FINAL REPORT - Abschlussbericht

1 General Information

DFG reference number: BE2547

Project number: 24-1

Project title: Keeping meristems at balance: stem cell maintenance and termination in flowers with two meristem types

Name of the applicant: Annette Becker

Official address: Institute of Botany, Justus Liebig University, Heinrich-Buff-Ring 38, 35392 Gießen, Germany

Name(s) of the co-applicants: n.a.

Names of the cooperation partners: Dr. **Katrin Ehlers**, Institute of Botany, JLU Gießen; Prof. Dr. **Alexander Goesmann** and Oliver Rupp, Bioinformatics and Systems Biology, JLU Gießen; Dr. **Knut Beuerlein**, Institute of Pharmacologgy, JLU Gießen; Dr. **Jörg Fuchs**, IPK Gatersleben, Germany.

Reporting period (entire funding period): October 1, 2020 until December 31, 2024.

2 Summary

Um die Entwicklung aller Blütenorgane gewährleisten zu können, müssen die Stammzellen des Blütenmeristems aktiv bleiben, bis der Fruchtknoten als letztes Organ initiiert ist. In vielen Blütenpflanzen existiert lediglich ein einziges Blütenmeristem, von dem die Blütenorgane in kreisförmiger Anordnung nacheinander nach außen abgegeben werden. Andere Pflanzen generieren allerdings ein zweites, ringförmiges Meristem, das durch eine verlängerte Aktivitätsphase noch Staubblätter generieren kann, wenn die Stammzellenaktivität im zentralen Blütenmeristem bereits eingestellt wurde. Diese evolutionäre Innovation führt zu einem flexibleren Wachstum, das eine Feinjustierung der Anzahl der Staubblätter bis spät in der Blütenentwicklung und damit Resourcenveschiebung zwischen weiblichen und männlichen Reproduktionsorganen erlaubt.
Wie dieses Ringmeristem auf molekularer Ebene gebildet wird, ist jedoch unklar, allerdings gehen wir von der Hypothese aus, dass das ursprünglich recht einfache genetische Programm der Terminierung der Stammzellenaktivität in Bezug auf Anordnung, Musterbildung, und Verbindung der Komponenten modifiziert wurde. Das Ziel dieses Projektes war es, ein

mechanistisches Verständnis der Bildung und Regulation zweier benachbarter Meristeme zu erlangen. Zudem wollten wir verstehen, wie das Ringmeristem in der Evolution entstanden ist und ob es mehrere unabhängige Ursprünge gibt. Wir haben daher zwei nach verwandte Arten innerhalb der Papaveraceae (Mohnartige Pflanzen) für eine vergleichende Studie ausgewählt: eine mit (*Eschscholzia californica*) und eine ohne Ringmeristem (*Pteridophyllum racemosum*). Geplant war, mit Hilfe von Laser Microdissection deren Meristeme zu isolieren, deren globale Genexpression mittels Transkriptomanalysen zu vergleichen. und Gene zu identifizieren, die zwischen die Arten und zwischen den Meristentypen unterschliedliche Aktivität aufweisen.
Im Rahmen dieses Projektes konnten wir zeigen, dass Ringmeristeme mehrfach unabhängig während der Evolution entstanden sind. Zudem konnten wir die Entwicklung der Blüten von *P. racemosum* detailliert dokumentieren, was insbesondere deshalb interessant ist, weil *P. racemosum* entweder die Schwesterart zu allen Papaveraceae oder die Schwesterart zu allen zygomorphen Papaveraceae (Fumarioideae) darstellt. Abschließend konnten wir die Transkriptome nutzen, um in Kooperation mit der AG Goesmann eine allgemein anwendbare Software Pipeline zu entwickeln, mithilfe die initiale Transkriptomqualität evaluiert werden kann, um sicher zu stellen, dass nur qualitative ausreichende Daten weiter analysiert werden. Dies ermöglicht eine sichere Dokumentation von Genexpressionsanalysen und erhöht deren Reproduzierbarkeit.

