DEVELOPMENTAL PATTERNING OF ASTERACEAE FLOWER HEADS

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DOCTORAL DISSERTATION
To be presented for public examination, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, in Auditorium B3, Forest Science Building, Latokartanonkaari 7, on the 20 August 2021 at 13 o’clock.

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ISSN 2342-5423 (print)
ISSN 2342-5431 (online)

Cover image: A combination of four images showing 1) a mature flower head of Gerbera hybrida; 2) a scanning electron micrograph showing a developing head meristem; 3) a light-sheet microscopic image showing expression of the DR5 reporter in the head meristem; and 4) a 3D model of a growing head meristem in the clockwise direction.
“如切如磋，如琢如磨。”
——《诗经·卫风·淇奥》
ABSTRACT

Inflorescences are flower bearing structures that display remarkable diversity in plants. Their architecture, referring to the number and arrangement of flowers, is considered as a key attribute to the reproductive success of plants. Asteraceae is one of the largest plant families, and the evolutionary success of this family has been largely attributed to their showy inflorescence structure, the flower head (or capitulum). A flower head combines up to a thousand individual florets and numerous leaf-like bracts onto a single receptacle, and the overall structure superficially mimics a giant solitary flower. Geometrically, the individual florets are arranged in left and right turning spirals following the consecutive numbers of the Fibonacci sequence. Such a pattern has fascinated interdisciplinary researchers over centuries. Elaborating *Gerbera hybrida* as a study system, this thesis aims at elucidating the molecular mechanisms underlying three key aspects in the development of a flower head: the phyllotactic patterning, the inflorescence patterning, and the patterning of floral organs.

This thesis first combined data from diverse microscopic methods with computational modeling and illustrated how the phyllotactic pattern is established during the growth of *Gerbera* flower heads. The patterning process was governed by the expansion and contraction of the organogenetic zone where new primordia arise. Earliest bract initia were found to pattern on a ‘naked’ head meristem, and to guide the emergence of Fibonacci spiral numbers. A critical character for the patterning process is the lateral displacement of auxin maxima pointing towards the older neighbor. Results from this thesis provided the first experimental basis for understanding how phyllotactic patterns are transited on a growing meristem.

This thesis then demonstrated how flower meristem identity genes *GhLFY* and *GhUFO* are co-opted to regulate flower head development. While *GhUFO* acts as the master regulator of flower identity, *GhLFY* has evolved two novel functions to regulate the determinacy of inflorescence meristem and the early ray floret initiation. The results provided novel insights to explain how the flower head structures are evolved and diversified.

This thesis lastly dissected functions of the *SEPALATA*-like *GRC*Ds in regulation of *Gerbera* flower and inflorescence development. In this study, the *GRC*Ds were shown to have evolved specialized functions in regulating floral organ identities, among which, *GRCD4* and *GRCD5* are two indispensable regulators for petal development. Moreover, *GRCD2* and *GRCD7* show redundant functions at the inflorescence level maintaining their determinacy. The results provide an example on how gene duplications could lead to specialized and redundant functions in regulation of a highly elaborated inflorescence structure.
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LIST OF PUBLICATIONS

This PhD thesis is based on three published articles, which are listed following the leading roman numbers:


* Equal contributions

AUTHOR’S CONTRIBUTION

I. TZ designed the project and experiments together with PE and PP. TZ performed all the microscopic imaging and most other experiments. The DR5 construct was made by SKB and THT, and the GhCLV3 in situ hybridization was performed by FW. MC, AO and PP constructed computational and mathematical models. TZ, MC, PE and PP analyzed the experimental data and modelling results. PP, TZ, MC, AO and PE wrote the manuscript.

II. TZ designed the experiments together with YZ, SKB, ST, VAA, THT and PE. TZ performed yeast two hybrid analyses, analyzed the growth in wild-type, and conducted expression and phenotypic analyses of gerbera transgenic lines (GhLFY and GhUFO RNAi) and Pingpong cultivar. YZ cloned constructs, performed the wild-type expression analyses, Arabidopsis-related experiments, and phenotypic analyses of 35S:GhUFO and GhUFO RNAi gerbera transgenic lines. SKB, ST and THT isolated the genes. YZ, TZ and PE wrote the manuscript with comments from all authors.

III. TZ performed most experiments, including cloning and identifying the genes, expression analyses in the wild-type, and phenotypic and expression analyses of the transgenic lines. IJ, SKB and ASR are involved in characterizing the transgenic phenotypes. KM identified GRCD7 and GRCD8 from the RNAseq data. THT constructed the GRCD4/5 single and double RNAi constructs. TZ and PE wrote the manuscript with comments from all authors.
ABBREVIATIONS

AXM: axillary meristem
CRISPR: clustered regularly interspaced short palindromic repeats
CZ: central zone
Evo-devo: evolutionary developmental biology
FM: flower meristem
FO: floral organ
FUM: floral unit meristem
IAA: indole acetic acid
IM: inflorescence meristem
LH: lateral head
Micro-CT: micro-computed tomography
NPA: naphthylphthalamic acid
PZ: peripheral zone
RNAi: RNA interference
RZ: rib zone
SAM: shoot apical meristem
SIM: sympodial inflorescence meristem
SSP: syncephalium subunit primordia
TF: terminal flower
TH: terminal head
1. INTRODUCTION

1.1 Inflorescence diversity and the Asteraceae flower heads

Inflorescences show extensive diversity in flowering plants. In this section, I will start with an overview of classification of the variety of inflorescence types. Special focus will be made to the evolution and unique features of Asteraceae flower heads, the main topic of this study. *Gerbera hybrida*, the study system of my thesis will be introduced in the end.

1.1.1 Diversity and classification of inflorescence types: open, closed and more

Plants in angiosperms develop a reproductive shoot system to complete their life cycles. This system may terminate either as a solitary flower (e.g., the water lilies) or an inflorescence, whose architecture has diversified extensively through evolution. In botany, the classification of inflorescence types usually considers two parameters: branching pattern of the shoot, and position of initiating flowers (Coen and Nugent 1994). Depending on the absence or presence of a terminal flower, inflorescences can be generally grouped as open (indeterminate) or closed (determinate) (Weberling et al. 1989). Open inflorescences harbor a monopodial main axis that grows continuously, with flowers forming on its flanks (Beniloch et al. 2007, Figure 1 A, B). In closed inflorescences, the growth of the main axis ends with a terminal flower and a new growing axis is re-established at the lateral position (Perilleux et al. 2014, Figure 1 C).

![Figure 1. Schematic figures show classification and meristem structures of different inflorescence types.](image)

(A) Examples of open inflorescences show an indeterminate axis with flowers formed on the flanks. The inflorescence meristem (IM) in most open inflorescences has a zonal composition. CZ: central zone; PZ: peripheral zone; RZ: nb zone; FP: flower primordia. (B) An exceptional case of open inflorescences exemplified by the Asteraceae flower heads (Open II, as per Bull-Herefufu and Claßen-Bockhoff 2010), which develops from a determinate floral unit meristem (FUM) while lacking the terminal flower. (C) Examples of closed inflorescences show determinate growth with main axis ending with a terminal flower. Termination of meristematic activities in the flower meristem (FM) resulted in fixed numbers of floral organs (FO).

The distinct growth patterns between open and closed inflorescences are determined by their early ontogeny. The aerial organs of plants are raised by a small group of cells located at the shoot tip, known as the shoot apical meristem (SAM). After floral induction, the vegetative SAM
undergoes a series of transitions; first transforming into a reproductive inflorescence meristem (IM) (Amasino 2010), which later produces flower meristems (FM) that give rise to floral organs (Irish 2010). Open and closed inflorescences show distinct growth fates of their apical meristems. The growth of SAM and the IM of open inflorescences is indeterminate, and the meristem develops with three active zones: the central zone (CZ) that maintains pluripotent cells; the peripheral zone (PZ) that produces new primordia continuously; and the rib zone (RZ) that develops into the stem (Kwiatkowska 2008, Figure 1 A). In contrast, FM and the IM of closed inflorescences show determinate growth, and a fixed number of floral organs is produced before meristem activity deceases by floral termination (Sablowski 2007, Figure 1 C).

