 continued).

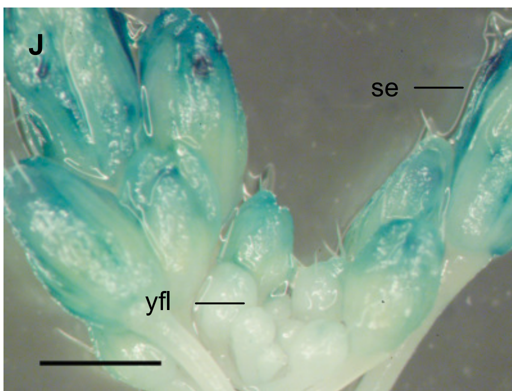
SRF4::GUS

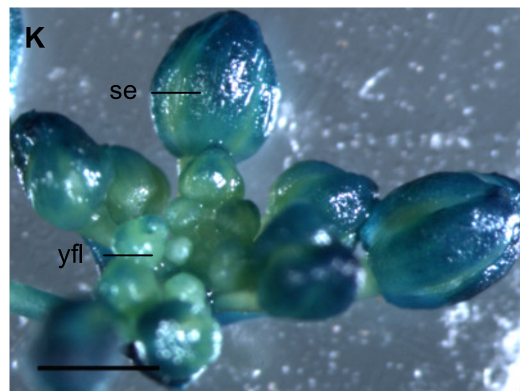






SRF6::GUS

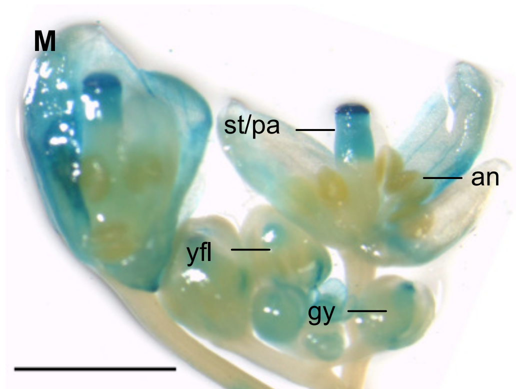




(Figure 3-5 continued).

SRF6::GUS

SRF7::GUS ***SRF7::GUS***



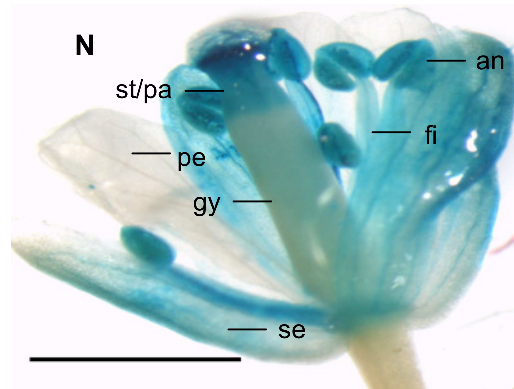


Figure 3-5. *SRF::GUS* expression of some selected lines in *Arab. th.* wt flowers. (A-D) *SRF1::GUSs* expression with short (s) construct (for construct details see Figure 3-4). **(E+F) *SRF1::GUSl*** expression with long (l) construct. **(G-I) *SRF4::GUS*** expression. **(J-L) *SRF6::GUS*** expression. **(M+N) *SRF7::GUS*** expression. Abbreviations: an- anther, co- connective, fi- filament, gy- gynoecium, mi- microsporocytes, ov- ovule, pe- petal, po- pollen, se- sepal, st/pa- style and papillar cells, yfl- young flowers;
Scale bar: 1 mm

3.2. Characterization of T-DNA insertion lines

A reverse genetic screen with T-DNA insertion lines was used for functional analysis. The advantage of T-DNA insertions is, that they do not transpose subsequent to insertion and they are chemically and physically stable through multiple generation compared to transposons (Radhamony 2005). The insertion lines were obtained from different sources (Table 3-2). Using right and left border primers to read sequence out of the T-DNA into the flanking genomic DNA, the location could be determined after sequencing. In addition the corresponding genomic primer oriented towards the T-DNA border ends was used (see Figure 3-6 and Table 2-1a,b). The insertion site was usually close to the predicted position. Not always an exact position could be identified (details are listed in Table 3-2a,b). Except for *srf7-2* (Wassileskija) all lines have wild type Col as their background.

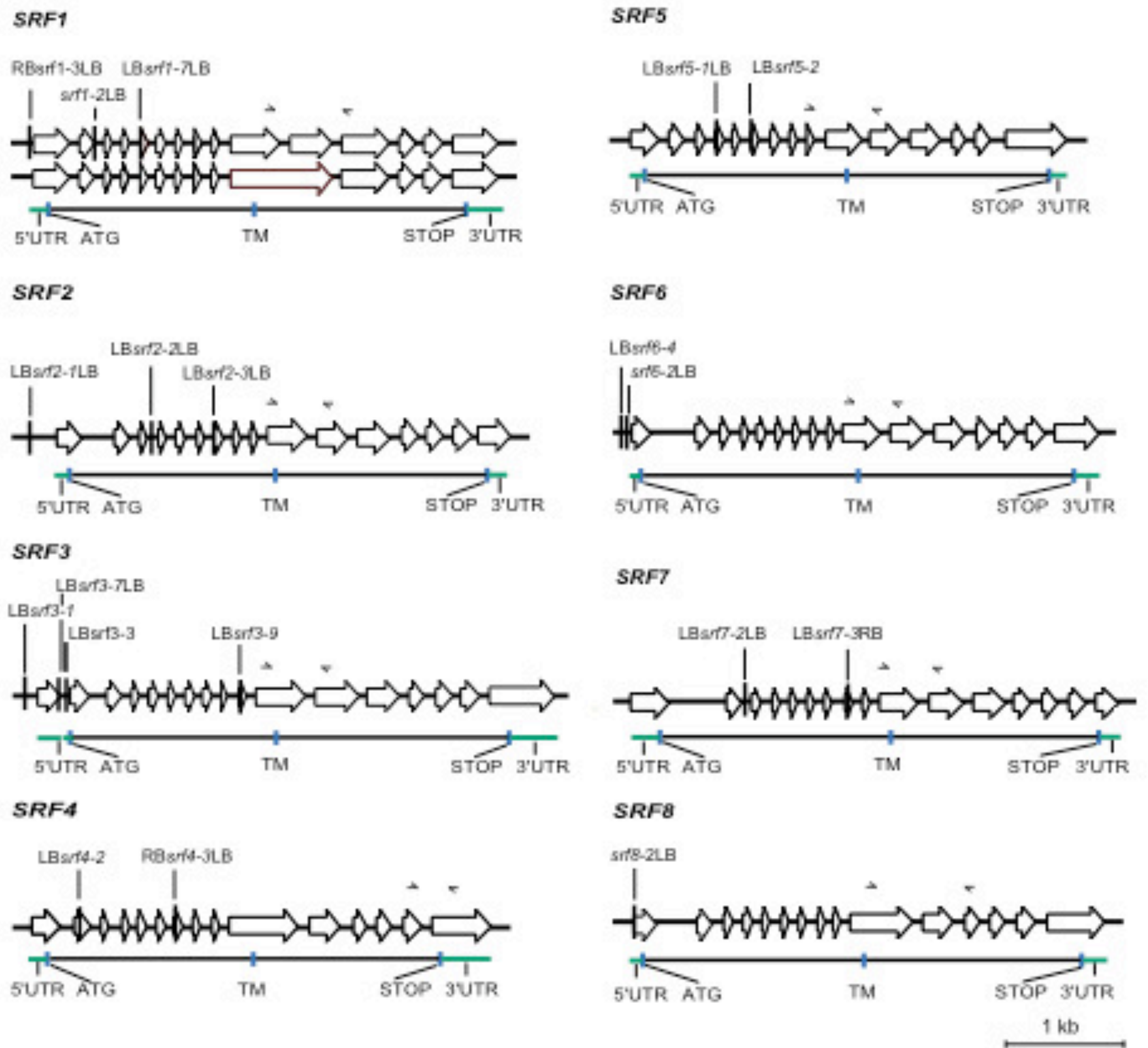


Figure 3-6. T-DNA locations within the *SRF* genome. Exons are illustrated as empty arrows. Little black arrows above the structure show the position of the used RT-PCR-primer. Abbreviations: LB - left border of T-DNA; RB - right border of T-DNA; TM - transmembrane domain; UTR - untranslated region.

