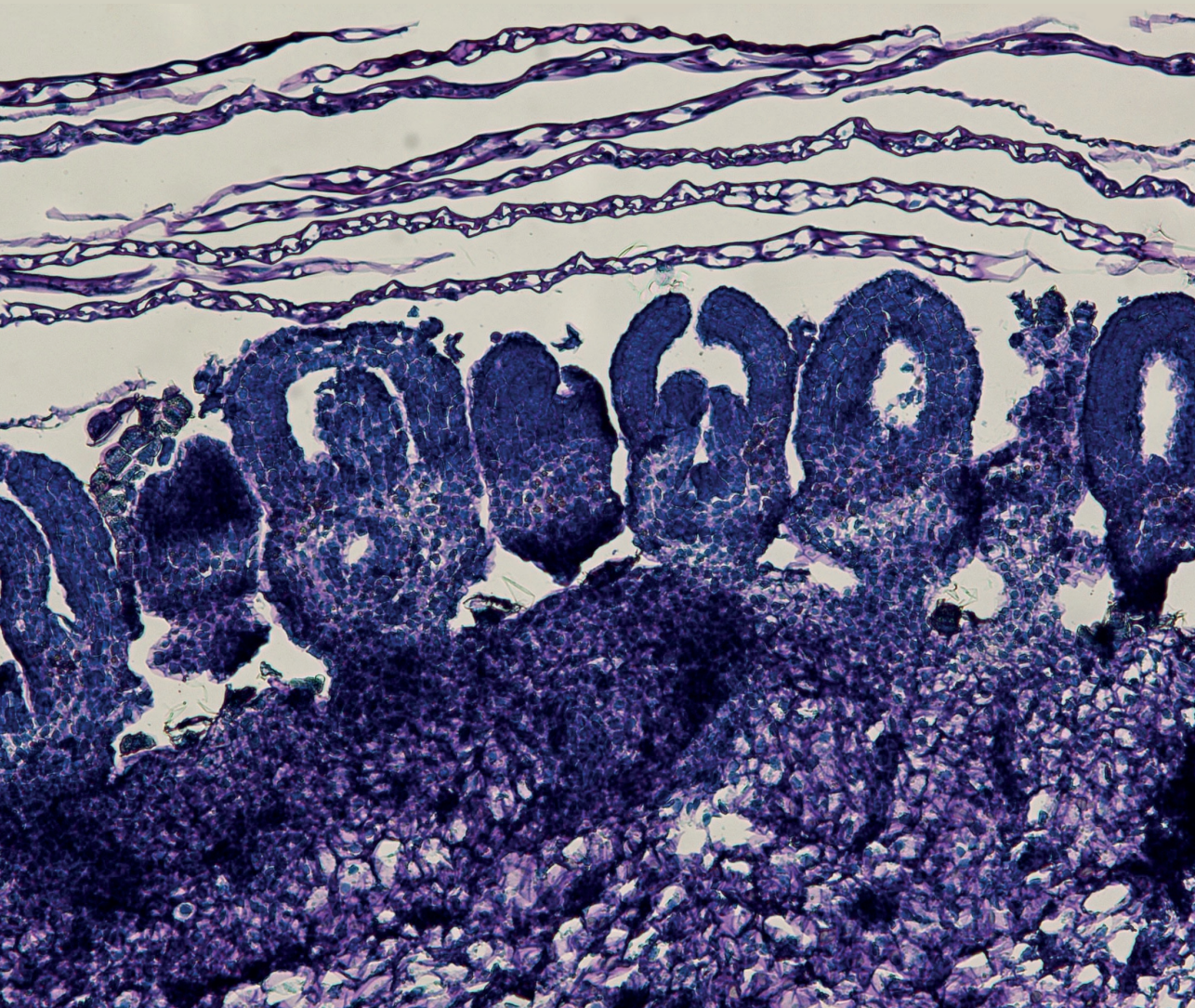
Elucidation of the morphological and genetic factors influencing fertility in Chrysanthemum

Annemarie Castricum



Propositions

1. The identity of a single floret in the Chrysanthemum capitulum is influenced by stress conditions.
   (this thesis)
2. Green flower colour in Chrysanthemum is responsible for reduced functionality of stigmas.
   (this thesis)
3. The 'adjusted p-value' criterium in multiple comparisons is sufficient to identify differentially expressed genes for a quantitative trait.
4. RNAi-mediated down-regulation is a better approach for functional studies in polyploid outcrossing species than CRISPR-Cas mutagenesis.
5. Genetic approaches are more powerful than differential expression analysis for candidate gene identification.
6. We should all become vegans.
7. It is (partially) our own fault that too few women are in high positions of power.

Propositions belonging to the thesis:

Elucidation of the morphological and genetic factors influencing fertility in Chrysanthemum.

Annemarie Castricum

Wageningen, 19 October 2022

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Elucidation of the morphological and genetic factors influencing fertility in Chrysanthemum

Annemarie Castricum

Thesis

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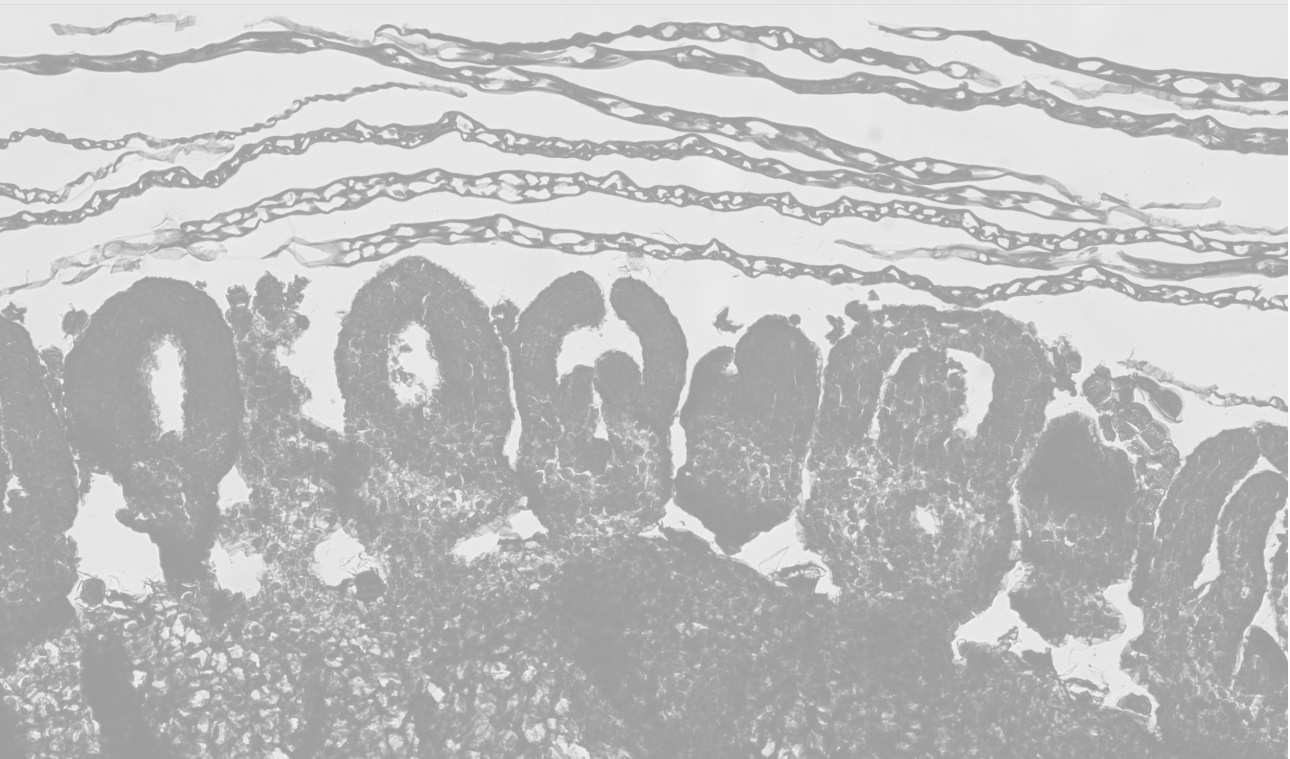
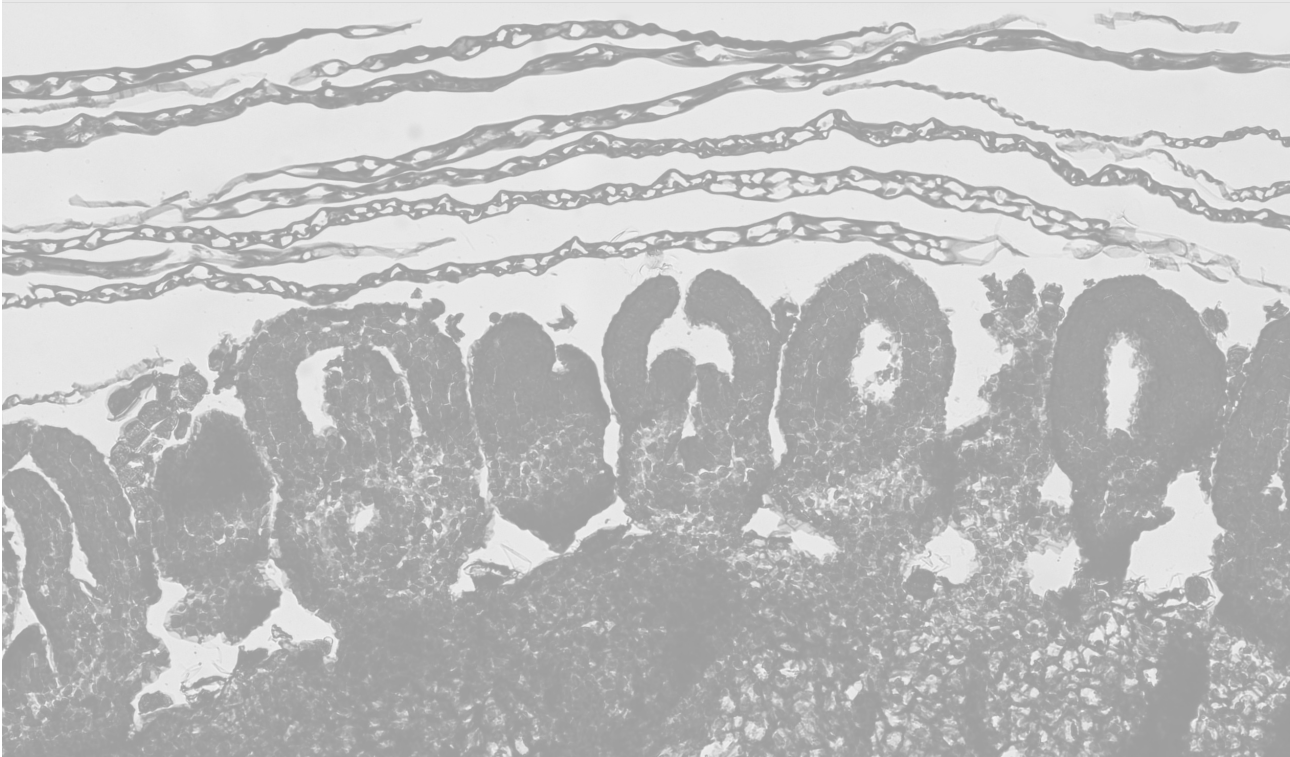