An interesting intersection between open and closed inflorescences is found in species with inflorescences that resemble single flowers, the structure known as pseudanthium (or false flower) (Prenner et al. 2009). Taken the Asteraceae flower head as an example, although it is classified as an open inflorescence due to the lack of a terminal flower, the growth of its IM is in fact determinate and it produces a fixed number of flower primordia (Uimari et al. 2004, Teeri et al. 2006b, Figure 1 B). From the ontogenetic point of view, flower heads are developed from the so-called floral unit meristem (FUM), which has a mantle-core configuration with reduced CZ activity similar to a FM (Kwiatkowska 2008, Bull-Hereňu and Clašen-Bockhoff 2010, Clašen-Bockhoff and Bull-Hereňu 2013). Bull-Hereňu and Clašen-Bockhoff (2010) proposed that such inflorescences should be classified as the ‘Open II’, in order to differentiate them from the classic open inflorescence with an indeterminate IM (Figure 1 B). Aggregation of flowers into a single reproductive unit has occurred multiple times in angiosperm evolution, and it has been viewed as an evolutionary innovation which adds up to the reproductive fitness of these species. Besides Asteraceae, the FUM type IMs has also been widely reported in diverse species, for examples in Euphorbiaceae (Prenner and Rudolf 2007), Dipsocoideae (Naghiloo and Clašen-Bockhoff 2017) and Nyssaceae (Clašen-Bockhoff and Arndt 2018). Studying the development and molecular genetics in species with such structures will enrich our overall understanding on the diversification of inflorescence structures.

1.1.2 Flower heads in Asteraceae – the evolution and unique developmental features

Asteraceae is one of the largest plant families comprising of more than 25000 species (almost 10% of all angiosperm species) (Mandel et al. 2019). Plants in this family are recognized by their showy inflorescence structure, the flower head that compresses hundreds of individual florets on a single receptacle but functionally mimics a single flower.

The evolutionary origin of the flower heads has been under a long debate. Asteraceae belongs to the well-supported MGCA clade: Menyanthaceae, Goodeniaceae, Calyceraceae, and Asteraceae (Lundberg and Bremer 2003). While Menyanthaceae and Goodeniaceae develop elongated inflorescences, compressed inflorescences are already present in Calyceraceae, the closest relative of Asteraceae (Harris et al. 1999). Harris et al. (1999) postulated that flower heads evolved as a result of a series of ‘condensation’ events, from which suppression of internode elongation of an elongated inflorescence led to the head formation. By close examination of inflorescence structures in selected Calyceraceae species, Pozner et al. (2012) found that reminiscent characters of the ancestral Goodeniaceae inflorescences, such as the terminal flower and peripheral branching units, are all present in the compressed
inflorescences of Calyceraceae (Figure 2 A). This painted a clear evolutionary route, where the authors further proposed that flower heads in Asteraceae may have evolved from further modifications of the Calyceraceae inflorescence by losing of the terminal flower and reduction of peripheral units into single flowers (Pozner et al. 2012).

Figure 2. Evolution and unique developmental features of Asteraceae flower heads. (A) Schematic presentations show inflorescence structures in Asteraceae and its close relatives. As proposed by Pozner et al. (2012), the head-like inflorescence structures first emerge in Calyceraceae, and a further loss of the terminal flower (TF) and peripheral branching units (BU) resulted in the final structure of Asteraceae flower heads. (B) Top view and a cross section of a mature flower head of *Gerbera hybridra*. Bracts and individual florets in different types (ray, trans and disc) are tightly packed on a single receptacle. (C) Scanning electron micrograph of a developing head at an early developmental stage. Flower primordia (FP), which form at the periphery of an enlarged IM, are arranged in a phyllotactic pattern that comprises of left and right turning spirals following consecutive Fibonacci numbers.

Apart from the general head-like structure, flower heads in Asteraceae show enormous diversity having evolved additional developmental features. For instances, flower heads can be either large or small with numbers of florets on a single head varying from one (e.g. in secondary heads of *Echinops*, Figure 2 A) to more than a thousand (e.g. in sunflowers). The extensive number of florets are tightly packed into a phyllotactic pattern consisting of left and right turning spirals that follow the Fibonacci numbers (1, 1, 2, 3, 5, 8, 13, 21, 34…). Different from the *Arabidopsis* inflorescence apex that has (3, 5) spirals, the phyllotactic pattern in sunflowers could reach a pattern with as much as (89,144) spirals. The pattern as such is often used as an iconic example to illustrate geometric regularity in nature (Elomaa 2019).

Another prominent feature leading to the head diversity is the evolution of different types of florets (recently reviewed by Fambrini and Pugliesi 2017). In many species, the margin of a head is often occupied by ray florets that develop showy ligules (Figure 2 B). Gain and loss of the ray florets has evolved multiple times in Asteraceae, and it has been shown to associate
with the pollination strategy of a plant (Sun and Ganders 1990, Kim et al. 2008). Moreover, in the individual florets of some species, highly modified floral organs with specialized functions have been evolved. For instance, pappus bristles in _Dandelion_ and pigmented spots on ray ligules in _Gorteria_ are two representative floral structures that promote seed dispersal and pollinator attraction, respectively (Cummins et al. 2018, Ellis and Johnson 2009). All these unique developmental features which deviate or are even absent in the traditional models like _Arabidopsis_ makes the flower head an excellent target for comparative _evo-devo_ studies.

1.1.3 _Gerbera hybrida_, a model system in studying Asteraceae inflorescence and flower development

_Gerbera hybrida_, originated from a cross between _Gerbera jamesonii_ and _Gerbera viridifolia_, is an ornamental crop species of the Asteraceae. Given by its showy, gigantic flower heads, _Gerbera_ is ranked among the most sold cut flowers, with thousands of cultivars displaying distinct floral traits that are available from the worldwide breeders (Teeri et al. 2006a). In the standard cultivar ‘Terra Regina’ used for molecular studies, the flower head (Figure 2 B) consists of 600–700 individual florets of three different types: the marginal zygomorphic female ray florets, central bisexual disc florets, and intermediate trans florets that are assembled on a single receptacle. Surrounding the entire structure, there are 80–90 leaf-like bracts providing sepal-like protective function in early development. The _Gerbera_ flower head represents many key developmental features in Asteraceae flower heads (as discussed in 1.1.2), for example, it develops from an expanded head IM that produces flower primordia in a phyllotactic pattern of 34 and 55 spirals in clockwise and counterclockwise directions (Figure 2 C). With the help of extensive sequence resources (Laitinen et al. 2005, Laitinen et al. 2006, Teeri and Elomaa unpublished) and the well-established _Agrobacterium_ mediated stable transformation system (Elomaa et al. 1993, Teeri and Elomaa 2003), _Gerbera_ has been serving as a model system for gene functional studies related to various aspects of inflorescence and flower development, including floral organ identity regulation (Yu et al. 1999, Kotilainen et al. 1999, Uimar et al. 2004, Broholm et al. 2008, Ruokolainen et al. 2010a), flower type differentiation (Broholm et al. 2008, Tähtiharju et al. 2012, Juntheikki-Palovaara et al. 2014), and floral organ differentiation (Kotilainen et al. 1999, Laitinen et al. 2007). The previous studies on _Gerbera_ inflorescence development have been mainly focusing on the late-stage flowers or the individual floret primordia, while the early patterning of the head-like IM is still poorly understood. This PhD thesis aims to deal with three patterning processes in early _Gerbera_ head development following the chronological order: phyllotactic patterning, IM patterning and FM patterning, all of which greatly contribute to the unique organization of the flower head. In the following chapters, each of the topics will be reviewed in detail.

1.2 Phyllotactic patterning in plants

The aerial organs in plants such as leaves and flowers are organized in a precise order, known as the phyllotaxis. The geometric beauty of phyllotaxis has drawn multidisciplinary interest over centuries. In this section, I will first briefly describe through the long history of phyllotaxis studies. It will be followed by a summary of the role of phytohormone auxin in regulation of phyllotactic
pattern in plants. The topic of transitions in phyllotactic patterns – the biological question exemplified in the development of a flower head – will be discussed last.