Table 3-2a. Location of the T-DNA-lines in the *SRF* genes

Accession No.	Gene	Vector	Insertion line	Reference-number	sequenced integration site
At2g20850	*SRF*1	pCSA110 [c]	*srf 1-2*	Garlic 182A08	intron 2
		pDAP 101	*srf 1-3*	Garlic 1297E4	4 bp before 5' UTR, promoter
		pROK2	*srf 1-7* [a]	Salk 087666	within exon 5
At5g06820	*SRF*2	pCSA110 [c]	*srf 2-1* [a]	Garlic 407 E2	295 bp before 5'UTR, promoter
		pCSA110 [c]	*srf 2-2*	Garlic 119 B10	spanning exon4 and intron 4
		pDAP101	*srf 2-3* [a]	Garlic 591 A03	exon 7
At4g03390	*SRF*3	pDAP101	*srf 3-1*	Garlic 797F7	237 bp before 5' UTR, promoter
		pCSA110 [c]	*srf 3-3*	Garlic 244C5	51 bp before ATG within 5'UTR
		pROK2	*srf 3-7* [a]	Salk 029908	within an 9 bp region in the 5'UTR localized 182 bp before the ATG
	SRF3	pROK2	*srf 3-9*	Salk 051048	exon 9
At3g13065	*SRF*4	pCSA110 [c]	*srf 4-2*	Garlic 230E8	exon 2
		pCSA110 [c]	*srf 4-3*	Garlic 253A9	exon 7
At1g78980	*SRF5*	pROK2	*srf 5-1* [a]	Salk 010161	exon 4
		pAC161	*srf 5-2*	Gabi 95A05	intron 5
At1g53730	*SRF6*	pCSA110 [c]	*srf 6-2*	Garlic 269D10	30 bp before 5'UTR and 100 bp before ATG, promoter
		pROK2	*srf 6-4*	Salk 062310	promoter (predicted)
At3g14350	*SRF7*	pD991	*srf 7-2* [a, b]	Wis mln 21.13	intron 2 and exon
		pAC161	*srf 7-3*	Gabi 040G01	exon 8
At4g22130	*SRF*8	pROK2	*srf 8-2*	SALK 111750	within the 5'UTR, 89 bp before ATG

All lines except *srf3-9* are homozygote. Complex insertion means that there exist two left borders (LB) in a head to head (corresponding to the 5'-end or the RB) configuration. In the other lines either LB or right border (RB) could be defined (see Table 3-4).

[a] complex insertion [b] WS (Wassileskija) background [c] homozygote *qrt* mutant background

Table 3-2b. Sequence alterations and homologies at the T-DNA integration site

T-DNA line	Junction type/ T-DNA configuration	Gene sequence deletion (-)/ insertion (+) (bp)	T-DNA deletion (-)/ insertion (+) (bp)	Homo-logy (bp)	Map position of the T-DNA integration
srf1-2	_-LB		-260^{LB}	5	8985923-8985927
srf1-3	RB-LB	-7^{g}; $+7^{rb}$; $+2^{lb}$	-264^{LB}; -178RB		8986465-8986471
srf1-7	LB-LB	-2^{g}; $+15^{lb_l}$; $+27^{lb_r}$	-92^{LB_l}; -79^{LB_r}		8985521-8985522
srf2-1	LB-LB	-19^{g};	-271^{l}; -252^{r}	4^{l}; 8^{r}	2112571-2112576
srf2-2	LB-LB	-41^{lb_l}; $+8^{lb_l}$; $+54^{lb_r}$	-266^{LB_l}; -247^{LB_r}		2113784-2113852
srf2-3	LB_LB	-3^{g}; $+5^{lb_l}$; $+46^{lb_r}$	-270^{LB_l}; -248^{LB_r}		2114239-2114241
srf 3-1	LB-_		-284^{LB}	3	1495122
srf3-3	LB-_	+11	-232^{LB}		1494603-1494626
srf 3-7	LB-LB	$+4^{lb_l}$; $+19^{lb_r}$	-72^{LB_l}; 93^{LB_r}		1494723-1494731
srf3-9	LB-_	+12	-108^{LB}		1493143-1493144
srf4-2	LB-_	+20	-248^{LB}		4187762
srf4-3	RB-LB	$+3^{lb}$;$+101^{rb}$	-267^{LB}; -145RB		4188634
srf5-1	LB-LB	$+13^{lb_l}$;	-82^{LB_l}; -74^{LB_r}	3	29715553-29715554
srf5-2	LB-_	+23	-133		29715303
srf6-2	_-LB	+19	-264		20065340-20065341
srf6-4	_-LB				20065260
srf7-2	LB-LB	$+1^{lb_r}$;	-6^{LB_l}; -10^{lb_r}	3	4786217-4786233
srf7-3	LB-RB	$+5^{rb}$	-22RB		4785384
srf8-2	_-LB	+26	-97^{LB}		11723656

Abbreviations: LB- left border, RB- right border; T-DNA left border sequence was found oriented to the left side of the gene sequence together with the RB (LB-RB) or without RB (LB-_); when classified as RB-LB or RB-_ the T-DNA integrated in reverse orientation with or without LB, respectively; no right border could be detected in the reverse configuration _-LB or in the head to head configuration LB-LB; (^{LB}) deletion of the left border sequence; (^{lb}) and (^{rb}) deletions or insertions at the left insertion or right insertion side, respectively; (^{lb_l}) and (^{lb_r}) deletions or insertions at the left or right integration side of the LBs; (^{g}) indicates deletions from the genomic sequence; (^{LB_l}) and (^{LB_r}) deletions of the LB at the left or right integration side sequence homologies can occure at the left (^{l}) or right (^{r}) integration side of a head to head T-DNA line *srf6-4* was sequenced until this sequence position. Further detailed analysis was impossible to detect, due to a corrupted sequence.