Chapter 1

General introduction



Chrysanthemum morifolium, formerly known as *Dendranthema grandiflora*, and hereafter referred to as Chrysanthemum, is a eudicot plant species from the Asteraceae family. The name "Chrysanthemum" is derived from the Ancient Greek meaning gold-flower (Encyclopaedia Brittanica, 1911). As a part of the *Asteraceae* family, Chrysanthemum is related to several economically important crops used for several purposes. Important species used for food include lettuce, sunflower, chicory and artichoke, while herbs such as Calendula, Echinacea and Chamomile are used in plant-based medicines. Several species of this family produce flavonoids and terpenoids used for their antiparasitic properties in medicine or pest control (Panda and Luyten, 2018). Additionally, several Asteraceae species, including Chrysanthemum, are mainly grown for their beautiful flowers, such as Gerbera, Dahlia, Tagetes and Zinnia. Chrysanthemum originates from Asia, where its flowers still enjoy great popularity, and the species was first introduced to Europe and the Netherlands in 1688 (Anderson, 2007). In addition to growing them for the beautiful flowers, Chrysanthemum is in Asia also used in teas and as an ingredient in medicines (Dong *et al.*, 2017; H. M. Yang *et al.*, 2017; L. Yang *et al.*, 2017; Ryu *et al.*, 2019).

In Europe, Chrysanthemums are commercially grown as cut flowers, pot plants, or annual garden mums for their ornamental value. Because Chrysanthemum naturally flowers in response to short days, it is mainly grown in autumn and used at traditional celebrations such as All Saints' Day. Additionally, it is commonly used as a grave flower because of its relatively long post-harvest performance. As a cut flower, Chrysanthemums are often used in bouquets and enjoy popularity because of the many flower types. In addition, advances in agricultural practices made it possible to grow and flower Chrysanthemum year-round by simulating short days in greenhouses using dark cloth, thereby expanding the market significantly. These days, the Netherlands is the top exporter of cut flowers, including the Chrysanthemum, which is the second most important cut flower in the world (Anderson, 2007; Spaargaren, 2015). In 2013, the global export of Chrysanthemum was 815 million dollars, with 64% originating from the Netherlands (Spaargaren, 2015). The largest exporters of Chrysanthemum are Colombia, the Netherlands, Vietnam and Malaysia (Anderson, 2007; Spaargaren, 2015).

Chrysanthemum cultivation and breeding challenges

Chrysanthemum is an obligate short-day annual plant with dark green foliage with a feathered leaf shape. Flowering is initiated under short-day conditions, and vegetative growth can be re-established under long-day conditions. This is an essential aspect of cultivation because it enables cloning from mother plants under long-day conditions. The resulting cuttings are then

rooted in long-day conditions to initiate new plants and transferred to short-day conditions when they are big enough to initiate flowering. Flowering is initiated similarly as in other species that respond to daylength. Arabidopsis, a plant that accelerates the switch to flowering under long-day conditions, measures daylength using *PHYTOCRHROME B* (*PHYB*). Under long-day conditions, expression of the florigen *FLOWERING LOCUS T* (*FT*) increases in the leaves, and the produced FT protein travels to the shoot apical meristem (SAM) to activate flower meristem identity genes such as *FRUITFULL* (*FUL*), *LEAFY* (*LFY*) and *APETALA 1* (*AP1*), thereby initiating flowering (Kobayashi and Weigel, 2007). In Chrysanthemum, this process seems similar, with florigen *FLOWERING LOCUS T-LIKE3* (*CsFTL3*) expressed under inductive short-day conditions when nights are long or flowering inhibitor *ANTI-FLOWERING T* (*CsAFT*) when nights are short (Oda *et al.*, 2012; Higuchi *et al.*, 2013).

Chrysanthemum breeding has always focused on producing many varieties with diverse flower types, colours and a good post-harvest performance (Teynor *et al.*, 1989; van Geest, Post, *et al.*, 2017; Mekapogu *et al.*, 2020). Chrysanthemum flower colours were initially white or yellow. But, owing to breeding efforts, there are also orange, purple, pink, red, bronze and green flower colours (Mekapogu *et al.*, 2020). Furthermore, Chrysanthemum with blue flowers have been created more recently by a Genetically Modified Organism (GMO) approach by insertion of a copy of *FLAVONOID 3',5'-HYDROXYLASE* (*F3'5'H*) into the Chrysanthemum genome allowing the production of delphinidin-based anthocyanins (Brugliera *et al.*, 2013; Noda *et al.*, 2017). With increasing global regulation of pesticide use, breeding for more pest- and disease-resistant varieties has also gained a lot of interest in recent years. Chrysanthemum is susceptible to several pests, including white rust, verticillium, fusarium and pythium (Su *et al.*, 2019). Additionally, there is a problem with thrips infestations in cultivation that cause silver spots and other deformations caused by suction. Moreover, as in many other crops, thrips can spread viruses such as the Tomato Spotted Wild Virus (TSWV). Additionally, Chrysanthemum has problems with viroids, including stunt and chlorotic mottle (Cho *et al.*, 2013). Breeding for resistance to these pests is of great importance for more sustainable cultivation in a world in which fewer and fewer pesticides are authorized for use. Also, other traits are subject to Chrysanthemum breeding, including plant architecture, daylength sensitivity and tolerance to environmental stresses such as salinity or heat.

Chrysanthemum is a vital economic crop and therefore breeding for better cultivars is essential. However, breeding progress has been hampered by low seed set, limiting the development of new varieties with desired traits such as disease resistance, innovative flower types or adaptation to climate change. This low seed set is most pronounced in varieties with

ornamental flower types. Studying the underlying cause of this low seed set and determining methods to increase it is unbelievably valuable from both a scientific and economic perspective and, therefore, the topic of this thesis. Below, an overview is presented on the general aspects that influence flower development and seed set in Chrysanthemum, ranging from agricultural practices to the molecular regulation of flower development in Chrysanthemum.