1.2.1 Phyllotaxis casting as a historic, interdisciplinary problem

Studies on the arrangement of leaves has a long history that can be traced back to as early as the ancient Greeks and Egyptians (reviewed by Alder et al. 1996). The term ‘phyllotaxis’, which was first brought up by Shimper (1830), is derived from two Greek words: ‘phyllo’ and ‘taxis’ referring to the leaf and arrangement, respectively. Phyllotaxis shows diverse patterns in plants, and systematic studies on phyllotaxis began with a series of works in 1830s by Schimper (1830) and Braun (1831), who recognized and introduced several essential parameters (e.g., divergence angle, parastichies) in order to describe phyllotaxis. In general, phyllotactic patterns can be classified into spiral, distichous/decussate or whorled, which are distinguished by divergence angles that separate the successive primordia (Figure 3 A). The spiral phyllotaxis is the most common type found in plants, and it has a characteristic with a divergence angle close to the golden angle (137.5°). In spiral phyllotaxis, the initiating primordia form close contacts that can be drawn into left and right turning spirals, called as parastichies, which follow consecutive numbers in the Fibonacci series. The eye-catching appearance and underlying mathematic properties have made phyllotaxis an interdisciplinary problem that puzzles not only botanists, but also mathematicians, physicists, and computer scientists.

Currently, it is widely accepted that phyllotaxis can be explained by the inhibitory field theory. As a basis for this theory, Hofmeister in his seminal work ‘Handbuch der Physiologische Botanik’ (1868) proposed that new primordia are initiated periodically in the largest available space that is left by the previous ones. It sets the basis for the currently well accepted inhibitory field theory to explain phyllotaxis, which was later refined by considering that each of the generated primordium produces an inhibitory effect that prevents initiation of future primordia in its vicinity (Snow and Snow 1962, Douady and Couder 1996, Figure 3 B). The ‘field’ can be biophysical, biochemical or a combination of both. Right after Hofmeister, Schwendener (1878) added up those primordia are interacting with each other through contact pressure, and this was put forward later by studies considering the inhibitory effects of different physical causes, such as mechanical buckling (Green et al. 1996). In parallel with the biophysical theories, Schoute (1913) argued that phyllotaxis is regulated by ‘chemical inhibitors’, which prototyped the initial field theory (Richards 1948) and inspired many of the current auxin-related studies (which are discussed in the next section). The nature of the inhibitory field theory has facilitated the use of diverse modelling approaches to simulate phyllotaxis (Adler 1974, Levitov 1991, Green 1992, Douady and Couder 1992, Douady and Couder 1996, Shipman and Newell 2005, Smith et al. 2006a, Pennybacker and Newell 2013). Apart from theoretical studies, the inhibitory field hypothesis is also supported by experimental results: both the initial microsurgical experiments (Snow and Snow 1931, Wardlaw 1949) and later laser ablation manipulations (Reinhardt et al. 2003) clearly demonstrated the existence of inhibitory effects from the older primordia.
1.2.2 Auxin regulates phyllotactic patterning in plants

In the most recent decades, it has been well established that the phytohormone auxin acts as a chemical signal that plays an essential role in phyllotactic patterning. Auxin is involved in the
regulation of diverse developmental processes of plant organs and tissues (updated in a recent book edited by Zažímalová et al. 2014). Presented in its natural form indole acetic acid (IAA), auxin is transported actively and in a polarized manner from cell to cell by efflux and influx carriers (Petrášek and Friml 2009). In Arabidopsis, mutations in genes encoding the auxin efflux carrier PIN FORMED 1 (PIN1) or blockage of auxin transport by the inhibitor Naphthylphthalamic acid (NPA) both resulted in a naked IM with abolished organ formation, the results that rang the first bell that auxin regulates organ initiation in the shoot apex (Okada et al. 1991, Galweiler et al. 1998, Vernoux et al. 2000). Reinhardt et al. (2000) tested out different phytohormones to rescue the ‘pin’ meristems and observed that organ initiation can be restored only when exogenous auxin was applied to the peripheral zone of the meristems, suggesting that local auxin maxima induce organ initiation in the shoot apex. By immunolocalization, Reinhardt et al. (2003) revealed that PIN1 proteins in the epidermal layers localize to the side of plasma membrane towards the site of incipient primordia with high auxin concentration (Figure 3 C). This was later confirmed by live imaging of auxin transcriptional reporter DR5 together with a PIN1-GFP fusion. Heisler et al. (2005) found convergence of PIN1 patterns towards the incipient primordium resulting in depletion of auxin, i.e. a chemical inhibitory field, around the primordium. The action of PIN1 is postulated to operate based on the concentration-based ‘against the gradient’ model in the meristem epidermis (Smith and Bayer 2009). This knowledge has been used to run computational models operating on a cellular mesh that form a ‘virtual tissue’, and the modeling results elegantly illustrated that the proposed simple, local sensing behavior is sufficient to reproduce diverse phyllotactic patterns at the tissue level (Smith et al. 2006b, Jonsson et al. 2006). The temporal resolution in understanding auxin actions on the meristem is further advanced with the development of the new auxin sensor DII, that compared to the commonly used DR5 reporter, shows auxin transcriptional output one step earlier by reflecting the degradation of signaling components (Vernoux et al. 2011, Brunoud et al. 2012). Just recently, Galvan-Ampudia et al. (2020) combined live-imaging data with computational modeling, which detailed the rhythmic patterning and dynamic transports of auxin in the SAM to a new level.

1.2.3 Transitions in phyllotactic patterns – a problem that requires further examinations

Phyllotactic patterning is a dynamic process and the pattern itself within a plant may change during the growth. For example, transition from a decussate into spiral pattern can be found in the Arabidopsis SAM, or from spiral to whorled in flowers of many species. In spiral patterns, the transitions link with changes of the number of parastichies that are observed. For example, from a lower order (e.g. 3,5) to a higher order (e.g. 55,89) in the sunflowers flower heads (Figure 3 E). Our current understanding on phyllotactic transitions is largely relying on modelling results. Systematic studies on phyllotactic transitions started with van Iterson (1907) by modelling of the packing of leaf disks of various sizes. He first showed how phyllotactic pattern changes with varying the ratio between the primordium size and the circumference of the SAM (Figure 3 F). This was later explained by an elegant work by Douady and Couder (1996), who demonstrated how changes in a single parameter $\Gamma$ (which only considers the size of inhibitory field of the primordium in relation to the meristem size) can recapitulate all observed changes in phyllotactic patterns. In this particular area, the experimental results have been lagging behind the theoretical advances. In sunflower, the transition has been shown to occur as a continuum from the vegetative shoot to the inflorescence (Couder 1998). In fact,
phyllotactic pattern changes have been observed from (2,3) to (3,5) in plants growing under different photoperiods (e.g. Erickson and Meichenheimer 1977), or by gibberellic acid treatments of the plants (e.g. Maksymowych and Erickson 1977). Besides these studies, little is known about how the transitions dynamically occur in the developing SAM or IM of the plants. Quoting Kuhlemeier (2007), he explicitly wrote ‘A major question is how higher order patterns such as those seen in sunflower heads are generated at all.’ These questions cannot be answered by studying traditional systems such as the SAM of tomato and the IM of *Arabidopsis*, as both of which do not show changes in meristem sizes and phyllotactic patterns over time. Multiple transitions occurring during the early patterning of flower heads make the head IM an excellent experimental system for study phyllotactic transitions.

1.3 Genetic regulation of inflorescence architecture

Since 1990s, the deployment of forward and reverse genetic tools in plants led to identification of key regulators of inflorescence patterning in diverse species. This applies particularly well to the two major types of inflorescences, racemes and cymes. In this section, the genetic regulation of their inflorescence patterning will be first reviewed. Discussion will then be extended to the experimental results and botanical hypotheses on how FM identity genes may involve in the development of pseudanthia.

1.3.1 Genetic regulation of racemose inflorescences

The well-studied plant model systems, *Arabidopsis* and *Antirrhinum* are two examples with simple racemose inflorescences. In both species, FMs initiate on the flank of an indeterminate IM that grows continuously. Such process is mainly regulated by a set of highly conserved genes, whose functions fall into two antagonistic categories: 1) the FM identity genes, represented by LEAFY (LFY, Weigel et al. 1992) and FLORICAULA (FLO, Coen et al. 1990), that are expressed in the FMs specifying the floral fates; and 2) the inflorescence identity genes, such as TERMINAL FLOWER 1 (TFL1, Shannon and Meeks-Wagner 1991) and CENTRORADIALIS (CEN, Bradley et al. 1996), which function at the IMs maintaining their indeterminacy. The genetic interplay between these two groups of genes determines the patterning logic of the racemose branching (Shannon and Meeks-Wagner 1993, Bradley et al. 1996, Bradley et al. 1997).