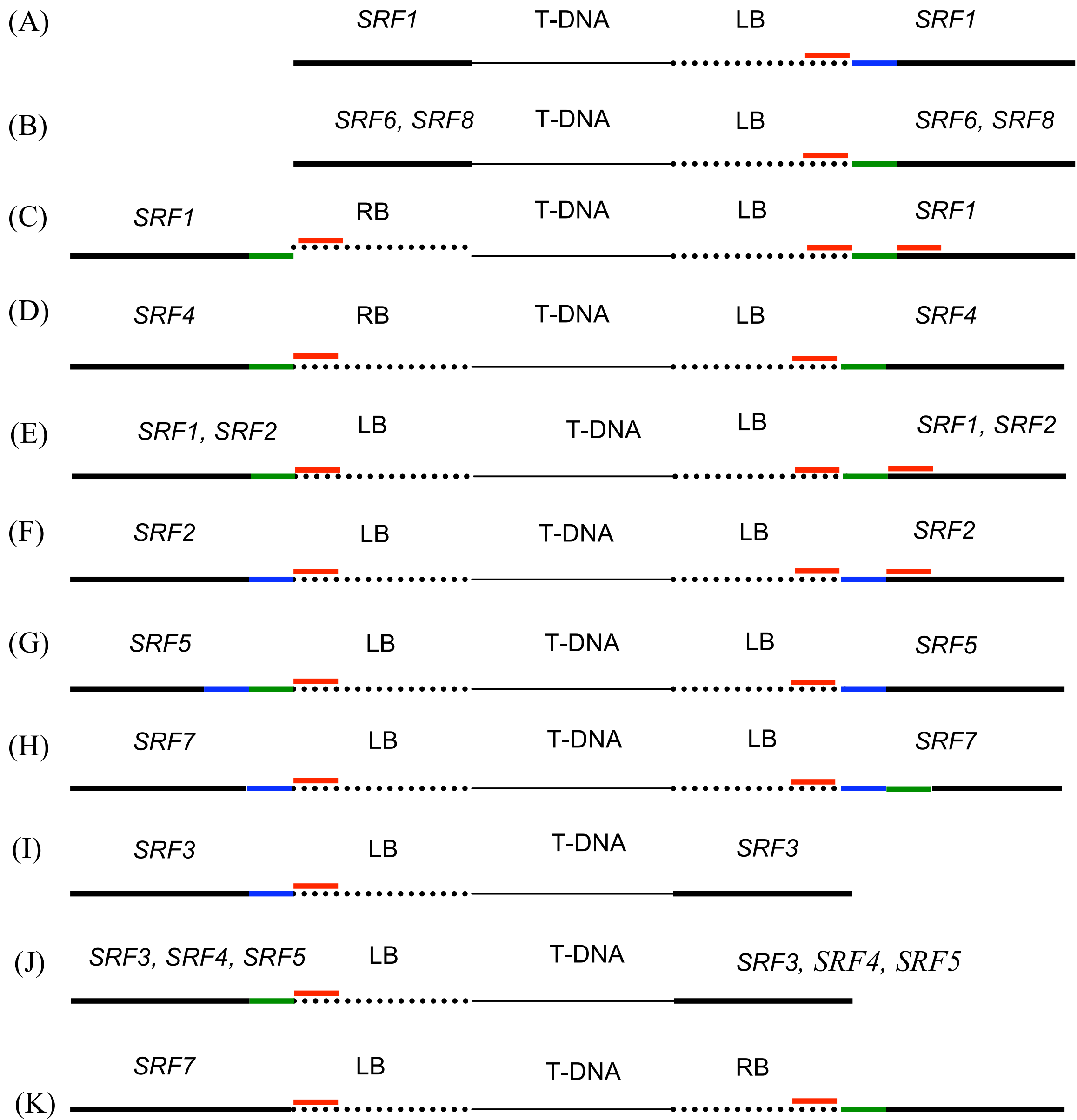


Figure 3-7. Schematic structure of the T-DNA integration. (A) *srf1-2;* (B) *srf6-2, srf8-2;* (C) *srf1-3;* (D) *srf4-3, srf3-7;* (E) *srf1-7, srf2-2, srf2-3;* (F) *srf2-1;* (G) *srf5-1;* (H) *srf7-2;* (I) *srf3-1;* (J) *srf3-3, srf3-9, srf4-2, srf5-2;* (K) *srf7-3*
The straight thick black line shows the genomic part. The straight thin black line represents the T-DNA region. The dashed black line display the left border (LB) or right border (RB). Blue line indicates a microhomology between short segments within target genomic sites and T-DNA. Red line represents deletions in the genomic, LB or RB sequence. Green line exhibits additional sequences.

All SAIL- former Garlic-lines created with the pCSA110-vector are homozygote for the *qrt* mutation (Rhee *et al.,* 1998). This mutation does not interfere with the general development of the plant. All plants were fertile with enough pollen to pollinate for the generation of double mutants. T-DNA lines with integration site at the beginning of the gene and within the exon were selected. To investigate if the lines are real knock-out mutants, RT-PCR from *srf1-2, 1-3, 1-7, 2-1, 3-1, 3-3, 3-7, 4-2, 4-3, 5-1, 5-2, 6-2, 7-2, 7-3* and *srf8-2* were performed. In all of the tested insertion lines a transcript was visible when using primer situated 3' from the insertion (see arrows in Figure 3-6). That means that after the T-DNA integration site the gene is still transcribed in the correct originally way. *Srf1-7, srf2-3, srf3-9, srf4-2, srf4-3, srf5-1* and *srf7-3* have their insertion in the exon of the extracellular region. But even these lines showed gene transcription analysed by the RT-PCR approach. The insertion sites of *srf1-3, srf2-1, srf3-1, srf6-2 and srf6-4* are located before the 5'UTR in the promoter, which can give an explanation for T-DNA gene transcription. The following Table summarizes the gene locations of the *SRF* insertion lines.

Table 3-3. Location of the T-DNA insertions in the *SRF* genes

Location of the T-DNA insertions	Genotype
Promoter	*srf1-3, srf2-1, srf3-1, srf6-2, srf6-4*
5' UTR	*srf8-2*
Exon	*srf1-7, srf2-3, srf3-7, srf3-9, srf4-2, srf4-3, srf5-1, srf7-2, srf7-3*
Intron	*srf1-2, srf2-2, srf3-3, srf5-2, srf7-2,*

Srf7-2 was detected spanning a region from intron 2 inside exon 3.

The size of the T-DNA vectors range from 4763bp (pDAP101), 5258 (prok2), 5799bp (pAC161), 5938bp (pD991) to 7541bp (pCSA110). All sequenced T-DNA insertion lines of the *SRF* genes are shown in Table 3-2a and 3-2b.

Figure 3-6 illustrates the results. In nine lines out of nineteen (47%) only one amplified DNA fragment with a left border junction could be detected. The identified LB was either inserted in the correct orientation according to the gene sequence (LB-_) or in the reverse orientation (_-LB). The right border (RB) could only be identified in three lines out of nineteen (app. 14%). In one line, the sole GABI line (*srf7-3*) left border and right border were present and in the correct orientation. In sixteen lines the RB was missing (84%). Seven insertion lines showed a head to head arrangement (LB-LB), where two RBs (5' ends or heads) were ligated.

Therefore two left-border sequences of tandem T-DNA copies was the most common configuration in this examination. The following Table 3.4 shows a small compilation of all T-DNA configurations. A lack of amplification with one of the T-DNA end primers could indicate that deletions removed either the LB or RB primer site from the left or right T-DNA ends, respectively. These observations fit to T-DNA insertion mutant collections (Castle *et al.,* 1993; Krysan *et al.,* 1996; Krysan *et al.,* 1999; Rios *et al.,* 2002). Table 3-2b gives a more detailed outline of the T-DNA flanking sequence results in connection with Figure 3-7.

Table 3-4. Integration pattern of the *SRF*-T-DNA junctions according to the genomic gene sequence.

Number of insertion lines	Insertion line	configuration
2	*srf1-3, srf4-3*	RB-LB
1	*srf7-3*	LB-RB
4	*srf1-2, srf6-2, srf8-2, srf6-4*	_-LB
5	*srf3-1, srf3-3, srf4-2, srf5-2, , srf3-9*	LB-_
7	*srf1-7, srf2-1, srf2-2, srf2-3, srf3-7, srf5-1, srf7-2*	LB-LB
(RB) right border; (LB) left border; (_) missing border sequence		

A significant bias of the integration sites within the genome was observed in the genome rich chromosome arm. Inside the individual genes the integration occurred in favor to 5', 3' UTRs and promoters (Alonso *et al.,* 2003; Qin *et al.,* 2003). T-DNAs in all homozygote lines analysed in this report were located either in the promoter region, in the 5'UTR or inside the gene.

Previous publications indicate that more than a half of the analyzed T-DNA lines contain tandem insertions or a second-site insertion that do not confer resistance (Rosso *et al.,* 2003). During T-DNA integration T-DNA or genomic rearrangements can occur (Takano *et al.,* 1997; Jakowitsch *et al.,* 1999; Kohli *et al.,* 2003; Windels *et al.,* 2003) The sequence results shown here corresponds to other investigations. All analysed lines have deleted parts in the T-DNA sequence (left or right border) and/or in the gene itself. It was frequently that additional sequences or overlapping/homologues sequences could be detected. A very huge part of the LB was usually absent, compared to the RB (if present) missing part. The presence of small deletions and unexpected DNA sequences at the sites of T-DNA insertions has been described by (Feldmann 1991) and (McKinney *et al.,* 1995). It is assumed that T-DNA integrates via left border invasion into a double-stranded breaks (DSB) (Tinland 1996; Somers *et al.,* 2004). That's why in most cases only LB border could be characterized.