In English

In order to ensure the development of all floral organs, the stem cells of the floral meristem must remain active until the ovary is initiated as the last organ. In many flowering plants, there is only a single floral meristem, from which the floral organs are released one after the other in a circular arrangement. However, other plants generate a second, ring-shaped meristem that can still generate stamens through an extended phase of activity even after stem cell activity in the central floral meristem has ceased. This evolutionary innovation leads to more flexible growth, allowing fine-tuning of the number of stamens until late in flower development and allows adjustment resource allocation between male and female reproductive structures.
How this ring meristem is formed at the molecular level is unclear, but we hypothesize that the originally quite simple genetic program for terminating stem cell activity has been modified in terms of arrangement, pattern formation, and connection of the components. The aim of this project was to gain a mechanistic understanding of the formation and regulation of two adjacent meristems. We also wanted to understand how the ring meristem evolved and whether there are several independent origins. For a comparative study, we selected two closely related species within the poppy family (Papaveraceae):

One species has a ring meristem (*Eschscholzia californica*), and the other does not (*Pteridophyllum racemosum*). Our plan was to isolate their meristems using laser microdissection, compare their global gene expression using transcriptome analyses, and identify genes with different activity levels between species and meristem types.
Within the scope of this project, we demonstrated that ring meristems arose independently several times during evolution. Additionally, we documented the development of *P. racemosum* flowers in detail. This is particularly interesting because *P. racemosum* is either the sister species to all Papaveraceae or to all zygomorphic Papaveraceae (Fumarioideae). Finally, in cooperation with the Goesmann research group, we developed a generally applicable software pipeline using the transcriptomes. This pipeline can be used to evaluate the initial transcriptome quality and ensure that only sufficient-quality data is analyzed further. This enables reliable documentation of gene expression analyses and increases their reproducibility.

3 Progress Report

The regulation of stem cell maintenance and termination is a crucial process for plants with their open plant architecture that allows the formation of new organs throughout the plant's life. The precise regulation of stem cell maintenance and termination in the FM is important for the successive and centripetal initiation of floral organs and contributes to the establishment of floral basic structure and the generation of flowers with fixed numbers of floral organs. The key regulators of shoot apical meristem (SAM) and FM stem cell homeostasis and termination are well characterized in *A. thaliana*. Thus, the genetic network regulating the FM in a plant with simple flower morphology has been elucidated with quite some detail.
However, across a broad phylogenetic range of angiosperms (including basal angiosperms, monocots, and several lineages within the eudicots) floral morphology is far more complex and includes variation in merosity, especially of petals and stamens. Several derived polystemonous flowers in eudicots deviate from a strict centripetal initiation of floral organs due to the innovation of a ring meristem in the androecium. However, very little is known about the formation and regulation of the ring meristem in terms of evolution and development. This project aims to elucidate how the ring meristem is formed and regulated, what the regulatory differences of the stem cells between the ring meristem and floral dome meristems are, and, as long-term perspective, how plants gained the ring meristem during the evolution.
We aimed to use the knowledge gained from *A. thaliana* and an alternative, "*Arabidopsis*-bias free" approach to understand how more complex floral morphologies develop in respect to floral meristem regulation. We planned to focus on our comparative analysis on two species

for detailed expression and functional studies of meristem regulators: *E. californica* (Papaveraceae) with a whorled phyllotaxis and variations in stamen number caused by the prolonged activity of the ring meristem and, *P. racemosum*, the earliest branching Papaveraceae, with a fixed number of four stamens and no ring meristem. While *E. californica* is a well-established genetic model organsim, with Virus-Induced Gene Silencing (VIGS) established to specifically down-regulate gene functions, *P. racemosum* is in a phylogenetically interesting position but no protocols have been established so far to work with this species on the molecular level. To elucidate the molecular evolution of the ring meristem, we will also perform sequence comparison and phylogenetic analyses in the context of Ranunculales and conduct VIGS experiments to analyze gene functions in *E. californica*.

We aimed to identify one set of molecular factors that have similar expression and function in the ring and the central floral meristem. More importantly, we were also interested in the genes (most likely transcription factors and receptor/ligands) that are different between the two meristem types and allow for the prolonged activity of the ring meristem when compared with the floral meristem. We hoped that our analysis will reveal the degree of conservation of the molecular network regulating the maintenance of two types of meristems in the flower. This knowledge would be useful not only in the context of developmental biology, but also, as this ring meristem is a novel structure, we hope to understand how this novelty has originated.