Flower types

Chrysanthemum has a typical composite 'flower' consisting of a capitulum (flower head) with many florets surrounded by the involucral bracts, giving the appearance of one single flower (Fig. 1A). In Chrysanthemum, these florets are composed of the less attractive-looking disc flowers and the showy female ray flowers that develop a long petal (ligule) (Fig. 1B). So, in principle, the florets are the flowers, and what is often regarded as the Chrysanthemum 'flower' is a complete inflorescence (capitulum) including all flowers (florets). Therefore, we refer to the individual flowers as florets to avoid confusion. In addition, we will refer to the inflorescence structure as a 'flower' or capitulum. The large variation in flower types of ornamental Chrysanthemum results from the combination of differences in the disc-/ray-floret ratio and morphological differences in these floret types (Fig. 1C). Interestingly, the first double-flowered type, containing an additional rim of ray florets, was described as early as 910 A.D. and either originated by spontaneous mutation or because of breeding efforts (Anderson, 2007). The most basic flower types can be categorized based on the percentage of disc florets. daisy is the 'original' flower type with mostly disc florets and one to a few rows of ray florets (Fig1C), while decorative flower types are the opposite, with only a few disc florets and many ray florets (Fig. 1C). Anemone flower types are daisy types with extensions of the ligules of disc florets, resembling a cushion in the center of the inflorescence (Fig. 1C). decorative types can be further divided into Spider, pompon and Spoonflower types, which have specific shapes of the ray floret ligules (Fig. 1C). decorative flower varieties can have varying amounts of disc and ray florets and are sometimes classified as 'half-decorative' if the disc/ray-floret ratio is between that of daisy and decorative types or other types, such as the half-pompon type (Fig. 1C).

Breeding for these beautiful flower types is highly desired, but a decrease in seed set is an increasing problem hampering progress. Decreased seed set appears associated with specific traits that breeders select for, because it is especially pronounced when highly decorative varieties are crossed (see Fig. 2). This can partially be explained by the fact that ray florets do not develop functional anthers, as decorative flowers seem less fertile when used as the mother

plant for crossings by hand pollination. It has been suggested that the more decorative female ray florets are generally of lower quality than the hermaphrodite disc florets due to inferior ovaries (Anderson, 2007; Cockshull, 2019), but a proper comparison of the reproductive characteristics of the disc and ray florets of different Chrysanthemum cultivars has so far not been performed, nor has it been elucidated how the specification of disc- or ray- floret identity is exactly regulated in this species. To address these issues, it is important to first understand inflorescence- and flower development in model species such as Arabidopsis and to discuss what is currently known from Asteraceae species. The next paragraphs of this Chapter are therefore dedicated to explaining the current understanding of the regulation of floret identity and development. In addition, the challenges of performing genetic and molecular experiments in Chrysanthemum will be addressed.

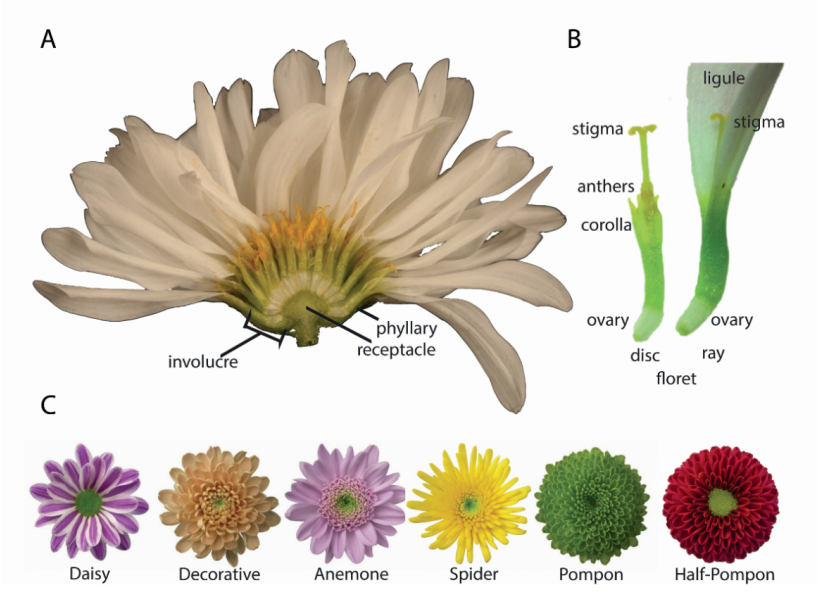


Figure 1. Introduction to the Chrysanthemum flower. (A) Chrysanthemum inflorescence/flower head, sliced in half to show capitulum structure. (B) Chrysanthemum disc and ray floret. On the left side is a disc flower and on the right side is a ray flower, showing only the basal part of the ligule. (C) Representation of Chrysanthemum flower types; daisy type, decorative, anemone, spider, pompon, and half pompon type.

Figure 2. Illustration of the seed set problem in Chrysanthemum breeding**.** The cross shown on top is of two daisy-type flowers, which resulted in many seeds. In contrast, the cross depicted at the bottom between a yellow decorative and a pink pompon type produced only three seeds.

Flower development

Composite flowers develop inflorescences with many florets tightly packed together to mimic a single flower. The disc florets are radially symmetrical, developing five short petals fused together, five stamens and one carpel. Ray florets display zygomorphic symmetry with three petal primordia fused to create one attractive ligule, no anthers, and one carpel. In Gerbera, there are also trans florets, which resemble a ray floret with shorter ligules and are considered trans florets based on their location on the capitulum (Laitinen *et al.*, 2006). Several Asteraceae species contain only disc florets, referred to as discoid, or only ray florets, referred to as ligulate. The species containing both floret types are referred to as radiate. For Chrysanthemum and other species that contain both floret types, ray florets are located at the rim of the capitulum to attract pollinators. Depending on the species, these ray florets can be female or completely sterile. Sunflower ray florets do not develop anthers nor a stigma (Coen *et al.*, 2002; Mason *et al.*, 2017). The largest part of the capitulum is covered with disc florets that are hermaphroditic and form seeds.

Floral meristem initiation

In general, flower development is determined by three factors, initiation of FM identity genes, initiation of floral organ identity genes and outgrowth of floral organs. Subsequently, flower fertility also depends on the functionality of the reproductive organs. In Asteraceae, the difference between disc and ray florets could be determined in several of these steps. Most insight into the development of the Asteraceae capitulum comes from research on Gerbera and sunflower (Teeri *et al.*, 2006; Shulga *et al.*, 2011; Zhao *et al.*, 2016; Zhang and Elomaa, 2021).

After the transition to flowering, the shoot apical meristem (SAM) converts into a large inflorescence meristem (IM), which develops into the capitulum. From the capitulum, floral meristems (FMs) are initiated, starting from the rim, and continuing inwards following the Fibonacci pattern in response to the formation of auxin maxima (Zhang *et al.*, 2021; Zhang, Wang and Elomaa, 2021). There is some debate on how the Asteraceae inflorescence structures have evolved, and this might be important for discovering the mechanism that determines floret identity. Three different theories have been proposed to explain the mechanism that has allowed Asteraceae species to develop this complicated inflorescence structure. The inflorescence structure might result from (I) a highly condensed raceme or cyme may be a (II) condensed structure combining cymose and racemose branching or (III) may have developed from a single meristem that subdivided into different primordia (Pozner, Zanotti and Johnson, 2012; Claßen-Bockhoff and Bull-Hereñu, 2013). Research in Gerbera suggests that the capitulum is the result of suppression of branching of the peripheral cymose combined with the determination of floral fate (Zhao *et al.*, 2016). Because each floret is a flower in itself and the regulation of its development probably follows that of other eudicot species. In Arabidopsis, the transition to flowering is regulated by floral integrators FT and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), which upregulate the FM-identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Golembeski and Imaizumi, 2015; Yamaguchi, 2021). TERMINAL FLOWER 1 (TFL1), on the other hand, represses *LFY* to maintain IM identity (Bradley *et al.*, 1997). Additionally, *LFY* upregulation is dependent on auxin maxima to release the repression of AUX/IAAs. The fact that auxin maxima are located at the site of FM initiation in *Matricaria inodora* (Zoulias *et al.*, 2019) suggests that the auxin-ARF-*LFY* regulatory module is conserved in Asteraceae.

Floral organ identity

In Arabidopsis, flower development is mostly regulated by MADS-box transcription factors that determine organ identity by being expressed in distinct regions of the flower meristem and regulating specific target gene sets. These so-called floral organ identity genes are induced by LFY (Yamaguchi, 2021). Flowers of Arabidopsis and many other species contain four floral whorls in which different floral organs develop. The original model for Arabidopsis flower development explained floral organ identity by the distinct expression of type A, B and C genes in the different floral whorls, and this model was later expanded with D- and E-class genes (Coen and Meyerowitz, 1991; Theissen and Melzer, 2007; Immink, Kaufmann and Angenent, 2010) (Table 1). The first and most outer whorl develops the sepals, and A-type genes

determine this identity. In Arabidopsis, type A genes are MADS-box gene *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the latter being the only ABC-gene that is not encoding a MADS-box transcription factor. The second whorl develops petals under the control of type A- and B-class genes. The two type B-class genes in Arabidopsis are *APETALA3* (*AP3*) and *PISTLLATA* (*PI*). The third whorl develops anthers; this identity is determined by a combination of type B and C genes. In Arabidopsis, *AGAMOUS* (*AG*) represents the C type. The fourth whorl develops the carpel/pistil, which is determined by C-type gene expression only. The expanded model includes ovule organ identity, determined by D-type gene function. Type D function is performed by two *SHATTERPROOF* genes, *SHP1* and *SHP2,* and *SEEDSTICK* (*STK*). Lastly, research showed the requirement of E-type MADS-box transcription factors in the development of all floral organs. Arabidopsis' E-function genes are four *SEPALLATA* genes (*SEP1-4*), of which the protein product forms heterotetramers with the other MADS-box identity proteins according to the floral quartet model (Theissen and Melzer, 2007).