3.3 Phenotype analysis

Confirmed homozygote T-DNA lines were used for the first phenotypic investigations of the *SRF* family if there is an altered phenotype in the insertion lines visible. Investigations refer to general aspects of plant structure, like morphology, habitus, dimensions of the whole plant and all single organs, growth rate (time from germination until siliques with seeds) and particular roots and trichomes from cauline and rosette leaves. The only visible phenotype was smaller rosette leaves of *srf4-2* and *srf4-3* than wild type when growing under normal greenhouse conditions like described in Material and Methods. Due to small dataset no statistical examination could be done. In (Eyueboglu *et al.,* 2007) more relevant data are already published and statistically proven to be significant (see also Figure 3-8). The leaf shape is not altered in the *srf4-2, srf4-3* mutants *or 35S::SRF4* plants. Other single, as well double-mutants showed no obvious visible phenotype. They were comparable with wild type. All single insertion lines were crossed into L*er* background, because from previously studies it is known that the *sub* phenotype is more obvious in L*er* background. A modifier gene in wt Columbia can probably cause this effect (Chevalier *et al.,* 2005).

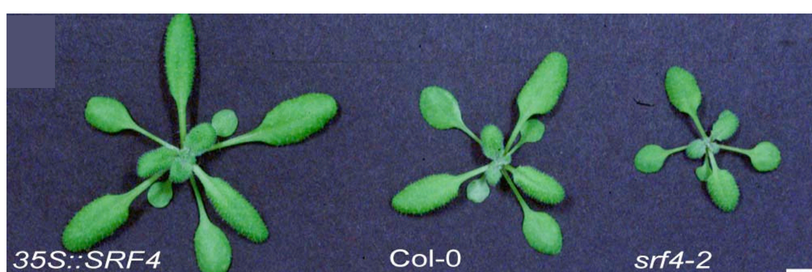


Figure 3-8. Phenotype of *Arabidopsis th.* leaves. The 35S::*SRF*4 (line 1–5), Col, and *srf*4-3 plants are shown. Leaves are enlarged and reduced, respectively. Scale bars: 0.5 mm. Picture from (Eyueboglu *et al.,* 2007)

3.3.1 Root analysis

To look also for different environmental conditions and other organs than the above ground organs, roots from *srf4-2/4-3* and *srf5-1/5-2* single mutants and double mutants (*srf4-2x5-1, srf 5-1 x 4-2, srf4-3x5-2, srf5-2x4-3, srf5-1x4-3* and *srf4-3x5-1*) were tested on standard 1% MS agar plates with 1% sucrose and concentrations ranging from 0,3-2% sucrose placed vertically under continuous light conditions (see Material and Methods). Concentrations over 1% were not further analysed, because this can cause stress condition and may not provide any functional indication. An excessive growth of adventitious roots was the general observation in all mutants as well as in wild type roots when growing on higher sucrose concentration.

At normal sucrose concentration (1%) *srf4-2* and *srf4-3* show significant (with a confidence interval of 95%) smaller roots than wtCol, while even *srf4-3* is highly significant (confidence interval = 99%) smaller than wtCol. A similar effect was visible when looking at the leave size. Leaves of s*rf4-3* were also smaller than *srf4-2* compared with wtCol. *srf4-2* and *srf4-3* mutant roots are highly significant (confidence interval = 99%) larger than wild-type roots grown on 0,3% sucrose plates. *Srf5-1* and *srf5-2* generate only significant larger

(confidence interval = 95%) on 0,3% sucrose plates, but highly significant (confidence interval of 95%) larger roots than wtCol when growing on 1% sucrose. Data are listed in Table 3-5.

The growth varies already between the reciprocal crossed double mutants. For example *srf4-2x5-1* means that *srf4-2* was the female cross partner and *srf5-1* provided the pollen (male component). When grown on 1% sucrose *5-1x4-2* shows smaller roots than wtCol but *srf*4-2x5-1 shows no different root length according to wild type.

Srf4-3x5-2 and *srf5-2x4-3* double mutants are both highly significant (α=1%) smaller than wtCol. S*rf5-1x4-3/srf4-3x5-1* double mutants are also highly significant smaller than wtCol and slightly smaller than *srf4-3x5-2/srf5-2x4-3* double mutants and a little larger than *srf5-1x4-2*.

When growing on 0,3% sucrose *srf4-2x5-1/5-1-4-2, srf4-3x5-2* and *srf5-1x4-3* double mutant roots are highly significant larger than wt. *srf4-2x5-1* and *srf4-3x5-2* display a bigger difference to the wild-type Col root length, which seems that the female (here *srf4-2* and *srf4-3*) components had an stronger effect on the growth. The growth effect on 0% sucrose plates was not so remarkable. Only *srf5-2* and *srf4-2x5-1* showed significant larger roots than wild-type.

The hypocotyle growth was investigated under dark condition like described in Chapter 2. The hypotcotyl growth did not show an aberrant phenotype to the wild type plant.

Table 3-5a. root length of *srf* single mutants grown on 0,3% sucrose MS plates					
plant line	wt	*srf*4-2	*srf*4-3	*srf*5-1	*srf*5-2
Mean±SD	1,20 ± 0,34	1,95 ± 0,84	2,03 ± 0,48	1,69 ± 0,46	1,39 ± 0,49
Observations	98	48	40	53	53
z-values		5,95 *	9,88 *	2,34	2,53

Table 3-b. root length of *srf* single mutants grown on 1% sucrose MS plates					
plant line	wt	*srf*4-2	*srf*4-3	*srf*5-1	*srf*5-2
Mean±SD	2,92 ± 0,59	2,66 ± 0,55	2,17 ± 0,49	4,09 ± 0,71	4,11 ± 0,62
Observations	52	62	67	66	66
z-values		-2,6	-7,32 *	9,8 *	9,45 *

Table 3-c. root length of *srf* double mutants grown on 0,3% sucrose MS plates					
plant line	wt	*srf*4-2x5-1	*srf*5-1x4-2	*srf*4-3x5-2	*srf*5-1x4-3
Mean±SD	1,20 ± 0,34	2,67 ± 0,69	1,71 ± 0,33	2,67 ± 0,49	1,58 ± 0,11
Observations	98	49	50	50	53
z-values		14,31 *	8,77 *	19,04 *	10,11 *

Table 3-d. root length of *srf* double mutants grown on 1% sucrose MS plates			
plant line	wt	*srf*4-2x5-1	*srf*5-1x4-2
Mean±SD	2,92 ± 0,59	3,00 ± 0,53	1,61 ± 0,3
Observations	52	69	72
z-values		0,87	-14,66 *

Table 3-4d continued.				
plant line	*srf*4-3x5-2	*srf*5-2x4-3	*srf*5-1x4-3	*srf*4-3x5-1
Mean±SD	2,28 ± 0,52	2,41 ± 0,66	1,87 ± 0,37	2,09 ± 0,42
Observations	66	49	72	65
z-values	-6,15 *	-4,04 *	-11,30 *	-8,53 *

Table 3-4. The mean ± standard deviation (SD) is shown. The mean value is given in cm. The z-value refers to two-tailed z-test. The critical z-values of a two-tailed z-test are 1,96 (P < 0,05)
and 2,58 (P < 0,001), respectively. * null hypothesis rejected (P < 0,001).

3.3.2 Pollen phenotype

The results from the ISH, GUS and AtGenExpress investigations have revealed a high expression of *SRF4* in wild type *Arabidopsis* pollen. A very specific pollen expression peak was also observed with *SRF5*, but not so highly expressed like *SRF4*. In order to address a possible function, T-DNA lines (*srf4-2, srf4-3, srf5-1* and *srf5-2*) were analyzed with DAPI staining.

The pollen showed no abnormalities in the pollen grain nuclei anatomy or nuclei division aberration (Figure 3-8). All three nuclei were detectable properly.