Further, trade-off in resource investment between male vs. female reproductive function has been a central theme in evolutionary biology and we had aimed to present a mechanism of molecular regulation of prolonged period of stamen production which will advance our understanding how plants regulate the resource allocation between the sexes on the molecular level. This project was supposed to finally provide a direct link between developmental genetics and ecology of male-female resource shifting.

These were our specific aims and work packages for the first, and already for the second funding phase. However, for reasons explained later in this report, we have not applied for a second funding phase.

WP1: LMD-RNASEQ ON FLORAL MERISTEMS TO OBTAIN TRANSCRIPTOMES FOR THE DIFFERENT MERISTEM TYPES

Previous studies in model plants have shown that the stem cell maintenance and termination of the FM are regulated by *WUS*, *CLV3*, *AG* and several other genes, which form different positive and negative feedback loops. Identification of orthologs of these genes in non-angiosperms implicate that the functions of the key meristem regulators may be extremely conserved during angiosperm evolution. However, in previous studies from the Becker lab, quantitative and qualitative genetic differences in meristem activity regulation between the ring meristem and the central floral meristem of *E. californica* were shown for *EcAG1*, *EcAG2, EcSTM,* and *EcCRC*. To obtain a general picture of which genes are involved into the regulation of two types of meristems, we will apply an “*Arabidopsis*-bias-free” approach to identify developmental regulators differentially expressed in the ring meristem and the central floral meristem in the following steps: (i) microscopic analysis of *P. racemosum* flower development (ii) Laser Capture Microdissection to collect materials of the different meristematic tissues from *E. californica* and the cells from the corresponding regions in *P. racemosum*; (iii) RNA extraction, library preparation, and RNAseq; (iv) assembly and digital gene expression calculation.

WP2: PTERIDOPHYLLUM RACEMOSUM GENOME SEQUENCING

We planned to obtain a medium-quality genome sequence for *P. racemosum* within this project to aid gene identification, transcriptome assembly, and transcript quantification. When we proposed this project, the most recent phylogeny of Ranunculales placed *P. racemosum* is sister to all other Papaveraceae, and our genome would have been be of interest to the research community, for example to those interested in the origin and evolution of alkaloid biosynthesis genes or the evolution of complex morphological traits, such as porous capsule fruits or zygomorphy. However, meanwhile, a new Ranunculales phylogeny was published placing *P. racemosum* as sister to the Fumarioideae, a Papaveraceae group including mainly zygomorphic species.

WP3: ANALYSES OF EXPRESSION PATTERNS OF CANDIDATE GENES FOR MERISTEM REGULATION

From the WP1, had planned to obtain a series of candidate genes that may regulate the stem cell activity in the meristems. We intended to perform gene expression analyses for the candidates in the two Papaveraceae species. By comparing the expression patterns of candidate genes, we would have then not only narrow the candidate genes down to some key regulators, but also identify genes showing differential expression patterns. This would have included genes that show conserved expression in meristems, genes expressed only in the central floral meristems, genes specifically expressed in the ring meristems of *E. californica*, and genes differentially expressed in the two types of meristems of *E. californica*. Based on the gene expression data, we would have been able to predict (i) which genes are required for the stem maintenance and determination in both types of meristems, (ii) which have specific functions in the regulation of the central meristem, (iii) which are specific for the formation and regulation of the ring meristem, and (iv) which contribute to the genetic differences in meristem activity regulation between the ring meristem and the central floral meristem, and (v) which are expressed differently.

WP3: FUNCTIONAL ANALYSES OF KEY MERISTEM REGULATORS

We planned to utilize VIGS and, in the second funding phase, transgenic technology with CRISPR-Cas9-mediated gene editing, together with careful phenotypic observations, to explore the function of genes that show different expression patterns. By integrating the expression and functional data of all the examined genes, we had hoped to confidently determine the functions of the selected genes in the two Ranunculales model species and construct regulatory networks for the regulation of the meristem types. Based on these results, we would then have elucidated the molecular mechanisms underlying the formation of the ring meristem and the regulatory basis of the two types of meristems within one flower. And by comparing the expression patterns and functions of these key meristem regulators in *E. californica* with their counterpart orthologs in *P. racemosum* we would have been able to detect regulatory differences between the meristem types (spiral vs. whorled, central FM vs. ring meristem). Most importantly, we would have been able to predict which genes are involved in the formation of the ring meristem, and whether changes in expression patterns or functions of certain gene(s) may have contributed to its molecular evolution.