Homologs/orthologs of these type A-E genes have been identified in several Asteraceae species, including Chrysanthemum. Their expression patterns have also been investigated in several studies, but it is challenging to determine which genes are differentially regulated between floret types and, therefore, might be responsible for the differences in identity. A summary of the published data is presented in Tables 1 and 2. Research in *Gerbera* showed that *GERBERA REGULATOR OF CAPITULUM DEVELOPMENT5* (*GRCD5*, E function) was explicitly expressed in ray florets and *CDEF1* (B function) in disc florets (Laitinen *et al.*, 2006; Zhao *et al.*, 2020). They also found an increased expression of *GERBERA AGAMOUS* versions *GAGA1* (C function), *GAGA2* (C function), *GRCD2* (E function) and *GDEF1* (B function) in disc florets (Laitinen *et al.*, 2006), and also the sunflower C-function gene *HELIANTHUS ANNUUS MADS-BOX* 59 (*HAM59*) appears specifically expressed in disc florets (Shulga *et al.*, 2015). *GERBERA DEFICIENS* (*GDEF1*) does not have a role in petal identity but has a redundant role in regulating stamen development in *Gerbera* (Broholm *et al.*, 2010). *GAGA1* showed classical C-type function, and silencing caused the conversion of stamen to petals and petals/carpels into sepal structures (Yu *et al.*, 1999; Kotilainen *et al.*, 2000). In addition to MADS-box genes, other Asteraceae transcription factors have also been associated with the development of either disc or ray florets. The TCP-family transcription factor *GERBERA HYBRIDA CINCINNATA1* (*GhCIN1*) might be involved in ray primordium development together with *GhCIN2* (Zhao *et al.*, 2020), as downregulation affected ray floret primordia at an early-stage by accelerating their development (Zhao *et al.*, 2020). Furthermore, *LEAFY* (*LFY*) expression in *Gerbera* was specific to ray florets in early-stage development and

therefore suggested to determine ray floret identity in *Gerbera* (Zhao *et al.*, 2016). This would be consequential to its evolutionary origins if ray florets are indeed derived from different branching systems. Based on all observations, *LFY* seems to have evolved a novel function in Gerbera floral development, as it regulates the ontogeny of outer ray florets (Zhao *et al.*, 2016).

The expression of the ABC-genes has also been determined in Chrysanthemum, where they observed that *AG2* (C function), *AP1* (A function) and *AP2* (A function) homologs were higher expressed in disc florets, while *AP3* (B function) had increased expression in ray florets (Wen *et al.*, 2019). The only common factor in the different studies between disc and ray florets over different Asteraceae species seems to be a higher expression of C-type genes in disc florets compared to ray florets. This is probably related to the impairment of anther development in the ray florets, which could imply that there is simply less tissue expressing C-type genes in ray florets. Apart from that, it is difficult to draw a specific conclusion from the existing data. The higher expression may be the result of more ligule tissue or of the development of anthers rather than the cause of the changed identity. The stage for these analyses is crucial for detecting reliable differences since disc and ray florets develop at different moments. Because gene expression of these homeotic genes differs over time, the detected differences may also simply reflect variation in developmental stage rather than being linked to the floret identity. In other words, with the resolution of currently published research, it is not possible to distinguish between the cause and consequence of the observed expression differences. Thus, it is still unclear if Chrysanthemum MADS-box genes contribute to a different development of the ray and disc florets, and if so, which gene(s) would be responsible. Other genes involved in the correct spatial upregulation of the floral organ genes, such as homologs of *UNUSUAL FLORAL ORGANS* (*UFO*), may also play a role. However, Chrysanthemum ray florets do develop anther primordia shortly after initiation arrest. Additionally, petal primordia develop in both floret types, and the difference becomes only pronounced during the outgrowth of these primordia. Thus, both these main differences between the floret types seem to be more related to organ outgrowth than to organ identity.

Floral organ outgrowth

Most research into Asteraceae floret identity has focused on the *TCP* (*TEOSINTE BRANCHED1/CINCINNATA/PROLIFERATING CELL FACTOR*) transcription factor *CYCLOIDEA* (*CYC*), a gene that seems responsible for ray floret identity in different Asteraceae species. *CYC* was first described in *Antirrhinum spp.*, where it was revealed to regulate the zygomorphic symmetry of the flowers via increased expression in dorsal regions

of the floral meristem, leading to differential dorsal and ventral petal development (Clark and Coen, 2002). *CYC* expression is regulated by the B-class MADS-box gene *DEFICIENS* (Clark and Coen, 2002). Therefore, it is likely that *CYC* homologs are responsible for petal outgrowth in both disc and ray florets in a different manner. In Asteraceae species, these *CYC* genes underwent several duplication events leading to three clades; *CYC1*, *CYC2* and *CYC3,* of which the protein products can function in complexes, resulting from the dimerization of CYC proteins within and between CYC clades (Tähtiharju *et al.*, 2012). In most Asteraceae species, *CYC2*-clade genes are important for determining ray-floret identity. In sunflower, insertion of a transposable element in the promoter of *CYC2* caused overexpression and, as a result, an inflorescence structure with only ray florets (Chapman *et al.*, 2012). These ray florets did develop anthers, but interestingly, these did not produce pollen. Downregulation of *RAY3* (*CYC2*) in *Senecio vulgaris* decreased ligule length but did not affect floret identity in either floret type (Garcês, Spencer and Kim, 2016). Research in *Gerbera* suggests that *CYC2* clade member *GhCYC3* is the candidate for regulation of ray floret identity since this is the only *CYC2* clade member exclusively expressed in ray florets. Its overexpression converts disc florets to ray-like florets with elongated petals and disrupted stamen development (Tähtiharju *et al.*, 2012; Zhao *et al.*, 2020). The *GhCYC3* promotor includes a TCP transcription binding site and two CArG boxes (Zhao *et al.*, 2020). *GhCIN1/2*, *GRCD5* (E-class) and *GAGA1* (C-class) were able to activate a *GhCYC3* reporter, suggesting that GhCYC3 acts downstream of the ABCE genes.

In Chrysanthemum, the overexpression of the two *CYC2* genes with the highest endogenous expression, *CYC2c* and *CYC2d,* only affects organ outgrowth (Huang *et al.*, 2016; Chen *et al.*, 2018). While *CYC2c* overexpression positively affects both disc- and ray floret ligules, *CYC2d* overexpression had a negative effect on ray floret ligule length. This suggests that, like *Senecio*, *CYC2* genes are determining ligule development but do not regulate the specification of ray floret identity. However, caution is needed in interpreting these overexpression experiments because transcripts accumulate in tissues and stages where they normally would not occur, and the observed phenotypes are not necessarily explaining the native function of CYC2. Comparisons between Chrysanthemum and close relative *Ajania pacifica* indicate an interesting role for *CYC2g* (Shen *et al.*, 2021). Ajania, which only has disc florets, does not express *CYC2g* because of mutations. Interestingly, the marginal florets do not develop a ray floret-like ligule; however, they also abort stamen resulting in female florets. Downregulation of *CYC2g* in *Chrysanthemum lavandulifolium* also affected ligule outgrowth, more towards disc floret morphology. However, the decreased expression did not prevent

stamen from aborting, thereby not completely changing floret identity. Three possible explanations for this are: (I) the expression was sufficiently reduced, still allowing stamen development, (II) the *CYC2* genes function in a redundant manner, and multiple genes need to be downregulated/mutated to observe an effect on stamen development, (III) Chrysanthemum *CYC2* genes are regulating ligule development rather than floret identity specification. Further research is necessary to distinguish between these possibilities. Thus, although evidence does suggest a key role for *CYC2* genes in ray floret identity in several Asteraceae species, there is no proof that it is causal for the determination of floret identity in Chrysanthemum. Even if the *CYC2* genes are controlling differential floret development, differentiation between the developing disc and ray florets would still depend on an upstream factor that assures a higher expression of the *CYC2* genes in the ray-floret primordia.