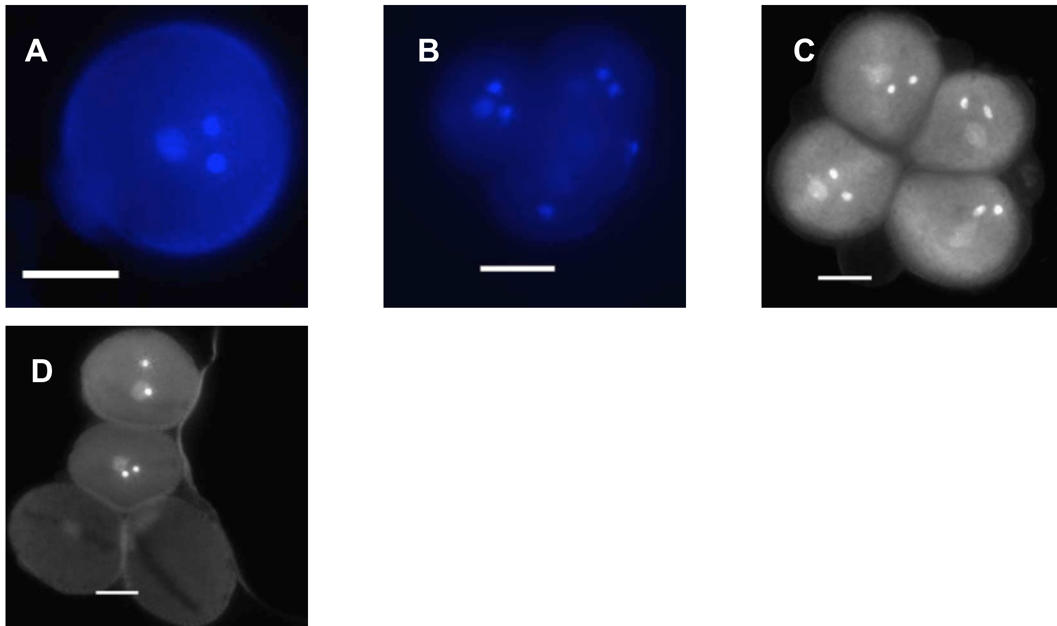


Figure 3-8. DAPI staining of wild type and mutant pollen. The presence of DNA was detected with DAPI, which specifically binds to DNA and fluoresces blue when exposed to ultraviolet light. (A) wild type Col, (B) *srf 4-2,* (C) *srf4-3* and (D) *srf*5-1. (A) and (B) fluorescence view with a UV-filter; (C) and (D) white light view. Scale bar = 10µm

4. Discussion

4.1 Expression analysis

To investigate the expression of genes different methods can be used; plenty of databases in web, like the AtGenExpress (microarray-based techniques) data at www.genevestigator.ethz.ch or http://bar.utoronto.ca are available. Expressed sequence tags (ESTs) is an useful approach as well to exploit the spatial and temporal expression patterns of genes, including mRNA and/or protein distribution in different cell types, during development, pathogen infection, or in different environments (Cooke *et al.,* 1996).
In this work ISH was performed in detail. GUS-promoter analyses were only done with few *SRF* members with little experiments. The anlysis of the AtGenExpress Data were used to get first impression of the expression level of the *SRF* family.
The expression detectable in petals and ovules with the *in-situ* method were maybe not as high like mature pollen, like indicated in the AtGenExpress data, but also existing.

4.1.1 *In-situ* Hybridization analysis

ISH analysis can give more precise insight into the spatial and temporal mRNA expression within the plant structure. For all *SRF* candidates an expression in the anthers and ovules were detectable. In *SRF2, SRF4, SRF5, SRF6, SRF7, SRF8* the expression was detectable from early flower stages until later stages. According to this expression analysis it seems that all our *SRF* gene family members are involved in anther and ovule development. In *SRF1* and *SRF3* the expression was very low compared to other expression profiles of the family members. Therefore the signal was difficult to identify. Another important fact to address is the time point of examination.

Young stage organs show a very high cell proliferation rate and consequently a high RNA content in their small cells, compared to older stage organs. Thus, there is a high-density fraction of cells additional to the organ-color (f.ex. yellow pollen), which has already a strong autonomous-coloring. All these aspects make it difficult to distinguish between strong and weaker expression.

The observation of *SRF* gene expression in flowers indicates for an activity of this gene family through the whole flower developmental stages. In *SRF2, SRF4, SRF5, SRF7* and *SRF8* the expression starts at the IM, through FM, young developing organs until older flower stages. They are specially acting in cell proliferating tissues of developing organs. The remaining members *SRF1* and *SRF3* seem to be only active in the later developmental stages (see above). A possible function during the anther development is supported by the observation of *35::SRF2/3/4/5/7* plants, as they show sterility (Eyueboglu *et al.,* 2007).

EXCESS MICROSPOROCYTES1 (EMS1) also known as *EXTRA SPOROGENOUS CELLS (EXS)*, a putative LRR-RLK is strongly expressed in the young anthers and ovaries. *EXS* transcripts are also present in the infloresence meristem and in the developing floral meristems. A strong expression continued in the young organ primordia, which is focused in the microsporangia and in the distal and chalazal regions of the ovules (Canales *et al.,* 2002). *EMS1/EXS* and the putative ligand *TAPETUM DETERMINANT1 (TPD1)* pay key roles in anther cell fate determination (Jia *et al.,* 2008). *Haiku 1 (IKU1)* and *Haiku (IKU2),* encoding LRR-RLKs are expressed in the endosperm nuclei of developing seeds. They are involved in endosperm proliferation and cellularization, where their mutants show reduction in endosperm growth resulting in reduced seed size (Luo *et al.,* 2005).

SERK1 and *SERK2* show a complex expression pattern throughout development. After Expression in anther primordia expression in the sporocytes ceases and continued in the tapetum. Double mutants of *SERK1* and *SERK2* are male sterile due to the failure in tapetum specification (Albrecht *et al.,* 2005). All these mentioned examples of genes involved in anther and/or pollen development could give an idea of the possible function of the *SRF* genes.

In some analyses (see examples in Figure 3-1) a sense expression was detected. For *SRF1, SRF4* and *SRF5* only a slight sense expression was visible. However *SRF7* shows a quite strong sense expression in the ovules. A possible explanation for sense expression detection could be the existence of small RNAs (sRNAs) (21to 24 nucleotides) actually described as MPSS mRNA targets (Meyers *et al.,* 2004; Lu *et al.,* 2005; Lu *et al.,* 2006), sRNA Contig sequences (Vaughn *et al.,* 2005), SAGE targets (Velculescu *et al.,* 1995; Velculescu *et al.,* 1997), small anti mRNAs or as miRNA targeted transcripts in Arabidopsis (Adai *et al.,* 2005). The small RNA population in plants is very complex, because in addition to produce microRNAs (miRNAs) that play critical roles in various developmental, stress and signaling responses, plants also produce a complex amount of small interfering RNAs (siRNAs). Among ~77'000 different small RNAs that have been sequenced from Arabidopsis so far, miRNAs seems to have only a proportion of <10%, while siRNAs numbers around 70'000. siRNAs can inhibit the translation of target mRNAs. Heterochromatic modifications with resulting transcriptional silencing is also caused by siRNAs (Vaughn *et al.,* 2005). Inflorescences were used to generate the MPSS libraries, which are known to be a rich source of small RNAs (Lu *et al.,* 2005). MPSS mRNA targets or anti miRNA were compiled at the internet page from SAIL (http://signal.salk.edu/cgi-bin/atta) or at http:://mpss.udel.edu/at (Meyers *et al.,* 2004; Lu *et al.,* 2005; Lu *et al.,* 2006). sRNA Contig sequences are also present at the transcriptome platform of SAIL, which represent the total amount of miRNA and siRNAs together (Vaughn *et al.,* 2005). Serial analysis of gene expression (SAGE) is another method for generating profiles of the mRNA expression in a given cell type (Velculescu *et al.,* 1995; Velculescu *et al.,* 1997). Small anti mRNAs in the reverse orientation of the cDNA sequence are defined aswell in the databases. Additional potential miRNA targeted transcripts could be found by computational prediction of miRNAs in Arabidopsis. These data shows intergenic regions or intron designations of miRNA precursur candidates corresponding to the sequence within the transcript of interest in the Arabidopsis genome (Adai *et al.,* 2005).