*LFY* encodes a plant specific transcription factor (Moyoud et al. 2011). As the master regulator of flowering in *Arabidopsis*, its function is both necessary and sufficient to confer floral identity. *lfy* mutants display severe defects in flowering, with flowers converted into either cauline leaves or inflorescence shoots (Shultz and Waughn 1991, Weigel et al. 1992), while ectopic expression of *LFY* transformed both apical and axillary shoots into single flowers (Weigel and Nilsson 1995). The robustness of *LFY* functions relies on its genetic interactions with other FM and inflorescence identity genes (Figure 4 A). In inflorescence apices, *TFL1* plays a predominant role by repressing the expression of *LFY* and *APETALA1* (*AP1*, Bradley et al. 1997). During floral initiation, *LFY* integrates signals from both the photoperiod and the phytohormone pathways, and its expression marked the first sign of flower development (Blázquez et al. 1997, Yamaguchi et al. 2013, Yamaguchi et al. 2014). In the floral anlagen,
LFY upregulates *AP1* expression by directly binding to its promoter, and *AP1* in turn activates *LFY* (Bowman et al. 1993, Wagner et al. 1999). As a result, the expression of *TFL1* is excluded from the FM and thus the floral fate is established (Liljegren et al. 1999). *LFY*-dependent activation of *AP1* also relies on the co-regulators *LATE MERISTEM IDENTITY 1/2 (LMI1/2)*, which form a feed-forward genetic regulatory loop securing the floral fate in a robust and irreversible way (Saddic et al. 2006, Pastore et al. 2011). Once FM identity is established, *LFY* activates the expression of floral organ identity genes in floral patterning, through direct binding to their promoters (Parcy et al. 1998, Winter et al. 2011). As a transcription factor, *LFY* forms homodimers (Hames et al. 2008, Siriwardana and Lamb 2012), and its functions also require participation of transcriptional co-regulators. For instance, UNUSUAL FLORAL ORGANS (UFO), SEPALLATA3 (SEP3), and WUSCHEL (WUS) have been shown to either physically interact or bind closely with *LFY* onto the genomic region of downstream genes, and activate their expressions (Lee et al. 1997, Chae et al. 2008, Liu et al. 2009, Lohmann et al. 2001).

![Figure 4](image.png)

**Figure 4.** Genetic regulatory networks in patterning of racemose (A) and cymose (B) inflorescences. (A) In *Arabidopsis*, *LFY* is the master regulator of flowering that regulates both floral initiation and later floral patterning. Meanwhile, *TFL1* maintains the indeterminacy of the inflorescence meristem (IM). (B) In *Petunia* and tomato, the *LFY* homologs *ALF/FA* are ubiquitously expressed, whereas *UFO* homologs *DOT/AN* are flower specific defining the floral identity. Moreover, separation of the sympodial IM (SIM) from the apical FM has been shown to be regulated by the WOX-like genes *EVG/S*.

### 1.3.2 Genetic regulation of cymose inflorescences

Cymose inflorescences show distinct growth habit as compared to racemes. Genetic studies carried out in two nightshade species, *Petunia* and tomato, revealed that although a similar set of FM identity genes are involved, their expression and functions in cymose branching are different from the racemose species (Figure 4 B, reviewed by Castel et al. 2010, Park et al. 2014). In both species, the *LFY* homologs *ABERRANT LEAF AND FLOWERS (ALF)* and *FALSIFLORICA (FA)* are still necessary for proper flowering (Souer et al. 1998, Molinero-Rosales et al. 1999). However, *ALF* and *FA* are no more sufficient for defining the flower identity, as *ALF* shows a ubiquitous expression pattern and overexpression of *ALF* does not alter the inflorescence development (Souer et al. 1998). By analyzing floral mutants showing similar phenotypes with *alfifa*, it was later found that the FM identity in cymes largely depends upon functions of the *UFO* homologs *DOUBLE TOP (DOT)* and *ANANTHA (AN)* (Souer et al. 2008,
Lippman et al. 2008). Similar to alifa mutants, mutations in DOT and AN completely abolished flowering. Moreover, ectopic expression of DOT terminated the inflorescence as a single flower in Petunia (Souver et al. 2008). Unlike ALF/IFA that are ubiquitously expressed throughout the development, the expression of DOT and AN are specific to FMs. By swapping the regulatory regions between Arabidopsis and Petunia LFY and UFO homologs, Kusters et al. (2015) showed that the flower-specific expression of DOT likely originated from changes in cis-regulatory promoter regions, while ubiquitous expression of ALF could be a consequence of altered upstream regulatory networks.

The development of cymose inflorescences also has an additional step by forming the sympodial IM (SIM), which re-establishes the growing axis that later reiterates as a terminal flower. In Petunia and tomato, two close homologs of the WUSCHEL-RELATED HOMEBOX (WOX) family genes, EVERGREEN (EVG) and COMPOUND INFLORESCENCE (S) were shown to promote SIM separating from the terminal FM (Rebocho et al. 2008, Lippman et al. 2008). EVG/S act also upstream of DOT/IFA, and their expression in SIM are a prerequisite for the induction of DOT/IFA for the meristem to acquire floral fate. By analyzing transcriptome data in meristem maturation, Park et al. (2012) showed that branching activity in tomato inflorescences correlates with quantitative expression levels of S and AN. These results further warrant functional significance of the genetic interplay between EVG/S and DOT/AN in regulation of the cymose branching.

1.3.3 Botanical hypothesis on the genetic regulation of pseudanthial inflorescences

The growth of pseudanthial inflorescences share similarities with single flowers in terms of determinacy and histological configuration of the meristems (Bull-Hereřu and Claßen-Bockhoff 2010, as reviewed in 1.1.1). Prenner et al. (2009) postulated that genes regulating flower development may play a role in controlling of the pseudanthium development. This is supported by experiments investigating expression patterns of LFY homologs in species with pseudantal inflorescences. The first example is the cyanthium in Euphorbia species (Euphorbiaceae). A cyanthium has a ‘hybrid’ flower/inflorescence nature that combines five male inflorescences and one female terminal flower into a single structure (Prenner and Rudall 2007, Claßen-Bockhoff and Frankenhäuser 2020). Using protein immuolocalization, Prenner et al. (2010) detected that LFY proteins are distributed first in the cyanthium IM and later in the male IM and subsequent FMs. Another example lies in the Asteraceae flower heads. Ma et al. (2008) reported the isolation and expression pattern of DFL, the LFY homolog in Dendranthema lavandulifolium. Using RNA in situ hybridization, DFL transcripts were found initially at low level in vegetative SAM and leaf primordia, while later the expression of DFL increased dramatically in the developing IM after the reproductive transition. The extended expression of LFY homologs across the entire IMs of both the cyanthium and flower head in fact resembles LFY expression in the stage 1–3 FMs of Arabidopsis (Parcy et al. 1998). Lines of evidence listed above suggest that functions of the FM identity genes may play a role at the inflorescence level in pseudanthial inflorescences. Characterization of their possible functions in species with flower-like inflorescences would shed light on how such structures have been evolved and established.
1.4 Genetic regulation of floral organ identities

Plants have evolved diversified forms in their floral organs. The genetic regulation of floral organ identities is described by the ABCE model in Arabidopsis and its derivatives other species. In Gerbera, although functions of B and C genes are largely conserved, the E genes have evolved specialized functions in regulating specific floral organs and the inflorescence patterning.

1.4.1 The ABCE model in regulation of floral organ identities

The genetic regulation of floral organ identities is best understood in the model system Arabidopsis. In its flowers, there are four whorls of floral organs: sepals (whorl 1), petals (whorl 2), stamens (whorl 3) and carpels (whorl 4) from outside in (Figure 4). The genetic regulation of floral organ identities in Arabidopsis has been conceptualized as the classic ‘ABCE’ model (Krizek and Fletcher 2005). In whorl 1 and 2, A function genes AP1 and APATHAL2 are expressed, and their functions are required for sepal and petal identities (Mandel et al. 1992). In whorl 2 and 3, B function genes APELATA3 (AP3) and PISTILLATA (PI) are expressed and participate in regulation of petal and stamen identities (Jack et al. 1992, Goto and Meyerowitz 1994). In the inner two whorls, the C function gene AGAMOUS (AG) is expressed specifying their identities (Yanofsky et al. 1990). In addition to the ABC genes, four E function genes SEPALLATA1/2/3/4 (SEP1–4) are expressed in all floral whorls, presenting functions that are redundant but indispensable for proper organ identities (Pelaz et al. 2000, Ditta et al. 2004). With the exception of AP2, all ABCE genes encode MADS domain proteins that form higher order protein complexes following the ‘floral quartet’ model to activate downstream genes in a whorl-specific manner (Figure 5, Honma and Goto 2001, Theissen and Saedler 2001).