Other mechanisms could also be involved in differential specification/development of disc and ray florets, either in a pathway that would act parallel to the *CYC2* genes or in the regulation of *CYC2* gene expression. For example, the outgrowth of organs may be influenced by the size of the floral meristem, which could be influenced by homeodomain transcription factors such as *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*)/*PROTODERMAL FACTOR 2* (*PDF2*) (Kamata *et al.*, 2013, 2014). The spatial signal that could be (directly or indirectly) responsible for differential *CYC2* expression may also be provided by variation in hormone concentrations. Hormones such as auxin, gibberellin and brassinosteroids (BR) interact to regulate the expression of target genes, which is in Arabidopsis also associated with the growth of tissues (Oh *et al.*, 2014). Hormone concentrations or the homeodomain transcription factors could regulate *CYC2* expression and thereby influence floret identity, or a hormone gradient in the capitulum could directly result in differential outgrowth of organs within florets, leading to a particular floret type (José Ripoll *et al.*, 2015; Zoulias *et al.*, 2019). In the latter case, a certain hormone threshold would be required to get outgrowth of organs because Chrysanthemum normally does not exhibit intermediate floret types. Additionally, particularly BR plays a significant role in the outgrowth of organs. It is involved in the outgrowth of anthers and has an important function in male fertility by regulating the expression of key genes involved in Arabidopsis anther and pollen development (Ye *et al.*, 2010). Also other hormones can play a role in specific floral organ development. For example, ethylene induces the development of female flowers in cucumber, and cytokinin increases the number of female flowers in Jatropha (Wang *et al.*, 2010; Pan and Xu, 2011). A recent review describes the role of TCPs as transcription factors and mediators of hormone activity and as key players in the promotion of hormone signalling (Nicolas and Cubas, 2016). Thus, while

hormones may act upstream of *CYC2* to define its expression domain, they are most probably also acting downstream to regulate organ outgrowth. [26,42]. Therefore, still, a lot of research must be performed to elucidate the mechanism that determines floret identity and to obtain more insight into the mechanism(s) that regulate the disc/ray-floret ratio.

Table 1. Type ABCDE genes in extended flower model of Arabidopsis. Colours used in genes correspond to the colours of the gene types.

Whorl	Gene type	Genes	
1 sepals	A+E	AP1, SEP	
2 petals	A+B+E	AP1, P.I., AP3, SEP	
3 stamen	B+C+E	PI, AP3, AG, SEP	
4 carpel	C+E	AG, SEP	
5 ovules	C+D+E	AG, SHP, STK, SEP	

*SHP=SHP1 and SHP2, SEP=SEP1, SEP2, SEP3 and SEP4

Table 2. Current knowledge of differential expression between disc and ray florets of organ identity genes in several *Asteraceae* species. The colours used are based on homology with type A-E class genes of the Arabidopsis model of Table 1.

Disc	Ray	Both	Species
GDEF1		*GDEF2*, *GGLO1*, GAGA1, GAGA2, GRCD2,	Gerbera (unique) (Laitinen *et al.*, 2006)
	GhCYC3, GRCD5, LFY		Gerbera (Pozner, Zanotti and Johnson, 2012; Zhao *et al.*, 2020)
GAGA1, *GAGA2*, *GRCD2*, *GDEF1*	*GRCD5, GRCD3 (AGL6like), GRCD1(AGL2like)*		Gerbera (increased) (Laitinen *et al.*, 2006)
SVP, WUS	AP2/ERF-TF, CYC2CM1-4, *MADSCM1*		Chrysanthemum (Liu *et al.*, 2016)
GA2, *AP1*, *AP2*	*AP3*		Chrysanthemum (increased) (Wen *et al.*, 2019)
HAM59		*HAM45*, *HAM75*, *HAM92*, *HAM31*, *HAM2*, *HAM63*, *HAM91*, *HAM137*	Sunflower (Shulga *et al.*, 2011)

The functionality of the reproductive organs

Reproduction is influenced by several factors, including the development of a functional stamen producing viable pollen, the development of a functioning carpel and pollen/pistil interaction. The most notable difference between disc and ray florets is the lack of stamen in ray florets.

Many factors are known to affect male fertility, and the best researched and detrimental factor influencing male fertility is heat stress (Xu *et al.*, 2017). This affects both stamen development and pollen fertility (Hedhly, Hormaza and Herrero, 2005; Xu *et al.*, 2017). Some research has been done on pollen development in Chrysanthemum with a focus on producing varieties that disperse no or little pollen because this is an undesirable trait for vase life. Some varieties, like Kingfisher, do not produce pollen because these are aborted during development (Wang *et al.*, 2014; Wang, Zhong, Huang, *et al.*, 2018). Other studies reported low pollen fertility with about 5-30 percent viable pollen, which was proportional to their seed set (Sun *et al.*, 2009; Wang, Zhong, Wang, *et al.*, 2018). These studies indicate that male fertility is not optimal or severely affected in particular genetic backgrounds.

While in some Asteraceae species, such as sunflower, ray florets do not develop a pistil (Coen *et al.*, 2002; Mason *et al.*, 2017), others show reduced fertility of ray florets in comparison to the disc florets (Coen *et al.*, 2002; Pu *et al.*, 2020). Chrysanthemum develops a pistil in both disc and ray florets, and no differences between these florets have been reported. The pistil consists of stigma, style with transmitting tract and the ovary (de Folter, 2020). Chrysanthemum stigma morphology is quite different from Arabidopsis as it develops two lobes (Katinas *et al.*, 2016). Furthermore, Chrysanthemum stigmas are semi-dry, and this influences pollen ingrowth (McInnis *et al.*, 2006; Allen, Lexer and Hiscock, 2010; Rejón *et al.*, 2014). Research in other species has shown that heat stress also impairs stigma receptivity (Hedhly, Hormaza and Herrero, 2003, 2005). Transmitting tract tissue consists of an extracellular matrix, which is a mixture of polysaccharides, glycoproteins, and glycolipids through which pollen tubes can grow (Pereira *et al.*, 2021). *SHORT INTERNODE/STYLISH* (*SHI*/*STY*) and *NGATHA* (*NGA*) genes are major regulators of both style and stigma development (de Folter, 2020). Asteraceae develop an inferior bi-carpellary dry ovary, which develops a single indehiscent seed (Marzinek, De-Paula and Oliveira, 2008; Vijverberg *et al.*, 2021). Research in dandelion has shown that the ovary consists of two different tissues; micro-morphological data show that the carpel/ovary is surrounded by a layer of another cell type that was interpreted as the floret receptacle (Vijverberg *et al.*, 2021). This suggests that also the functionality of female reproductive tissues is very different from other species and could

influence the reproductive success of the Asteraceae. Furthermore, a study focusing on the self-pollination of a specific line to create a more homozygous Chrysanthemum plant detected several problems in fertility, including in ovules (Wang, Zhong, Wang, *et al.*, 2018).

Pollen-pistil interaction is an especially important mechanism following the prevention of inbreeding depression through self-incompatibility and impeding interspecific crosses that produce sterile offspring (Bedinger *et al.*, 2017). Recent research in ragweed (Senecio) has shown that self-incompatibility in Asteraceae might be different from sporophytic self-incompatibility (SSI) known from Brassicaceae and that this could be a result of the semi dry stigmas as compared to the dry stigmas (Allen *et al.*, 2011). Several studies have focused on self-incompatibility and interspecific crosses in Chrysanthemum, but how fertilization leads to successful seed set in case of a compatible interaction has hardly been studied in Chrysanthemum. However, problems with seed set have been reported before by Anderson *et al*, who noted that it was essential to do reciprocal crosses to distinguish self-incompatibility from fertility issues (Anderson *et al.*, 1989).

Chrysanthemum breeding and research

Chrysanthemum breeding differs a lot from that of many edible crops in that it is not possible to perform hybrid breeding in which two inbred lines are used to produce a superior hybrid. This classical approach cannot be applied because Chrysanthemum, like many ornamental crops, is originally a self-incompatible, highly heterozygous polyploid. In addition to complicating breeding, this also highly affects research progress, specifically at the level of genetics and molecular biology.

Chrysanthemum is an outcrossing species, meaning that it deploys several mechanisms to reduce the chance of inbreeding. First, Chrysanthemum is protandrous, which means that anthers ripen before the stigma is receptive. Additionally, it has sporophytic self-incompatibility to prevent inbreeding (Ronald and Ascher, 1975; Allen *et al.*, 2011), which is more stringent than gametophytic incompatibility because protein similarity of the pollen surface is scanned at the stigma (Cartwright, 2009). Recognition initiates a response preventing the pollen tube from growing into the stigma. Several years ago, Anderson and Ascher (Anderson and Ascher, 1996) discovered a locus overcoming this self-incompatibility, resulting in pseudo-self-compatibility, a trait that is now widespread in Chrysanthemum breeding. Nonetheless, many breeders believe that problems in seed set are caused by self-incompatibility, which might be more pronounced in some subgroups that contain a more limited gene pool.

While several botanical species of Chrysanthemum are diploid, decorative varieties have ploidies ranging from diploid to heptaploid (Yang *et al.*, 2006; Wang, Jiang, Chen, Fang, *et al.*, 2013; Wang, Jiang, Chen, Qi, *et al.*, 2013). For example, *Chrysanthemum morifolium*, the cultivated variant, is hexaploid, but displays aneuploidy (Dowrick, 1953). Aneuploidy is when there are missing or extra chromosomes, thereby resulting in an unbalanced amount. This could result from crossing with Chrysanthemum with a different ploidy level. Although little is known about the ancestry of cultivated Chrysanthemum based on genomic research, there are several theories about the original wild species that were crossed. Research using chloroplast DNA and the sequence of the nuclear *LFY* gene suggest that *C. indicum, C. zawadskii, C. dichrum, C. nankingense, C. argyrophyllum*, and *C. vestitum* were involved in hybridization events leading to the modern cultivated Chrysanthemum (Ma *et al.*, 2020). The tetraploid *Chrysanthemum indicum* is derived from the polyploidization of a closely related species, *C. lavandulifolium* (Yang *et al.*, 2006). This complicated origin from different botanical species makes genetics challenging, as it influences the mode of inheritance.