In the future vegetative stages can be analyzed to look for other expression than flowers. Especially the root or the rosette leaves, where a phenotype of *srf4* was investigated. A little disadvantage of the *in-situ* analysis is the non-quantitative examination. Other experiments must be used additional, for example quantitative RT-PCR analysis.

4.1.2 AtGenExpress data

The GeneChip array data (Schmid *et al.,* 2005) were used for analyzing the expression profiles of the *SRF* genes, which are publicly available from the Nottingham Arabidopsis Stock Centre Transcriptomics Service (NASCArrays). These data comprises 79 samples covering various growth condition and developmental stages. These results demonstrate a wide range of expression pattern. In the *SRF*4 and *SRF*5 profiles there is an obvious high expression in the mature pollen.
Already characterized RLKs involved in pollen regulation are found in the tomato genome. The pollen-specific receptor kinases LePRK1 and LePRK2 interact with a novel peripheral membrane protein that is essential for normal pollen tube growth and that again are involved in regulating local responses (Kaothien et al., 2005). Kim *et al.,* 2002 confirmed with RT-PCR pollen expression for five of Arabidopsis candidates, where two of them are clearly homologues of LePRK3. *BRASICA CAMPESTRIS PROTEIN 1 (BCP1)*, which is expressed in the diploid tapetum and in haploid developing pollen encodes a small anther-specific protein from *Brassica campestris* and *Arabidopsis thaliana.* This gene is essential for completion of pollen development (Xu et al., 1995). The mRNA of the *RECEPTOR LIKE KINASE IN FLOWERS1* (*RKF1,* At1g29750), a LRR-RLK, is highly expressed in early flower primordia and during stamen development in Arabidopsis flowers (Takahashi et al., 1998).

Additional *RKF1* promoter directed high GUS expression in pollen grains. The putative extracellular domain of *RKF1* contains 13 tandem repeats of leucine-rich sequences.

According to these results there are few examples of LRR-RLKs, which have a reasonable function in pollen development. Also *35S::SRF2, SRF3, SRF4, SRF5* and *SRF7* plants exhibit sterility (Eyueboglu *et al.,* 2007), therefore these genes seems to be involved in the anther- tapetum and/or pollen development. For the AtGenExpress data, Col-0 ecotype was used, contrary to wild-type L*er* background used in the *in-situ* experiments.

4.1.3 Promoter-GUS studies

In this first pilot study the GUS expressing transgenic lines were also used to confirm the *in-situ* Hybridization (ISH) results. In this experiment only a few whole flowers were analysed, for that reason more sample probes should be taken to find probably additional expression, which was probably overseen.

SRF1, SRF6 and *SRF7* show expression in the filaments, in the connectives of the stamina, in the style of the gynoecium and in the ovules. Also in sepals an expression was vsible.

SRF4 shows a very specific expression in the developing microsporocytes and mature pollen, but new observations indicate also expression in the sepals, petals, filaments, anthers and whole gynoecium (Christine Skornia, personal communication) .

The expression results obtained with the promoter-GUS examination indicate again for a wide range of expression throughout the flower developmental stages, like already expected from the ISH experiments and AtGenExpress data.

Flower sections will be the next more detailed analysis, to define more precisely the expression and for comparing with the ISH results.

Also other organ tissues like leaves or roots should be analysed.
It can serve as a pre-experiment to create *in-situ* Hybridization experiments, which are known to be much more exactly. For further analysis a cyclin dead box (CDB) DNA sequence could be integrated which direct the degradation of the GUS protein at the end of mitosis phase during cell cycle (Donnelly *et al.,* 1999). It avoids a non-specific staining.

4.2 Reverse genetic approach by using T-DNA lines

The aim of the study represented here was to find possible functions for the eight *SRF* family members. Is the function of the members comparable or quite different to SUB. For a confirmation of possible phenotypes, insertion lines with at least two different independent alles were used (except *srf8* with only one insertion line).

Although many Arabidopsis knockouts mutagenized with insertion elements, such as the T-DNA of Agrobacterium or transposons have already been obtained, few of them display phenotypes that provide a direct indication to gene function (Bouche *et al.,* 2001). Presumably partial and complete functional redundancy besides the ability of higher plants to adapt their physiology to various stresses and constraints without undergoing morphological changes and by the difficulties to detect slight physiological alterations and/or weak reduction of fitness could explain the lack of phenotypic modification in some cases. It also appears that many mutations are conditional and/or do not alter plant morphology even in the presence of severe physiological defects (Bouche *et al.,* 2001). A further discussion point is the average number of independent inserts (multiple insertions) which is 1,5 per diploid genome (Feldmann 1991). It could mean that somewhere else in the genome one more insertion exists.

Not only a further T-DNA, also complex arrangement of T-DNA, existence of vector backbone sequences, chromosomal duplication and rearrangements or a combination of these events could be present in the genomes of transformed host plants (Jorgensen *et al.,* 1987; Veluthambi *et al.,* 1988; Tax *et al.,* 2001). Some studies showed the existence of truncated T-DNA regions. T-DNA regions beyond the border repeat were also found to be stably integrated into plant genomes at high frequencies (Kononov *et al.,* 1997). 75% of the transgenic tobacco plants contain binary vector backbone sequences integrated into the plant genome. Therefore it is possible that the mutations and the observed mutant phenotype caused by the insertion of backbone sequences independent of the T-DNA (Koncz *et al.,* 1989; Errampalli *et al.,* 1991; Feldmann 1991). T-DNA integration is not site-specific and appears to distribute randomly throughout the plant genome. But is most likely that intergenic regions are more susceptible to T-DNA integration (Alonso *et al.,* 2003; Rosso *et al.,* 2003). The most frequent T-DNA integration event exhibit regions of homology (or microhomology) at T-DNA/plant DNA junctions or contain short filler DNAs at their junction (Gheysen *et al.,* 1991; Mayerhofer *et al.,* 1991), which was also found in these analysis (see Table 3-2b). Longer microhomologous regions were usually observed near the 3'end (LB) of the T-DNA. (Feldmann 1991) and (McKinney *et al.,* 1995) reported that T-DNA integration could be accompanied by small deletions and unexpected DNA sequences in the plant DNA and T-DNA sequences at the site of T-DNA insertion. Like observed in this study deletions in the T-DNA were more severe at the T-DNA 3'-end (LB) compared with its 5'-end (RB), because the RB is protected by the VirD2 protein during the integration process (Gheysen *et al.,* 1991; Mayerhofer *et al.,* 1991).

The results from *SRF* gene project are conforming to the reports of (Rios *et al.,* 2002), where only 8% of insertions resulted in the amplification of two DNA bands with the left and right T-DNA border primers.

By contrast, two left-border sequences of tandem T-DNA copies were found in 32% of tagged genes, whereas two right border–gene junctions were detected in 5% of mutant loci. Only one amplified DNA fragment carried either a left (41%) or a right (14%) T-DNA border junction could be detected in this study.

The data in the present report and mentioned publications indicated that a significant proportion of insertion loci contained tandem T-DNA copies facing the insert junctions with their left border sequences. This fusion between two right border ends ("heads") is a precise fusion, whereas the left border junctions ("tails") often exhibited imprecise fusion and/or contained filler DNA ((Tzfira *et al.,* 2004). The occurrence or detection of mainly LB sequences can be explained by the fact that the integration starts with the LB and couldn't integrate completely due to a stop of the integration process. It was found that ~ 40% of all T-DNA junctions harbored filler DNA (Windels *et al.,* 2003). A model described in (Tzfira *et al.,* 2004) explains the "head to head" configuration with the attachment of the VirD2 molecule at the 5'-end (RB) by following connection of two T-DNAs. Why mostly the LB could be detected without another LB or RB could be explained with this theory, that probably the other LB was more truncated than the detected LB or that the insertion procedure was interrupted. All analyzed lines had deleted parts in the T-DNA sequence (left or right border) and/or in the gene itself. Consistent with the data presented here, a genome-wide T-DNA insertional analysis revealed a bias for T-DNA integration in favor to intergenic regions, promoters and 5' and 3' untranslated regions (Alonso *et al.,* 2003; Rosso *et al.,* 2003). 90,000 T-DNA lines reported in (Rios *et al.,* 2002) revealed that 54% of T-DNA inserts landed in exons, 23% in introns, and 23% in promoter or 5' untranslated mRNA coding sequence.