WP4: ANALYSIS OF THE MOLECULAR EVOLUTIONARY MECHANISM UNDERLYING THE ORIGIN OF THE RING MERISTEM

It appears that the ring meristem may have multiple origins in angiosperms as groups known to exhibit this feature are embedded in different clades of the angiosperm phylogeny (table 1). In Ranunculales, the ring meristem is a characteristic feature of the Papaveraceae, which may originate prior to the divergence of the family. However, as *P. racemosum* represents the earliest branching Papaveraceae and has no ring meristem this scenario is not supported by recent phylogenies, rather, the ring meristem may have originated in the Papaveraceae after the Pteridophylloideae split from the rest of the lineage. By performing molecular evolutionary analyses (via sequence comparison and phylogenetic analyses, and *in silico* protein characterization) in Ranunculales, we had hoped that to reveal the genes responsible for the origin of the ring meristem and further investigate which kind of mutations have taken place in these genes on the genomic level by comparing basal and core eudicot meristem regulators via phylogenetic placement and selection analyses.

Description of the project-specific results and findings

WP1:

(i) microscopic analysis of *P. racemosum* flower development: *P. racemosum* is an understory species endemic to the higher altitude Japanese forests and thus impossible to grow in greenhouse conditions. We have established the growth conditions for *P. racemosum* to maintain the plants, but we were never able to breed them. We ordered plants from a dutch perennial specialist and in our hands, they spent the winter outside and were brought into the greenhouse for material collection only. Together with Dr. Katrin Ehlers, a researcher in the Becker group, the PhD student D. Kong characterized the floral morphogenesis of *P. racemosum* employing scanning electron microscopy and described stages of flower development of this enigmatic species. The results are published in Kong D, Ehlers K, Becker A (2024) Floral morphology and development of *Pteridophyllum racemosum* Siebold & Zucc. (Papaveraceae), Botany Letters, DOI: 10.1080/23818107.2024.2352773

(ii) LCM to collect materials of the different meristematic tissues from *E. californica* and the cells from the corresponding regions in *P. racemosum*: LCM was done by the PhD student D. Kong on the two *E. californica* meristem types of two developmental stages based on the protocol developed with the help of Dr. Knut Beuerlein, JLU. This process was challenging and time-consuming because the meristems consist of few cells only and are next to each other, aggravating tissue collection.

(iii) RNA extraction, library preparation, and RNAseq. Many rounds of LCM and RNA extractions were done by D. Kong, however, the RNA integrity (RIN) value was often too low for proceeding with library preparation. After some optimization, library preparation was done for 16 samples, each pooled from many LCM sections (2 developmental stages, 2 meristem types, 4 replicates). RNA extraction was done by Andrea Weisert, a technician in the Becker lab, library preparation and RNAseq was done at the WGGC in Cologne, in collaboration with Dr. Kerstin Becker. Library preparation was done with the standard low-input kit also used in Kivivirta et al., 2019.

(iv) Assembly and digital gene expression calculation was done by Oliver Rupp, JLU, showing several problems with the initial dataset: high rRNA content and consequently a small number of reads mapped to a unique position in the genome (between 15.5 Mio and 29 Mio reads with an average of 20 Mio reads). Further, and more expected, the sample correlation heatmap showed clustering of samples not related to replicates.

WP2: *P. racemosum* genome sequencing: Genome size measurements with flow cytometry using leaf tissue was done by our cooperation partner Dr. Jörg Fuchs, IPK Gatersleben, Germany and determined the genome size to 623 Mb for the haploid genome.