There are two types of inheritance in polyploids, disomic and polysomic, which are determined by how polyploidy was initiated, and the amount of time passed since. When an increase in ploidy level originates from a doubling of very similar chromosomes, each can pair with any of the others. Therefore, recombination can occur between any of these, resulting in polysomic inheritance. Polyploid species that show mainly polysomic inheritance are also referred to as auto-polyploids. Potato is a popular example of an autotetraploid crop. Occasionally, local rearrangements occur in one pair, inhibiting recombination in that region between the other chromosomes that did not undergo this rearrangement, resulting in local disomic inheritance. Other polyploid species originate from interspecific crosses of related species, such as *Chrysanthemum morifolium*. In these species, the dissimilar chromosomes from the parents do not pair, and recombination therefore only takes place between the original parent's chromosomes, which is disomic inheritance. Therefore, these behave as diploids with more chromosomes, for which several chromosomes are very similar. Polyploids with disomic inheritance are also referred to as allopolyploids. Wheat is a popular example of an allohexaploid crop. Recombination between chromosomes originating from different original species can sporadically occur and, in time, allows for more pairing and recombination until these species develop a more polysomic inheritance type. For this reason, many polyploid crops exhibit neither mode of inheritance completely. As previously mentioned, *C. morifolium* most likely originated from crosses between different wild species, and the assumption was that Chrysanthemum inheritance would be disomic. However, recent studies revealed that

Chrysanthemum has mostly polysomic inheritance with possible partial disomic inheritance in some populations (van Geest, Voorrips, *et al.*, 2017).

Chrysanthemum is highly heterozygous, and this is most likely a result of the combination of outcrossing and a high ploidy level. Because Chrysanthemum is hexaploid, there is less selection on individual alleles since there are five more copies that can potentially fulfil the function of that specific gene. The abundance of alleles could give rise to an above-average amount of non-functional alleles and losses of chromosome fragments, giving problems in certain crosses and preventing offspring from obtaining a higher range of homozygosity. Therefore, Chrysanthemum breeding is performed by crossing highly heterozygous parents and selecting the desired offspring from highly varying seedling batches. Then, the selected seedlings are propagated by moving them to long-day conditions to take cuttings for further selection from a higher number of clones.

Genetic approaches that can be employed in Chrysanthemum

Chrysanthemum's highly heterozygous hexaploidy nature makes it suitable for applying mutation induction in breeding efforts. Mutation induction is a commonly used breeding strategy in many crops used to induce genetic variation. Mutation induction is often used in plants that exhibit many of the required traits to change a single trait, such as flower colour, where a variety of colours can be created in an elite cultivar. The techniques used are EMS or radiation that create different mutations ranging from SNPs to the deletion of a part of a chromosome (Broertjes, 1966; Yamaguchi, 2018). For example, research has shown that several yellow-coloured mutants were created by radiation that broke part of a chromosome, including the gene *CAROTENOID CLEAVAGE DIOXYGENASE 4a* (*CCD4a*), which is required for degradation of carotenoid (Yoshioka *et al.*, 2012). While mutation induction is an effective breeding strategy, it is most valuable to expand upon already successful varieties. To develop more advanced new varieties, it is better to use a wide range of genetic tools and gain more insight in the involved loci by use of markers. However, the use of genetic tools was highly challenging in Chrysanthemum due to its highly heterozygous hexaploidy nature and the inability to produce homozygotes. Until recently, it was impossible to construct a qualitative genetic map and perform subsequent genetic analysis or produce a reliable genome sequence. Therefore, the possibility to use marker-assisted selection (MAS) was limited, and constructing a genetic map was the general tool for genetic analysis.

The genetic map is used to perform statistical analysis to predict loci that influence quantitative traits. While statistics can predict linkage between markers that distinguish the

parental alleles and the phenotype, the power is significantly increased using a map that includes positions between markers (Würschum, 2012; Akond *et al.*, 2019). A genetic map is constructed by producing a population that segregates genetically and calculating the recombination frequencies between markers for each progeny. In diploids, the recombination frequency is calculated for each marker pair. Markers that exhibit linkage, having a recombination frequency lower than 0.5, are grouped and ordered based on the frequency to construct a genetic map for each linkage group. A linkage group represents a chromosome, which is not wholly synchronous since some regions on a chromosome exhibit low recombination frequencies. Therefore, these regions take up limited space on a linkage group. Constructing a genetic map for a polyploid species is more complicated, and tools have only recently been developed (Bourke, van Geest, *et al.*, 2018; Bourke, Voorrips, *et al.*, 2018; Su *et al.*, 2019; Song *et al.*, 2020). Assembly of a polyploid genetic map uses markers that segregate as a diploid as basis and uses more complicating segregating markers to couple homeologs to one chromosome. In a bi-parental population of Chrysanthemum, the result would be 108 homeologs, 54 from each parent. Depending on the polyploid's inheritance type, the method of combining these differs. The resulting genetic map can then be used for genetic studies in several ways, such as quantitative trait loci (QTL), bulk segregant analysis (BSA) and genome-wide association study (GWAS).

In addition to a genetic map, the availability of the genome sequence can largely facilitate genetic analysis. However, the Chrysanthemum genome is huge, with an estimated size between 7.9 and 9.4 Gb (Su *et al.*, 2019) (Nakano *et al.*, 2021). In addition, it is extremely difficult to sequence and assemble a heterozygous genome, especially for crops with a higher ploidy level. As a consequence, only diploid Chrysanthemum species have been recently sequenced, including that of *C. nankingense* (3.07Gb) (Song *et al.*, 2018), *C seticuspe* (2.72Gb) (Hirakawa *et al.*, 2019; Nakano *et al.*, 2021), *C. makinoi* (3.1Gb) (van Lieshout *et al.*, 2021) and *C. lavandulifolium* (2.6Gb) (Wen *et al.*, 2022). These reference genome sequences can be used for QTL analyses, GWAS, and other (subsequent) research approaches. In addition, it might be possible to use these as a basis for sequencing and assembling the hexaploid genome of *C. morifolium*.

The availability of genetic maps and genome sequences provides straightforward positional information for the markers. This allows for interval mapping, an approach that greatly enhances the statistical power of QTL analysis. However, QTL analysis in a polyploid species is complicated because interactions between alleles can affect the severity of a phenotype. For example, a combination of positive and negative alleles can bring about

different dose effects in the progeny, and a larger QTL population is therefore required for polyploids. Fortunately, newly available tools can develop models that can distinguish the effect of individual homeologs. Another tool used in breeding that depends on a genetic map with positional marker information is GWAS, which is similar to QTL analysis but can be performed based on a large number of varieties and not perse on a segregating population. Different varieties are generally genetically more diverged and therefore contain a higher number of recombination events between markers, resulting in a better estimation of loci controlling the trait.

The recent development of tools to perform QTL and GWAS analysis in Chrysanthemum and the construction of diploid genome sequences opens up more possibilities for marker development and MAS to identify variation already present in the germplasm. MAS can be used to select seedlings in early stages for essential traits and enable better use of greenhouse space by discarding unwanted seedlings. Additionally, MAS can be used to determine the allele dose of potential parent plants to help in choosing crossing combinations. An increased dose of desirable alleles will result in offspring with more of the desired alleles. In conclusion, recent advances in genetic techniques and the development of genome sequence are of immense value for research and subsequently breeding of Chrysanthemum using MAS.

Outline of this thesis

Chrysanthemum breeding efforts are hampered in flower types with increased ornamental value because of low seed set. We hypothesized that this low seed set resulted from a decrease in the disc/ray-floret ratio in these flower types. Therefore, we aimed to investigate differences in fertility between floret types and determine possible reasons for reduced fertility in ray florets. Additionally, we set out to identify potentially causal genes and to determine the mechanism responsible for the disc/ray-floret ratio to assist in possibilities for MAS.

In **Chapter 2,** we investigated seed set in varieties with high, medium or low amounts of disc florets. The analysis showed a correlation between fewer disc florets and a low seed set, confirming our hypothesis. Since equal pollen amounts were used, and therefore pollen quantity did not cause low seed set, we further investigated differences in reproductive organs of disc and ray florets. We discovered variation in stigma morphology between floret types and varieties. Ray florets more commonly showed less opening of the stigma lobes, a phenotype reminiscent of a developmental stage before maturation. This low stigma quality resulted in low pollen ingrowth. Additionally, an extensive analysis with almost nine thousand crosses of 189 varieties confirmed that stigma quality combined with the ratio of disc/ray florets is an essential factor determining seed set.