The advantage or viability of T-DNA usage to get some insight into the gene function was tested and proved with mutant lines with known functions. They showed different correlations between T-DNA locations within the gene and the observed phenotype.

For example a line with the insertion in the 5' leader sequence of *TERMINAL FLOWER 1 (TFL1)* gene showed no *tlf1* phenotype (Larsson *et al.,* 1998). In contrast a line with an insertion in the 5' leader of the *WAX2* gene showed the expected *glossy* phenotype (Chen *et al.,* 2003).

Also in other insertion locations like intron and exon (early or later in the gene) show the expected phenotype. One cannot say that insertion outside the open reading frame (ORF) cannot cause a phenotype (Rhee *et al.,* 2003).

Nevertheless in one case of *TRANSPARENT TESTA (TT5)* locus the insertion sits in the 3' region at the end of the gene and displays no *tt* phenotype (Burbulis *et al.,* 1999; Winkel-Shirley 2001; Rosso *et al.,* 2003).

For accurate analysis backcrosses and when the phenotype is known, co-segregation analysis will be needed for exclude other influencing parts in the genome, like mentioned before. Maybe a Southern Blot analysis should be done to test if more than one insertion line exists in the gene. Another possible approach to look for an eventual phenotype in the other *SRF* members than *SRF4* is to test different wild type backgrounds. Further analysis with different environmental conditions must be tested for example for light, temperature, stress or pathogens.

4.3 Redundancy aspects in the SRF family?

To eliminate a possible redundancy, double mutants of the respective close related members were created. The double mutant investigation should uncover the possible redundancy within the *SRF* gene family members. But the double mutants reveal no obvious phenotype. When assuming a redundancy for example of *SRF4* and *SRF5,* where the global correlation analysis of *SRF* transcripts revealed a possible redundant effect (Eyueboglu *et al.,* 2007), the double mutants should show a phenotype. *Srf4-2x5-1, srf4-3x5-1* and *srf4-3xsrf5-2* double mutants showed similar effects like *srf4-2* or *srf4-3* single mutants in leaves.

This result let presume a different function, because when they act in the same process, one expect an additive or a similar effect of the single mutant leave phenotype. The leaf size in the double mutants was smaller like the single *srf4* mutant. The same case was observed in *srf4/srf5* roots, where smaller roots or like wildtyp root length could be measured. This concludes that at least the analysed gene pair combinations have no redundant function.

Evidences for a divers function of the *SRF* members are the sequence differences in the SRF protein structure for example in a part of the kinase domain, where the substrate binds. Also the global *SRF* coexpression analysis do not provide for a redundancy affect (except *SRF4/SRF5)*.

There exists few examples of closely related LRR-RLK gene family members that they are functional redundant. For instance *SERK1/SERK2* or the *BAK1 (SERK3)* homologes (Li, Wen *et al.,* 2002; Nam *et al.,* 2002; Albrecht *et al.,* 2005). Trippel mutants might be the next experiment when looking at the gene function of the family. It could be possible that a third gene family member leads to a phenotype and therefore they are involved in the same pathway (Eyueboglu *et al.,* 2007).

4.4 Phenotypic investigations

The morphological form and shape of the *srf* mutant plants organs resembles the wild type phenotype, except *srf4*. Analysis of the rosette leaf size of *srf4-2* and *srf4-3* insertion lines show reduced size compared to wild type plants and *SRF4::*35S overexpressing lines (Eyueboglu *et al.,* 2007). Additional *srf4* roots showed also a reduced length compared to wildtyp.

All other analysed single *SRF* gene mutants (T-DNA) exhibit no obvious phenotype. There are different reasons for the absence of phenotypes in the T-DNA lines. They are no real knock-out mutants, because all mutants still show RNA transcripts and for this reason they probably produce still active proteins.

To test if the produced RNA amount was enough or in excess, a quantitative RT-PCR might be performed in future experiments.

Additional to the already described facts before, plants have the ability to adapt their physiology to various stress and constraints without undergoing morphological changes, even when they have profound physiological defects. To identify conditional phenotypes, the mutants have to be tested under a wide range of environmental conditions (Meissner *et al.,* 1999).

Other possibilities for screening the gene function are expression analysis (like described in the chapters before) or gain-of-function. It is especially useful for genes that have redundant and multiple functions during development. The observed result from *srf4-2* and *srf4-3* with a reduced leave size (Eyueboglu *et al.,* 2007) should be proved that the phenotypic characteristic is indeed controlled by the gene of interest. This can be carrying out with a couple of backcrosses to wild type. To further verify that the insertional mutation causes the phenotype, one can complement the mutation by introducing a wild-type copy.

4.4.1 Root phenotype

The root morphology was investigated on MS media plates place vertically under continuous light conditions and different sucrose concentrations.

Sugars like sucrose, glucose and fructose have been implicated to control many aspects of plant development, including hypocotyl elongation, primary and lateral root growth and the development of adventitious roots (Zhou *et al.,* 1998). Like reported in (Bingham 1993; Kurata 1997; Kurata *et al.,* 1998; Cano-Delgado *et al.,* 2000) sucrose stimulate elongation growth of the hypocotyls and the root.
In addition sugars affects gene expression, cytoplasmic Ca^{2+} concentration, cell division, seed germination and flowering (Jang *et al.,* 1997; Pego *et al.,* 1999; Riou-Khamlichi *et al.,* 2000; Furuichi *et al.,* 2001; Ohto *et al.,* 2001).

When adding higher concentrations of glucose (>2%) to the growth media seedling growth was suppressed (Zhou *et al.,* 1998).

It is evident that not only hormones, such as auxin and ethylene, but also sucrose play an important role in root development. Sucrose and/or glucose induce the initiation of adventitious roots. (Takahashi *et al.,* 2003). Sucrose is the main transported form of assimilates, but it also regulates a variety of processes such as photosynthesis and carbon or nitrogen storage. The effects of high sucrose levels are mediated directly by modulation of gene expression (Martin *et al.,* 1997). Root architecture is mostly affected by the carbon availability in the plant. The carbon status of root cells involved in elongation and branching processes is thought to determine the root structure. Changes in elongation rate and/or branching are associated with changes in sugar concentration (Freixes *et al.,* 2002). Under normal growth condition with 1% sucrose, roots of *srf4-2, srf4-3, srf5-1* and *srf5-2* showed length differences to wild type. *srf4-2* roots are smaller and *srf5-1* longer, one can expect that in the double mutant the single mutant effect is compensated. All double mutants show smaller root length compared to wildtype, except s*rf4-2xsrf5-1* double mutants showed no difference to wild type. In this case the negative growth affect of *srf4-2* is suppressed by *srf5-1.* To conclude a function of *srf5-1* as a modifier is speculative, due to the fact that double mutants created with the reverse cross or other *srf4* partner the roots were smaller, like the single *srf4* mutants. But so far we do not know any function of *SRF5.* The longer root length of *srf5* mutants grown on 1% let assume, that it acts as a negative root length regulator. The last observation with smaller roots in *srf5-1x4-2* let argue back to the leave phenotype observation, where in the double mutants the same growth defect like in the *srf4* mutants could be detected. From these observations it can be conclude that *SRF4 and SRF5* function in different processes during the plant development. Probably both have its own effect, but do not act together at the same time or developmental stage during plant growth.The phenomenon of different phenotype of plants with the same genotype can be explained with a segregation distortion event.