![Diagram of Arabidopsis flower and ABCE model](image)

**Figure 5.** The Arabidopsis flower and the ABCE model in regulation of the floral identities. The flower consists of four different whorls: sepal, petal, stem and carpel. ABC genes are expressed in specific whorls and a certain combination with participation of the E function genes defines each whorl’s identity. At the molecular level, ABCE function proteins form higher order protein complex in order to function.
1.4.2 Genetic regulation of floral organ identities in *Gerbera*: E-function as an exception

The genetic regulation of floral organ identities in *Gerbera* has been found to follow a modified ABCE model. While B and C function genes show largely conserved functions in regulating floral organ identities (Yu et al. 1999, Broholm et al. 2010), the *Gerbera* E-function genes, the *GERBERA REGULATOR OF CAPITULUM DEVELOPMENT* genes (*GRCDs*), show diverged functions in regulating the flower head development. There are five *GRCD* members (*GRCD*1–5) that have been identified (Laitinen et al. 2006), and their functions set a great example of how a gene family is duplicated and subfunctionalized. Different from the redundant E functions provided by the *SEP* genes in *Arabidopsis*, *GRCD1* and *GRCD2* are the two *GRCD* genes that have been functionally characterized, and they show specific E functions that regulate floral organ identities in a whorl-specific manner. Down regulation of *GRCD1* converted staminodes into petal-like structures in ray flowers, suggesting that *GRCD1* is required for proper development of ray flower whorl 3 (Kotilainen et al. 2000). In anti-sense *GRCD2* lines, homeotic changes in style and stigma were observed, indicating *GRCD2* is indispensable for whorl 4 identity (Uimari et al. 2004). Furthermore, *GRCD2* was shown to regulate the determinacy of meristem fate in both FMs and IMs, suggesting that *GRCD2* has evolved a novel function in regulating inflorescence development (Uimari et al. 2004, Teeri et al. 2006b). By a large-scale protein-protein interaction study, Ruokolainen et al. (2010a) showed that while *GRCD1* and *GRCD2* form specific protein complexes with other *Gerbera* MADS box proteins, *GRCD4* and *GRCD5* show broad range of interactions indicating that both of them may provide general E-class function in *Gerbera*. In this thesis, I conducted functional analyses for the transgenic *GRCD4* and *GRCD5* plants and identified additional members of the *GRCD* gene family. Altogether, those data improve our understanding on how the E function genes regulate IM and FM patterning in *Gerbera*. 
2. AIMS OF THE STUDY

The flower heads in Asteraceae combine multiple developmental innovations that greatly facilitate the evolutionary success of this plant family. My PhD thesis elaborates Gerbera hybrida as an experimental system, aiming at elucidating the molecular mechanisms underlying three major aspects of the growth of flower heads. The results aim to provide in-depth insights on the evolution and development of this unique type of inflorescence. The specific aims of the thesis are:

I) Individual florets on Gerbera flower heads are arranged in a phyllotactic pattern consisting of left and right turning spirals in consecutive Fibonacci numbers. The first aim of this thesis was to investigate the formation of such patterns to understand how the patterning process is associated with the overall growth of the head. At the mechanistic level, it focuses on revealing roles of the phytohormone auxin in different developmental stages of the head.

II) The structure of flower heads resembles a single solitary flower, and the early development of the IM of flower heads shares similarity with flower meristems. The second aim of the thesis was to identify gerbera homologs of flower meristem identity genes LFY and UFO and characterize their functions in early flower head ontogeny.

III) Gerbera E-function GRCD genes show highly specific functions in regulation of floral organ identity. The third aim of my thesis is to first identify possible missing GRCD genes by mining RNA sequencing data. Functions of GRCD4 and GRCD5 were to be characterized since they were previously hypothesized as general E-function genes.
3. MATERIALS AND METHODS

The materials and methods conducted in this thesis have been described in the publications I, II, and III. Details of each element can be found with the help of the following table.

**Table 1.** Materials and methods used in publications I, II, and III. Those methods marked with asterisks were conducted mainly by the co-authors in the corresponding publications.

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4. RESULTS AND DISCUSSION

4.1 Growth and phyllotactic patterning of Gerbera flower heads (I)

In this thesis, multiple microscopic techniques were optimized to analyze growth and phyllotactic patterning of Gerbera flower heads. The experimental results revealed many interesting perspectives on the patterning process and how it associates with overall development of the head structure. Experimental outputs from this part of thesis work were combined with computational modeling, which lead to a unifying 3D model to explain the fascinating phyllotactic pattern observed in Gerbera flower heads.

4.1.1 Phyllotactic patterning in Gerbera: a three-phase process

Combining traditional 2D SEM imaging with 3D high-resolution micro-CT scanning, this study focused on revealing the growth dynamics during the early patterning of Gerbera flower heads. It begins with an initial ‘naked’ meristem located at the base of the rosette (Figure 6 A) and ends with a flower head bud that is fully packed with floret primordia (Figure 6 B). Since the growth of a gerbera plant and its flower heads are stereotypical under standard greenhouse conditions, it let us to collect a set of samples and reconstruct a developmental sequence to illustrate the growth of Gerbera flower heads (Figure 6 C). On a growing Gerbera head, there are three active domains characteristic: 1) the undifferentiated central zone, which is marked by the expression of Gerbera ortholog of CLAVATA3 (GhCLV3); 2) the organogenetic zone, where the organ primordia are initiated and 3) the outer zone bearing the initiated bracts and floret primordia (Figure 6 C). Different from SAM or IM of Arabidopsis and tomato whose sizes remain constant, phyllotactic patterning in Gerbera is governed by the expansion and contraction of the organogenetic zones and can be further characterized into three phases.

The first phase is characterized by the emergence of first bract primordia into a ring-like pattern at the rim of head meristem (stage I to II). During this phase, the patterning process was well revealed under confocal microscopy by examining transgenic Gerbera lines expressing the auxin reporter DR5rev:3xVENUS-N7 (Heisler et al. 2005). For the first time, the results demonstrated how the rapid transitions in phyllotactic patterns could occur on a growing meristem (discussed in detail in the next section). The second phase of patterning is marked by the formation of a primordia lattice with zigzagging lines (stage III to V). This is due to a gradual dissociation of the organogenetic zone from the head margin. As a result, new primordia form closer to the center of head meristem compared to the older ones. The zigzagging lines in phyllotactic patterns have been previously characterized by the initial disc packing experiments as a consequence of packing of leaf discs (van Iterson 1907, page 265–269), and more recently this pattern was thought to act as a template for successive patterning of primordia (Hotton et al. 2006, Pennybacker and Newell 2013). In the third phase of patterning (stage VI to XI), the organogenetic zone started to shrink in size and spiral numbers decreased following the reverse Fibonacci order (Figure 6 B). Compared to previous works that have mainly focused on the patterning of florets (the third phase), the results presented in this study cover the entire phyllotactic patterning process of Gerbera heads.
Figure 6. Growth and phyllotactic patterning of Gerbera flower heads. (A, B) Scanning electron micrographs show the beginning (A) and the end (B) of phyllotactic patterning of Gerbera heads. The shoot apex (located below the ground) contains one terminal head (TH), one lateral head (LH), three leaves (1, 2, 3) and one axillary meristem (AXM) that later reiterates a similar growth pattern. In the final pattern (B), the number of spirals decrease following the reverse Fibonacci numbers from outside in. (C) A growing contour of Gerbera head based on longitudinal sections of a developmental series from micro-CT data. Growth can be traced by individual landmarks (bracts, florets and active ring). (D) Confocal microscopic image shows a ‘naked’ head meristem expressing the DR5rev:3xVENUS-N7 reporter, with 13 DR5 maxima arranged in an approximately ring like pattern. Note that the gaps separating these maxima are not equal in sizes. (E) A diagram of showing how the pattern progresses following an expanding head meristem. New primordia are inserted first into the center of gaps, then moved towards the older neighbor that recreates a long (L) and short (S) segments. (F) Comparison of the patterning rhythm between Arabidopsis IM and Gerbera ‘naked’ meristem.