In **Chapter 3,** we performed transcriptome analysis of flower mutants with an altered disc/ray-floret ratio to identify candidate genes involved in determining the ratio and, therefore, floret identity. Two varieties with mostly ray florets and only a limited number of disc florets had a similar mutant with more disc florets. Therefore, we decided to use the two mutants to find differentially expressed genes in both mutants to identify candidate genes for regulation of floret identity. We first determined the stage at which floret identity becomes visible and collected flower buds in the stages prior to and post this stage. In the differential expression analysis, we focused on differentially expressed genes common in both mutants in at least one of the investigated stages prior to the visible differentiation of florets. We discovered Homeodomain leucine zipper (HD-ZIP) IV transcription factors and several genes related to the Brassinosteroid signalling pathway as putative candidates for involvement in the altered mutant phenotypes.

Chapter 4 also describes the search for genes that regulate the disc/ray-floret ratio but using a genetic approach. We performed a quantitative trait loci (QTL) analysis on a population

between a pink daisy type and a green pompon type. Two QTLs were discovered, one on linkage group 1 (LG1) and one on LG4. Interestingly a genome-wide association study (GWAS) on a large set of varieties verified both QTLs. Furthermore, the region associated with the trait could be narrowed down considerably by the GWAS approach facilitating the selection of potential candidate genes. Interestingly a Chrysanthemum *PDF2* homeolog was discovered on chromosome 1 that was also differentially expressed. Additionally, the region on chromosome 4 also included all Chrysanthemum *CYC2* homologs implicated in floret identity in several Asteraceae species.

In **Chapter 5,** we describe the functional characterization of several candidate genes identified in the differential expression analysis of Chapter 3. Plants were transformed with RNAi constructs for *GIR1*, *HOTHEAD* (*HTH*), *DWARF1* (*DWF1*), *RICESLEEPER2* (*RSL2*) and *PROTODERMAL FACTOR 2* (*PDF2*) or overexpression constructs of *GLABRA2-INTERACTING REPRESSOR* (*GIR1*), *WIP DOMAIN PROTEIN2* (*WIP2*) and *TINY2*. Most plants transformed with the *RSL2*-RNAi and *WIP2*-ox constructs did not show the expected downregulation and overexpression. Therefore we could not determine the possible involvement of these genes in floret identity. Additionally, *HTH*, *GIR1* and *TINY2* overexpression did not yield the expected flower phenotype. However, plants with downregulation of *DWF1* and *PDF2* did show the expected increase in the number of disc florets. *DWF1* encodes for a protein involved in Brassinosteroid (BR) synthesis we expected that its decrease would either induce disc floret development or inhibit ray floret development. Additional experiments with Brassinazole treatment, a BR inhibitor, resulted in a similar phenotype with more disc florets in one of the tested varieties providing more evidence for the role of BR in determining the disc/ray-floret ratio in Chrysanthemum. The reduction of *PDF2*, an HD-IV ZIP transcription factor involved in the outgrowth of trichomes in Arabidopsis, seems to affect floret identity as well.

Chapter 6 discusses the obtained data and the consequences of this study for Chrysanthemum breeding. First, it infers the effect of the differentially expressed genes of Chapter 3 and the possible link between floret identity and stigma quality, thereby affecting the reproductive success of ray florets. Next, it discusses the overlap of the RNAseq, QTL and GWAS results and the evidence for the role of *PDF2* and Brassinosteroid in the regulation of floret identity. Lastly, we discuss possible strategies to combat the problem with low seed set in the breeding of Chrysanthemum.

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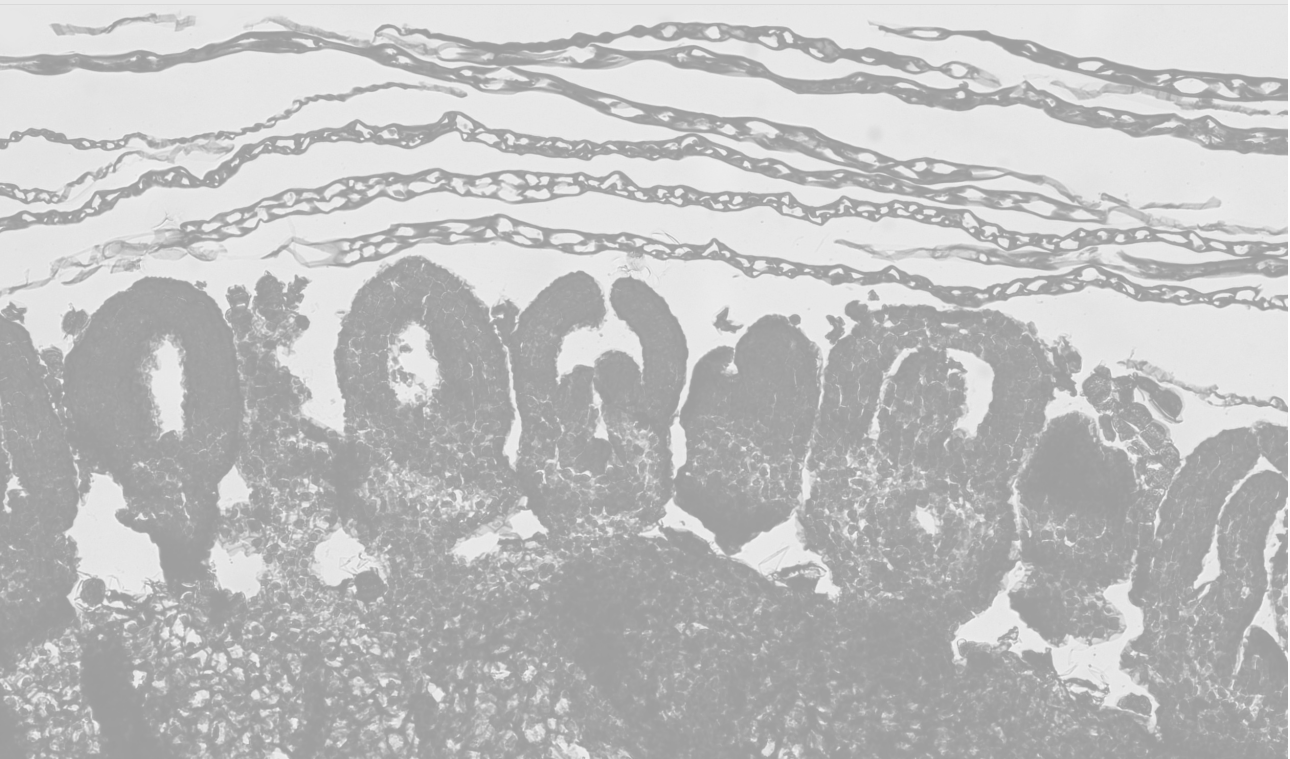

Chapter 2

Identification of morphological factors underlying seed set reduction in Chrysanthemum varieties with high ornamental value

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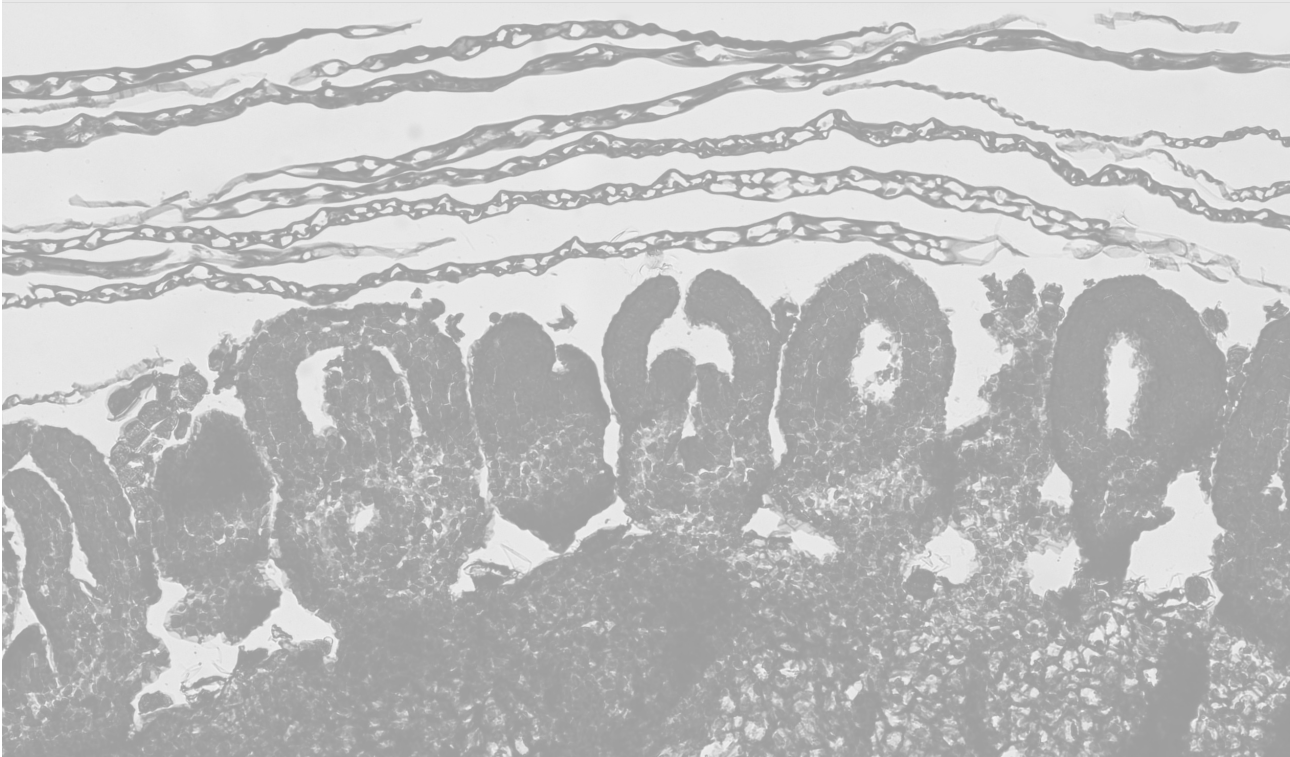