Segregation distortion (SD) is often attributed to pollen-pistil incompatibilities (Lord *et al.,* 2002), gametic competition (Lu *et al.,* 2002), negative epistatic interactions (Li, Z. *et al.,* 1997) or gamete abortion (Sano 1990). Törjek 2006 described, that an SD region in the *Arabidopsis* genome is associated with reduced fertility and is predominantly caused by an epistatic interaction of two loci situating on chromosomes IV and V.

Already characterized genes that are involved in root development are LRR-extensins (*LRXs*) or *CLV3/EMBRYO SURROUNDING REGION (ESR)-* related (*CLE*).

LRX1 is an extracellular protein of Arabidopsis consisting of an LRR-domain and a structural extensin domain and it is specifically expressed in root hairs. They are presumed to be cell wall localized and may be critical regulator of cell shape specification, for example LRX1 is required for proper root hair cell morphogenesis and elongation (Baumberger *et al.*, 2001). Also SUB/SCM is required for root hair specification (Kwak *et al.,* 2005), which indicates that other members of this SRF family could be involved in this process.

CLE peptides are involved in another process within roots or SAM. The mutated peptides investigated in the study of (Oelkers *et al.,* 2008) arrested the activity of the root apical meristem and lateral root meristems, which results in reduced root growth. The receptor complex CLV1/ClV2 with their peptide CLV3 is required for limiting the number of stem cells at the shoot apical meristem (SAM).

It has been also shown that the *AGAMOUS*-Related MADS-Box Gen *XAANTAL1 (XAL1/AGL12)* has an important role in root development as well as in flowering transition. *xal* mutants have a short-root phenotype with a smaller meristem, lower rate of cell production, and abnormal root apical meristem organization. Additional they showed abnormal periclinal divisions at the Quiescent Center (QC) and deformed columella cells (Tapia-Lopez *et al.,* 2008).

4.4.2 Pollen development investigations

The pollen phenotype in *SRF4* and *SRF5* were more closely examined, because of the investigated AtGenExpress data (Figure 3-4). Among all other members, there was a remarkable difference to other tissue expression, especially in *SRF4.*

Approximately one third of the *SRF*-insertion lines were in *qrt*-mutant background, like *srf4-2* and *srf4-3*. All insertion lines were fertile, showed no abnormality in the pollen structure (data not for all mutants shown). There is also no deviation in the structure and size of the three cells in the young developing microspores (see Figure 3-8). The studies about No Pollen Germination 1 (NPG1) indicate that the *qrt* background has no affect on the pollen development (except the tetrad separation) or germination. It means that the *qrt* mutants do not affect the general pollen development of the *srf* genes.

The development of anthers including tapetum, microsporocytes or pollen is very complex, where several LRR-RLK are involved. Therefore male fertility depends on the interaction of many developmental steps in the anthers and male gametophytic tissue. For example the RECEPTOR-like PROTEIN KINASE 2 (RPK 2) regulates the anther development. The phenotype of the T-DNA insertion lines *rpk2-1* and *rpk2-2* are sterile, because of defects in anther dehiscence and pollen maturation (Mizuno *et al.,* 2007).

SERK1 and *SERK2* are also genes, which are involved in anther development (see previous sections) (Albrecht *et al.,* 2005).

LAT52 is a candidate of ligand molecule for the *Petunia* LRR-RLKs LePRK2 and it is expressed in pollen itself and not the pistils. It is suggested that LePRK2 is required for proper pollen growth rather than pollen-pistil interaction (Torii 2004). Other known LRR-RLK involved in pollen development are described already before.

4.5 Conclusion and outlook

4.5.1 Other possibilities of getting mutants

TILLING (Targeting Induced Local Lesions In Genomes) is another tool involving traditional chemical mutagenesis followed by high throughput screening for point mutations (McCallum *et al.,* 2000a, b; Perry *et al.,* 2003; Till *et al.,* 2003; Henikoff *et al.,* 2004). Gene disruption is a dominant tool for getting knock-out mutants that helps in understanding biological function of the numerous uncharacterized open reading frames (ORFs). Gene knock-out systems offer a direct way to determining function. Mutagenesis allows only partial analysis of genes that function at multiple stages of development or genes that are functionally redundant or highly pleiotropic. Additionally it is difficult to identify genes required in the gametophytic generation. Thus Activation tagging, enhancer traps and promoter traps in gene tagging studies is complementary to loss of function studies (Radhamony 2005). *SRF*::*35S* plants were already characterized by B.E. and partly reported in (Eyueboglu *et al.,* 2007). Results from successful application of double stranded RNA mediated interference (RNAi) approaches in *Arabidopsis* (Wang *et al.,* 2002; Masclaux *et al.,* 2004) will provide new insights for sequence specific inhibition of gene function in *Arabidopsis* (Tuschl 2003). RNAi is a nice compromise, if no phenotype with T-DNA lines could be obtained, especially when the mRNA of the T-DNA is still expressed and no real knock out were obtained.

4.5.2 Potential functions of the *SRF* members

SUB/SCM is the first characterized member of this family. It is required for the correct forming of the plant organs by coordinating correct cell division and cell number. Additional *SUB/SCM* influences root hair patterning (Kwak *et al.,* 2005; Kwak *et al.,* 2007).

To receive possible function of the gene family members, analysis of global expression profiles and the Gene ontology (GO) term enrichments of co-expressed genes provide evidences for distinct functions of the gene family (Eyueboglu *et al.,* 2007). Therefore it is an useful strategy to get some idea about genes, where the function is still unknown and where mutants do not display any obvious phenotype. These findings also serve for the non-redundant effect within the closest related *SRF* gene pairs.

For example *SRF4* seems to be involved in pectinesterase activity processes. Pectinesterase (PE) is a ubiquitous cell wall associated enzyme that modifies plant cell walls. It is found in all higher plants as well as in some bacteria and fungi. PE modulates the cell wall during fruit ripening and plays a role in cell wall extention during pollen germination and pollen tube growth, abscission, stem elongation and root development. PE has also been shown to play a role in a plants response to pathogen attack.

Also *SRF7* seems to be also involved in cell wall biosynthesis and cellulose synthase activity processes (Eyueboglu *et al.,* 2007).

In this work the expression was analysed with *in-situ Hybridization* and GUS-promoter reporter genes were performed. *In-situ Hybridization* analysis revealed that all *SRF* genes are expressed in the anthers and ovules. *SRF2,4,5,7* and *SRF8* mRNAs are also expressed from the beginning of flower development in the IM, flower primordia and young developing organs like sepals, petals, stamen and ovules. Probably this gene family is involved in ovule and/or anther development.

Arguments for a function during anther development are the observation of *35S::SRF2/3/4/5/7* plants, which shows sterility and the co-expression analysis, which indicates for a possible function of SRF4 during Pectinesterase activity like described before.

Phenotypic analyses were made with T-DNA lines. *srf4* mutants showed smaller leaves compared to wildtype, thus SRF4 is a positive regulator of leaf size (Eyueboglu *et al.,* 2007). Cell size analysis indicates that the size defect is caused by a cell expansion effect rather than a cell division effect during the leaf development (Banu Eyuboglu and Christine Skornia, personal communication).

Already characterized genes, that are involved in organ growth processes are for example *ARGOS, AINTEGUMENTA (ANT), BRI1* and *ARGOS-like (ARL)*.

ARGOS regulates cell proliferation and organ growth in an Auxin dependent pathway upstream of *ANT.* Reduced expression of *ARGOS* results in smaller leaf size and overexpressing *ARGOS* plants display larger leaves (Hu *et al.,* 2003)).

ARGOS-LIKE (ARL), a homologe to the *ARGOS* gene, is a regulator of cell expansion-dependent organ growth. It is suggested that it acts downstream of the BRI1. *arl* mutants exhibit smaller cotyledons and leaves (Hu *et al.,* 2006).

The root analysis of *srf4* T-DNA lines also indicate for a positive growth regulator during root development, because mutants display smaller roots grown on 1% sucrose.

All other mutants showed no obvious phenotype, so far.

These results provide a lot of indications to promote the study of this gene family for getting further fascinating results out of this family.

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