4.1.2 Phyllotactic transitions on an expanding head meristem

As discussed in section 1.2.3, transitions in phyllotactic patterns are a biological question that requires closer examination. An intriguing element of the phyllotaxis in Gerbera flower heads is how the first bracts can be arranged in a (8,13) pattern with one (for the lateral head) or only a few (for the terminal head) preceding leaves. This organization lacks a proper primordia lattice that is postulated by many disc-packing models (as compared to Figure 3 F). Developmentally, the transitions happen on an expanding head meristem that is morphologically ‘naked’ (novisible bulges of primordia), while auxin maxima are patterned in concert with the meristem expansion (Figure 6 D, E). By analyzing 56 randomly collected Gerbera meristems at this critical stage, the patterning process was found to include many interesting features. Particularly, for the patterning of first 13 auxin maxima, they are initiated far away from the head center and form a ‘ring’-like pattern at the periphery of head meristem (Figure 6 D). The auxin maxima are not uniformly distributed within the ring; instead, the angular gaps in between them are bimodal, and can be categorized as long (L) and short (S) in a specific order (Figure 6 E). Following the expansion of the head meristem, new auxin maxima are inserted in bursts fitting in the longer gaps, and they gradually tilt towards the older neighbor that subdivides the gap into longer and shorter gaps again (Figure 6 E). As a result
of the meristem expansion, the number of auxin maxima on the ring increased following consecutive Fibonacci numbers. These results altogether provide new experimental basis on how phyllotactic transitions occur in a developing meristem. At the same time, they indicate some new aspects in interpreting spiral phyllotaxis. As the relation of our results to the previous literatures on modeling of phyllotaxis has been thoroughly discussed in the paper (I), here I would like to extend the a few more points from the experimental side.

**Golden angle, permutations, and local interaction:** Spiral phyllotaxis is commonly described as a sequential progress, with consecutive primordia separated by the golden angle (137.5°). Deviation from this strict angle (known as ‘permutation’) has been recently brought into attention, as it can be both observed in simulations (Douady and Couder 1996, Mirabet et al. 2012, Refahi et al. 2016) and real plants (Bensnard et al. 2014, Landrein et al. 2015, Refahi et al. 2016). It was shown that the enlarged size of the meristem or loss of a secondary inhibitory field (cytokinin) either increased the rate of permutations or even led to co-initiation of organs in *Arabidopsis* reducing the robustness of phyllotaxis (Bensnard et al. 2014, Landerein et al. 2015). The patterning process of auxin maxima on an expanding *Gerbera* meristem, however, provides a different perspective on this. Instead of a clear divergence angle, the robustness in transitions is reflected by the geometric arrangement of auxin maxima into a ring with long and short gaps, that can be further translated into Fibonacci numbers of spirals (a main character of spiral phyllotaxis). In a recent hypothesis paper by Godin et al. (2020), the authors proposed that spiral phyllotaxis is a geometric canalization process with emphasis on local interactions of primordia, where robustness is defined. Our results are in line with this thought, the ‘ring’ presentation of model 1 in (I) (Figure 6 E), which is rooted from Himner (1931), is in fact an abstraction of the geometry with simple large and short intervals, where the robustness of the entire pattern is reflected.

**Tempo and rhythm:** Spiral phyllotaxis is also described as an iterative process, with consecutive primordia separated by a certain time, the plastochrone. In *Arabidopsis*, this time interval equals to 10–14 hours by the rhythmic patterning of auxin, along an IM that remains constant in size (Galvan-Ampudia et al. 2020). In *Gerbera* ‘naked’ head meristems, however, the number of auxin maxima in randomly collected samples showed strong preference to the Fibonacci numbers, indicating that new auxin maxima are initiated in bursts when the head IM constantly expands. Theoretically, increased meristem size (assuming primordium size remains constant) will lead to reduced plastochrones (Douady and Couder 1996), while our result suggested that the rhythm itself could be more complicated in case of transitions. If we compare phyllotactic patterning with music, the transition of phyllotaxis occurring on the expanding *Gerbera* head meristem cannot be merely treated as a simple increase of tempo for a plain note, instead it sings a rhythm that include many harmonies with notes at designated places (the longer gaps within the ring) (Figure 6 F).

**Lateral displacement:** One unique component in transitions occurring on the *Gerbera* ‘naked’ meristem is that the DR5 signals of bract initia show lateral displacement, with a positional bias towards the older neighbor. How is this mechanistically possible? As a first step, investigating the PIN1 expression and localization corresponding to the DR5 signals in the bract initium on the *Gerbera* head meristem will give further indications. Moreover, we have recently developed a laser ablation method for *Gerbera* head meristem, which allows targeted ablations with a
precision of 2–3 cells (Zhang et al. 2021). Performing similar experiments as done in Arabidopsis (Reinhardt et al. 2003, 2005) by removing the older, younger or both neighbors and by observing their effects on DR5 and PIN1 actions could help to better expose the nature of the lateral displacement.

4.1.3 An integrated 3D model for phyllotactic patterning of the Gerbera flower head

Computational modeling is often used to complement experimental results, and in many cases, it helps to get a more comprehensive understanding of a biological process by filling the gaps where the resolution of experimental data is not adequate (Prusinkiewicz 2012). In this thesis, experimental data obtained from multiple microscopic methods were combined and integrated into a unifying model to explain the phyllotactic patterning in Gerbera flower heads (Model 3, I). The model operates on a growing contour obtained from longitudinal sections of micro-CT data, where primordia can be used as landmarks to interpolate the growth in between different samples (Figure 6 C). Thanks to the radial symmetry of the heads, it can be translated into 3D shape by rotating 360 degrees. What constitutes a good model is that it allows extensive validation, for example, the model 3 has been validated from different perspectives in the paper (I). By comparing the model with real Gerbera flower heads at different growth stages, the model captures the development at a primordium level resolution (Figure 8 in I). Meanwhile, computational models can also predict scenarios which promote future experiments. For example, the model 3 predicts that loss of radial symmetry in the active ring – a key assumption in most modeling studies in phyllotaxis – does not alter the final pattern of the heads. It was later proved by investigating the heads with aberrant shapes (e.g. oval shaped active ring) where the observed patterns are consistent with model predictions. By changing the parameters, we anticipated that the model can also be used to simulate extreme cases, for instance, the phyllotactic patterning in fasciated flower heads with active ring in irregular shapes. Lastly, the integrated model also provides a starting point to collect data and construct further models to analyze other objects that show similar structures – for example, to understand development of individual flowers with many floral organs.

4.2 Co-opting FM identity genes in regulation of Gerbera IM patterning (II)

The flower head overall resembles a single flower, and this is also reflected by its early ontology that develop from a determinate IM. In this study, the Gerbera orthologs of two major FM identity genes, GhLFY and GhUFO, were isolated and their functions in inflorescence patterning were characterized. While GhUFO acts as the master regulator of FM identity in Gerbera, GhLFY has evolved novel roles in defining head IM as a determinate structure and also functioning in early initiation of peripheral ray florets. These results altogether provide new insights on how the head structure is patterned, evolved and genetically regulated.

4.2.1 Recruitment of FM identity genes in regulation of flower head development

LFY and DOT have been shown to act as the master regulators of FM identities in Arabidopsis and tomato, respectively (Weigel et al. 1992, Souer et al. 2008). In these species, both genes are necessary and sufficient to assign floral fate to the meristems. In Gerbera, their orthologs
GhLFY and GhUFO are similarly maintained as single copies. RNA in situ hybridization revealed that while GhUFO is expressed specifically in FMs, the expression domain of GhLFY spread across the undifferentiated IM and individual flower primordia (Figure 7 A). By analyzing phenotypes of transgenic Gerbera lines with down-regulated GhLFY and GhUFO expression, it was found that both genes are still necessary for FM identity. However, GhLFY RNAi lines showed an additional phenotype with an indeterminate IM. Moreover, by overexpression of GhUFO, the head IM was transformed into a giant flower-like structure producing numerous floral organs in whorls. Altogether, the results indicated that the IM in a head is already a determinate structure, as proposed by the FUM hypothesis, and that the FM identity gene GhLFY has been recruited to the inflorescence level to maintain its determinacy.