Andrea Weisert, technician in the Becker group isolated high molecular weight (HMW) genomic DNA from *P. racemosum* using several methods with the Macherey Nagel nucleobond HMW DNA isolation kit being the most successful. The genome sequencing was done at the West German Genome center in Düsseldorf in cooperation with Dr. Tassilo Wollenweber, using ultra-low input PacBioHiFi sequencing chemistry producing two successful runs on a RevioSMRT cell producing almost 2 Mio reads resulting in a 50 X genome coverage with an N50 length of around 20 kb. The cleaned assembly has 74 contigs with a N50 length of 13 Mb. The embryophyte BUSCO values are at 98.9 %. O. Rupp has done the genome assembly and all analyses of the *P. racemosum* genome so far. This genome is currently being analyzed and will be published together with several other reference genomes resulting from the DFG funded "RanOmics" project to understand genome evolution in the hyperdiverse Ranunculales order. It is already available to researchers of the larger "RanOmics" community (Becker et al., 2024) from a BLAST server set up by O. Rupp incorporating all high quality Ranunculales genomes.

<u>WP3</u>

The transcriptome data we obtained in WP1 showed, after initial quality control that the read counts for the individual transcriptomes were in principle too low for standardized differential gene expression analysis such as DeSeq2. Moreover, as the genes in focus for this work, transcription factors and receptors/ligands are generally show low transcript levels, it is more difficult to obtain reliable and reproducible expression data for them. We have still analyzed the data keeping in mind their poor quality and lumped all LMC generated data together (Fig. 1, M3). for Weighted Gene Coexpression Network Analysis (WGCNA) with the *E. californica* expression atlas generated in the Becker lab in cooperation with the Joint Genome Institute of the Department of Energy, USA (Fig. 1).

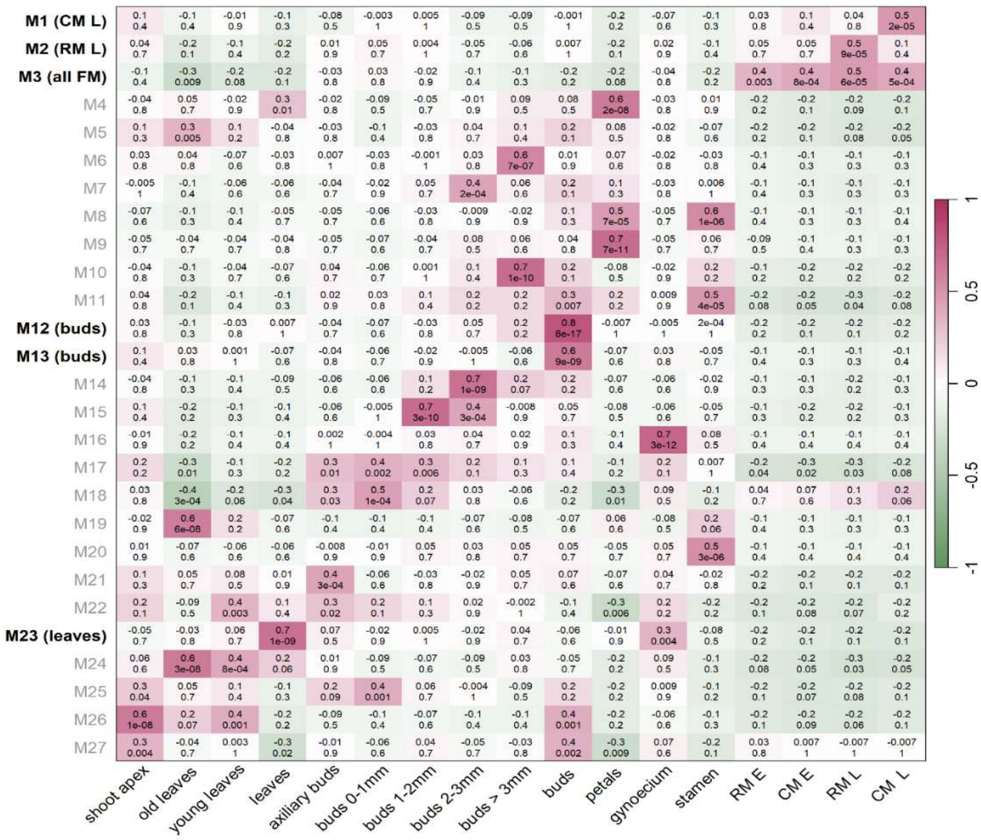

Fig. 1) Weighted gene co-expression network analysis (WGCNA) reveals several genes co-expressed genes specific for the *E. californica* meristem. This is a WGCNA membership plot calculated from *E. californica* expression atlas transcriptome data combined with the low quality meristem transcriptomes from this project.