The spatiotemporal expression of FM identity genes has been considered as a key factor determining inflorescence architecture. LFY and UFO together are thought to play an essential role, and the place where they first co-express predicts a rising flower. In Arabidopsis, LFY and UFO are first co-expressed in the peripheral zone of the IM, which later gives rise to a FM (Bláquez et al. 1997, Lee et al. 1997). In petunia, instead, ALF and DOT are first expressed together at the terminal FM (Souer et al. 1999, Souer et al. 2008). At the molecular level, LFY and UFO physically interact with each other, and experimental data suggests that it relates to post-transcriptional modification of LFY that affects its transcriptional activities (Chae et al. 2008). Interestingly, ectopic expression of an active form of UFO fused with a transcriptional activator VP16 was sufficient to induce ectopic FM formation on the leaves of Arabidopsis (Risseeuw et al. 2013). In our study, the power of LFY and UFO on flowering was further illustrated by the GhUFO overexpression phenotypes. Precocious expression of GhUFO confers floral fate to the whole head meristem, where B and C function genes are expressed in similarly way than in FMs, and later develop into whorls of floral organs in a correct order.

Lastly, the loss of IM determinacy phenotypes observed in the GhLFY RNAi lines also raised a question: how does GhLFY function at IM to maintain the determinate growth? Highly likely, it involves genetic interactions with other FM and inflorescence identity genes. Among these, orthologs of AP1 and GhTFL1 (isolated in paper III) are the most apparent candidate worth testing. Our preliminary data showed that all GSQUA genes (the gerbera AP1/SQUA-like genes) are expressed in the undifferentiated Gerbera head IM, and overexpression of GhTFL1 led to indeterminate head IM, similar to those in GhLFY RNAi lines (Zhang et al. unpublished data). These results altogether indicate that a partial genetic program of the FM has been recruited to the IM of flower heads. Further experiments will help to clarify whether there are direct genetic interactions among these conserved regulators.

4.2.2 Ray florets have a distinct developmental origin as compared to the disc florets

The development of marginal ray florets is a key developmental innovation contributing to the evolutionary success of Asteraceae flower heads. Ray florets have been postulated to have evolved from a further modification of peripheral branching units that are found in the inflorescences of Calyceraceae, the sister clade of Asteraceae (Pozner et al. 2012, Figure 2 A). In most Asteraceae species with heterogamous heads, it was found that ray florets show delayed initiations as compared to the disc florets (Harris 1995). In wild-type Gerbera, ray florets are initiated exclusively from the axils of the last series of bracts, after several ‘whorls’
of trans florets have already initiated. Interestingly, *GhLFY* is expressed in the group cells located in the leaf axils that will later develop into ray floret primordia. In transgenic *GhLFY* RNAi lines, the ray primordia are initiated as enlarged oval shaped meristems that later bifurcates into two or three primordia. Such development resembles the branching syncephaliun subunit primordia (SSP) found in the inflorescence of Calyceraceae (Harris et al. 1999, Pozner et al. 2012), indicating that ray florets in *Gerbera* heads may share similar developmental origin as SSPs in Calyceraceae and their solitary presence as a single floret in Asteraceae heads depends upon proper *GhLFY* function. Our result is thus in support of Pozner's hypothesis, which further suggest that ray florets are distinct from disc florets by their initiations.

![Image](image.png)

**Figure 7.** Genetic regulation of flower head development by the FM identity genes and GRCDs. (A, B) Schematic presentation of *GhLFY*/*GhUFO* (A) and GRCDs (B) during IM patterning. Note that *GhLFY* and a subset of GRCDs are expressed in the undifferentiated IM. (C) Modified (A)BCE model of Gerbera MADS box genes in regulation of floral organ identities.

The evolution of ray florets is also considered as a main driven force of flower head diversity. Based on the type of florets they bear, flower heads can be generally classified as discoid (disc floret only), disciform (disc florets and reduced ray florets), radiate (both disc and ray florets) or ligulate (ray florets only) (Gillies et al. 2002). Genetically, the differentiation of flower types in flower heads is regulated by the *CYCLOIDEA(CYC)*-like genes encoding TCP domain transcription factors (see review Fambrini and Pugliesi 2017). The evolution of flower heads from radiate to ligulate can be explained by precocious expression of CYC-like genes in the disc florets (Broholm et al. 2008, Chapman et al. 2012, Fambrini et al. 2014, Juntheikki-Palovaara et al. 2014, Fambrini et al. 2018), whereas mutation or loss of function in a similar set of CYC genes led to radiate heads transformed into disciform heads (Mizzotti et al. 2015, Fambrini and Pugliesi 2019, Shen et al. 2021). An interesting remaining question is, how the
discoid heads evolved. It has been postulated that discoid heads evolved from radiate heads by losing the peripheral ray florets (Bremer and Humphries 1993). Bello et al. (2013) studied Anacyclus species which have both discoid and disciform heads and found that the discoid heads always lack ray primordia initiated from bract axils. Very recently, Fambrini et al. (2021) characterized a discoid sunflower genus Helianthus radula and found out that a mutation in HaCYC2c led to failed initiation of ray florets from the axils of involucral bracts. These results again emphasize the difference between ray and disc florets by their early initiation, and pinpoint further that CYC genes may genetically interact with LFY in the outgrowth of the ray floret primordia.

4.3 Dissecting functions of GRCD genes in regulation of Gerbera flower and inflorescence development (III)

In this study, a total of eight SEPALLATA-like GRCD genes were identified in Gerbera. Their expression patterns during inflorescence and flower development were analyzed in detail. Transgenic phenotypes indicated that while GRCD4 and GRCD5 are indispensable regulators for petal identity, progressive loss of all GRCD genes converted floral organs into leaf like structures. Additionally, GRCD7 was shown to act redundantly with its close paralog GRCD2 to regulate IM determinacy. The results illustrate how conserved genetic modules evolved to regulate the growth of a highly elaborated inflorescence structure.

4.3.1 Specialized E-function genes and modified (A)BC model in regulation of Gerbera flower organ identities

Previous studies demonstrated that GRCD1 and GRCD2 show specific functions in regulation of staminode and carpel identities, respectively (Kotilainen et al. 2000, Uimari et al. 2004). In this study, it was found that GRCD4 and GRCD5 are the two regulators required for proper petal development. Downregulation of GRCD4 and GRCD5 together showed specific phenotypes where petals were transformed into bract-like structures. These two genes were thought to act partially redundantly, as down-regulation of GRCD4 or GRCD5 alone led to only mild phenotypes: in GRCD4 RNAi lines the growth defect was restricted to petal epidermis, whereas GRCD5 RNAi lines show reduced ligule length in ray florets. The phenotypic differences indicate that GRCD4 and GRCD5 each have specific functions in regulating different phases of petal development. Interestingly, a follow-up study by Zhao et al. (2020) showed that GRCD5, but not GRCD4, binds to the promoter region of the ray specific GhCYC3, suggesting that GRCD5 acts upstream of GhCYC3 regulating petal elongation. Consistently, GhCYC3 was found to be significantly downregulated in petals of the GRCD5 RNAi lines.

Many of the GRCD4/5 double RNAi lines showed severe floral phenotypes. This was found as a cross-downregulation effect, as GRCD4 and GRCD5 share high sequence similarities with other GRCDs. By profiling expression of all GRCD genes in these lines, we found a correlation between the severity of phenotypes and the number of GRCDs being downregulated. Progressive loss of expression of certain GRCDs genes resulted in stepwise conversions of floral organ identities in a whorl–specific manner. Particularly, the staminode and carpel identities were found to be associated with the expression of GRCD1 and GRCD2/7 that
confirmed the results of previous studies on their functions. In the most severe cases, loss of expression in nearly all \textit{GRCDs} resulted in transformation of floral organs into leaves and also in floral reversion, indicating that the \textit{GRCD} genes act redundantly providing the E functions in \textit{Gerbera}. Although the resolution of our results is not as clear cut as in species where forward genetics can be applied, data presented in this paper allowed us to update a modified (A)BCE model for floral organ identities in \textit{Gerbera} (Figure 7 B, C). Targeted gene editing tools, for instances the CRISPR-Cas9 system, shall be developed in the near future to validate the proposed model.

4.3.2 \textit{GRCD2} and \textit{GRCD7} have a specific function in regulation of IM determinacy

Functional studies of the \textit{SEP}-like gene functions have been mainly conducted at the level of single flowers (Malcomber and Kellogg 2005). In \textit{Gerbera}, however, the expression analysis showed that four out of eight \textit{SEP}-like \textit{GRCDs} (\textit{GRCD2}/3/6/7) are expressed in the undifferentiated IM (Figure 7 B). In antisense-\textit{GRCD2} lines, their IMs show indeterminate growth producing infinite numbers of flower primordia (Uimari et al. 2004). Interestingly, \textit{GRCD7}, a close paralog of \textit{GRCD2} that shares more than 80\% identity at nucleotide sequence level, was also found to be downregulated in these lines. By characterizing the \textit{GRCD4}/5 double RNAi lines, we identified several lines displaying indeterminate IM phenotype and such phenotype always associated with down-regulation of \textit{GRCD7} or \textit{GRCD2}. These results altogether suggest that \textit{GRCD2} and \textit{GRCD7} play a redundant role in maintaining IM determinacy in \textit{Gerbera}. Phylogenetically, \textit{GRCD2}/7 groups with the \textit{Arabidopsis SEP}1/2, and the \textit{GRCD2} has been shown physically interact with several AP1/FUL-like GSQUA proteins in yeast 2- and 3-hybrid assays (Ruokolainen et al. 2010a, b). The IM functions of \textit{GRCD2}/7 might act through their specific interacting partners. Moreover, since \textit{GhLFY} RNAi lines display similar indeterminate phenotypes in their IMs, it would be interesting to test how \textit{GRCD2}/7 and \textit{GhLFY} were co-opted at the inflorescence level (as discussed in 4.2.1).
5. CONCLUSIONS AND FUTURE REMARKS

In this PhD thesis, I focused on three major aspects in the development of Gerbera flower heads: the phyllotaxis, the inflorescence architecture and the development of flowers. All these studied aspects greatly contribute to the uniqueness and evolutionary success of Asteraceae, the largest plant family of flowering plants.

Firstly, we incorporated experimental data with computational modelling, and demonstrated how the intriguing spiral phyllotactic patterns on the Gerbera flower head is formed. One crucial element that distinguishes Gerbera heads from traditional model systems (e.g. Arabidopsis and tomato) is that the patterning process is governed by expansion and contraction of the organogenetic zone, which can be attributed to the changing size of undifferentiated central domain of the meristem. An immediate question to be asked is, how the gigantic IM in flower heads has been evolved and genetically regulated? This question is exemplified in Gerbera and sunflower, as both species develop an undifferentiated IM that can reach a size of a few millimeters – about 10 to 20 times larger than an Arabidopsis IM (Marc and Palmer 1981, Zhang and Elomaa 2021). In this study, we also applied confocal in vivo imaging to study Gerbera head meristems, which allowed us to dissect the auxin patterning process on the ‘naked’ head meristem at cellular resolution. Our data reports for the first time how phyllotactic transitions occur along an expanding meristem. A critical character for the patterning process is the lateral displacement of auxin maxima pointing towards the older neighbor. Mechanistically, the cause for such movement is not clear. Introducing new fluorescence reporter lines, for instances DII based ratiometric R2D2 reporter (Liao et al. 2015), PIN1-GFP (Benkova et al. 2003, Heisler et al. 2005), or Gerbera specific markers will help to clarify this question. Moreover, the newly optimized confocal live-imaging protocol and laser-ablation method (Zhang et al. 2021) for Gerbera head meristem makes such a plan technically feasible.

Secondly, we illustrated how conserved FM identity genes, GhLFY and GhUFO, have been recruited to patterning of IMs of flower heads. While GhUFO acts as the master regulator of FM identity in Gerbera, GhLFY has evolved a novel function at the inflorescence level to maintain IM determinacy. An essential next step would be to further clarify how such function is achieved, and to what extend the genes or even whole gene networks regulating floral programs are co-opted to the IM level of flower heads. Our results also suggested that ray floret development shows distinct developmental origin as compared to the disc florets. The spotlight is now focusing on a small group of cells located in the axils of bracts. In addition to GhLFY, recent studies showed that these group of cells also express other interesting genes (Zhao et al. 2020, Basile et al. 2019). Targeted transcriptomic data, for examples using laser assisted microdissections, would help to clarify how genes are dynamically expressed during the ray floret primordium outgrowth.

Lastly, we analyzed the expression and functions of SEP-like GRCDs in Gerbera flower head development. Our results showed that GRCDs regulate floral organ identities in a whorl specific fashion, and GRCD4 and GRCD5 are two indispensable factors for petal identity. Most interestingly, we confirmed that GRCD2 and GRCD7 showed redundant functions in regulating IM determinacy in heads. The next step would be to explore how these two genes possibly act
together with GhLFY at the IM level, as well as more generally identify the gene regulatory networks affecting IM determinacy.

Taken together, this PhD thesis addresses several questions important at different stages of head development, and it also presents further hypotheses to be tested. Future studies will be facilitated by adapting new gene editing tools, availability of the *Gerbera* genome sequence, and optimized experimental toolsets in this thesis (microscopic pipelines) to further develop the *Gerbera* head meristem as a model system for understanding the development and evolution of flower heads in Asteraceae. Results from these works will greatly enrich our overall understanding on the regulation of inflorescence diversities in flowering plants.
6. ACKNOWLEDGEMENTS

The thesis was carried out at the Gerbera Laboratory, Department of Agricultural Sciences, University of Helsinki, Finland. This work is financially supported by the Doctoral Programme in Plant Sciences of University of Helsinki, the Academy of Finland, and the Finnish Cultural Foundation.

Firstly, I want to express my deepest gratitude towards my supervisor, Prof. Paula Elomaa. From the first day I entered the Gerbera lab, your endless guidance, supports, and patience throughout the years helped me grow towards a researcher that always keep independency in minds. Meanwhile, I would like to thank the other supervisor of my thesis, Prof. Teemu Teeri. Your enthusiastic attitude towards science inspires everyone in the lab and sets the standards for pursuing a research career for lifetime. Here, I also want to express my greatest thanks to Prof. Przemyslaw Prusinkiewicz from the University of Calgary, Canada. It was a true pleasure working with you to learn how biological data and modeling can be magically combined.

Secondly, I would like to thank my thesis committee members, Prof. Roosa Laitinen and Prof. Ari Pekka Mähönen. The lovely discussions we had in every year’s follow-up meeting greatly helped the work to move forward smoothly. At the same time, I want to thank Dr. Francois Parcy and Prof. Hongzhi Kong for your valuable suggestions and inspiring comments on the thesis draft. Here, I also want to thank the DPPS coordinator Karen Sims-Huopaniemi for your timely and excellent supports to solve our daily issues as a doctoral candidate.

Thirdly, I want to thank the coauthors of the articles included in the thesis: Yafei, Suvi, Feng, Anneke, Inka, Sari, Kati from Helsinki; Mik and Andrew from Calgary; and Victor and Tianying from Afflulo. These works simply cannot be finished without your timely input and efforts. Suvi, thank you for introducing me into the gerberology from day one! Yafei, special thank here for sharing the first authorship of LFY/UFO paper. It was an encouraging experience working with you, and I wish you will continue a successful career in CAU. Mik, thank you for your patience on numerous calibrations of the models again and again, which ultimately leads to the most beautiful one in our shared phyllotaxis paper! Feng, thank you for the charming GhCLV3 in situ figures, and I cannot wait to see the more exciting works you are doing to come out soon!

Fourthly, I want to thank all the members of the Gerbera lab for creating such friendly working atmosphere. Specially, I want to salute to our excellent technicians Anu, Eija and Marja, and the gardener Sanna for your help! Your unreserved supports are essential, without them, these works cannot be finished in such time frame.

Lastly, I would like to thank the sole supports and accompany from my family members: my wife Mengyi Sun and my 2-year-old son Zixuan Zhang. Mengyi, thank you very much for your understandings throughout the years. You are my true hero! I want also to thank my parents, Yuling Zhou and Guangming Zhang, for your unwavering supports for letting your son choose what he really likes to do!

Helsinki, August 2021

- Teng Zhang 张腾
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