<u>WP3</u>

In the WGCNA analysis D. Kong could identify genes active specifically in the floral meristem of *E. californica* (Fig. 1, module 3). The most significantly upregulated genes were the homologs of the Arabidopsis MADS-box genes *SEP1* (*EcAGL2*) and *APETALA3* (*EcDEF3*), the YABBY transcription factor *EcCRC* (Orashakova et al., 2009) and *EcICU2*, the homolog of the Arabidopsis gene *INCURVATA2*. The latter one encodes for part of a DNA polymerase interacting most likely with Polycomb group proteins. D. Kong carried out VIGS for all genes except for *EcCRC* as this was done in the Becker group, already many years ago (Orashakova et al., 2009). Silencing of *EcICU2* caused severe growth defects, already in the vegetative phase but had no effect on stamen number. *EcDEF* silencing lead to homeotic conversions of stamens into petal-like organs but had no effect on organ numbers and *EcSEP1* silenced plants showed a small and insignificant number of homeotic transitions of stamen into petals. Taken together, these functional analyses could not specify genes regulating ring meristem activity specifically.

WP4: ANALYSIS OF THE MOLECULAR EVOLUTIONARY MECHANISM UNDERLYING THE ORIGIN OF THE RING MERISTEM

It appears that the ring meristem may have multiple origins in angiosperms as groups known to exhibit this feature are embedded in different clades of the angiosperm phylogeny. My PhD student D. Kong and I have collected literature on ring meristem occurrences in flowering plants and discuss the topic of multiple independent origins of ring meristems in the publication “Kong D, Becker A (2021) Then there were plenty – ring meristems giving rise to many sta-men whorls. Plants, 10, 1140, DOI: 10.3390/plants10061140”.

4 Published Project Results

4.1 Category A – Articles in peer-reviewed journals, contributions to peer-reviewed conferences or to anthology volumes, and book publications

Kong D, Becker A (2021) Then there were plenty – ring meristems giving rise to many stamen whorls. Plants, 10, 1140, DOI: 10.3390/plants10061140

Kong D, Ehlers K, Becker A (2024) Floral morphology and development of *Pteridophyllum racemosum* Siebold & Zucc. (Papaveraceae), Botany Letters, DOI: 10.1080/23818107.2024.2352773

Lotz D, Rössner LH, Ehlers K, Kong D, Rössner C, Rupp O, Becker A (2024) Conservation of the dehiscence zone gene regulatory network in dicots and the role of the SEEDSTICK ortholog of California poppy (*Eschscholzia californica*) in fruit development. BMC Evodevo 15:16, DOI: 10.1186/s13227-024-00236-0

4.2 Category B – Any other form of published results, Contributions to non-peer-reviewed conferences

Kong, Doudou: poster presentation “Stamen galore – occurrence and regulation of stamen-producing ring meristems” at the Plant Biology Europe Conference in Marseille, France, 2023

Kong, Doudou: poster presentation “Stamen galore – occurrence and regulation of stamen-producing ring meristems” at the EuroEvoDevo Meeting in Naples, Italy, 2022

Software packages

A pipeline for quality control for bulk RNAseq experiments in plants for wet-lab biologists with limited bioinformatics expertise was generated in cooperation with Oliver Rupp of the Bioinformatics and Systems Biology Department at JLU, deposited at https://github.com/oliverrupp/rup and submitted for publication to the JoVe journal.

4.3 Patents (applied for and granted)

n. a.

Deutsche Forschungsgemeinschaft
Kennedyallee 40 · 53175 Bonn, Germany · Postal address: 53170 Bonn, Germany
Tel.: + 49 228 885-1 · Fax: + 49 228 885-2777 · postmaster@dfg.de · www.dfg.de