Summary

Chrysanthemum flowers with higher ornamental value show lower seed set compared to the original daisy-type flowers, and this complicates breeding programs. We investigated this problem and found it to be highly correlated with the ratio between disc and ray florets. There is a positive correlation between the percentage of disc florets and seed set. Therefore, we did a comparative morphological analysis to identify potential causes for the difference in fertility between the two floret types and discovered a high variation in stigma morphology. This difference in stigma morphology is mainly related to a reduced opening of the stigma lobes. This morphological characteristic is remarkably similar to the early stages of stigma development in varieties that develop a normal stigma, suggesting an arrest at a certain moment during stigma development. Additionally, we discovered a high variation in stigma receptivity using peroxidase assays. While we discovered that stigma morphology is floret-type dependent, with a general tendency for lower quality stigmas in ray florets, receptivity appears floret-type independent and seems determined for the entire inflorescence. Interestingly, we found a correlation between a higher percentage of disc florets per inflorescence and higher disc-floret stigma quality scores. While investigating the repercussions of the differences in stigma quality, we found that lower stigma morphology had a negative impact on pollen capture and ingrowth. Large-scale analysis showed that 20 percent of the crosses in our dataset did not yield any seeds. Three factors increased the chance of a cross not producing seeds, namely: low stigma receptivity, a low ray-floret stigma morphology score and a low percentage of disc florets. However, the variation in seed set was explained mainly by the interaction between floret-type ratio and stigma morphology. The correlation between the percentage of disc florets and disc-floret stigma morphology hampers the selection of suitable parents that will produce sufficient seeds in breeding programs aiming at obtaining highly decorative varieties. However, since ornamental varieties with better stigma quality exist, it should be possible to uncouple these traits. Additionally, since we did not find a coupling between floret type and stigma receptivity, the latter could be a more accessible trait to select for in relation to reproductive success.

Introduction

Chrysanthemum is one of the most important cut flowers in the world, and its popularity can be attributed to the wide range of flower phenotypes (Anderson, 2006; Spaargaren, 2015). Breeders attempt to develop new varieties with better characteristics, but progress in some

subgroups is severely hampered because of low seed set. This appears to be specific to flower types with a high number of ray florets, often considered to have a higher ornamental value.

Chrysanthemum is part of the Asteraceae family. In accordance with that, a Chrysanthemum flower head is not just one flower but an inflorescence structure with many florets clustered together, mimicking a single flower (Harris, 1995; Gillies, 2002). Chrysanthemum has disc and ray florets, with ray florets along the rim of the inflorescence, which is also known as the capitulum, and disc florets in its centre (Fig. 1A). The capitulum is enveloped by involucral bracts that protect the inflorescence structure during development (Anderson, 2006). Florets develop similarly to other flowers, such as those from Arabidopsis, except that sepal development arrests shortly after initiation to facilitate resemblance to a large flower. Disc florets are actinomorphic, tubular structures and develop both anthers with pollen and a pistil with functional stigma. Ray florets are zygomorphic female and lack anthers but form a long petal (ligule) in contrast to the disc florets. Floret primordia are first initiated at the rim of the capitulum and gradually develop inwards in spirals according to a Fibonacci pattern. Floret development follows this pattern, although disc florets grow faster than ray florets. Disc florets are protandrous, which means that the anthers produce pollen before the stigma is receptive to prevent self-pollination. As a result, disc florets in inner rows may still produce pollen, while for florets in outer rows, the anthesis stage that completes stigma maturation has passed. Chrysanthemum florets produce a bifurcate stigma with the reflexed lobes and sweeping hairs at the tip (Wetzstein *et al.*, 2014; Katinas *et al.*, 2016). Papillae tissue is located at the rim on the sides of the stigma lobes. As with other Asteraceae species, stigma peroxidases play a vital role in influencing stigma receptivity (McInnis *et al.*, 2006). Each floret has an ovary with one ovule and hence is able to produce a single seed. Although each of the hundreds of florets per flower head could potentially produce a seed, this somehow does not happen. Unknown factors inhibit seed set, which is most prominent in varieties with mainly ray florets, and although several studies observed limited seed set, none focused on finding the underlying cause. The absence of pollen in ray florets does not solely explain this effect, as the seed set remains lower when florets in flower heads of varieties with high ornamental value are hand-pollinated with high amounts of pollen. This suggests that the problem of the observed reduction in seed set in varieties with a high ornamental value stems from reduced fertility on the maternal side.

Several Asteraceae species exhibit reduced fertility or complete sterility of ray florets (Coen *et al.*, 2002). Sunflower ray florets, for instance, are not fertile (Chapman et al., 2012; Mason et al., 2017), and *Petasites tricholobus*, a cryptically dioecious species, produces sterile

carpels in male florets (Yu *et al.*, 2011). Floret types of *Artemisia annua*, on the other hand, differ only in elongation of the stigma lobes. Ray-floret stigma lobes were elongated compared to disc-floret stigma lobes, and ray florets surprisingly produced more seeds in the two varieties that were tested (Wetzstein *et al.*, 2014). While a *qualitative* reduction in fertility of ray floret female reproductive organs has not been observed in Chrysanthemum, there may be a *quantitative* reduction in fertility of ray florets (Pu *et al.*, 2020). Traditionally, research on Chrysanthemum seed set has been predominantly focused on self-incompatibility, where researchers also encountered fertility problems (Anderson *et al.*, 1989). They performed reciprocal crosses to distinguish self-incompatibility from fertility problems. Researchers attempting interspecific crosses also found reduced fertility. Although they encountered issues with pollen ingrowth, this was most likely a lack of pollen recognition and cellular signalling to guide the pollen tube to the egg cell (Sun *et al.*, 2009).

We hypothesise that the reduced seed set in Chrysanthemum varieties with high ornamental value is due to an increase in the number of ray florets that exhibit reduced fertility of female reproductive organs. Therefore, we performed a comparative morphological study on female reproductive organs of disc and ray florets. Our analysis revealed substantial variation in stigma morphology between florets of different cultivars and a general tendency for lower stigma quality in ray florets. We found that low stigma quality negatively impacted pollen ingrowth independent of floret type. We determined the percentage of disc florets and stigma quality of both floret types for 187 varieties and quantified their effect on seed set in almost 8.5 thousand crosses. Additionally, we identified other factors that contributed to the lack of seed set in 23 percent of the investigated crosses.

Results and discussion

Varieties with less disc florets produce fewer seeds

There is substantial seed set variability in different Chrysanthemum varieties, and it has been suggested that this is linked to their flower type (capitulum structure). To get more insight into the relationship between flower type and seed set, seed set data from an extensive collection of crosses performed at a Dutch breeding company were analysed. To facilitate the analyses, the flower types in the database were grouped into three categories based on the percentage of disc florets (Fig. 1A-C). daisy-type flower heads contain mostly disc florets and only ray florets at the rim of the capitulum (Fig. 1A), half-decorative types have both a large amount of disc and ray florets (Fig. 1B), and a decorative type contains mostly ray florets and only a limited

number of disc florets (Fig. 1C). A positive correlation between the number of disc florets and seed set was found. The highest seed set was reported in the daisy types, followed by half-decorative types, and the lowest seed set was found in decorative flowers (Fig. 1D). To further investigate the connection between the number of disc florets and seed set, a group of 189 Chrysanthemum varieties was phenotyped for the percentage of disc florets (Fig. 1E). In this data set, the percentage of disc florets was also linked to seed set, confirming the correlation between ray floret abundance and seed set discovered earlier. Both disc and ray florets develop female reproductive organs, including pistils, comprising stigma, style and ovary, while ray florets lack the male reproductive organs, the stamen (Fig. 1F). However, the difference in seed set was not due to a lower amount of pollen since hand pollinations were performed with equal amounts of pollen. In conclusion, ray florets produce fewer seeds, and this is likely due to differences in the female reproductive organs. This prompted us to investigate female organ characteristics that influence fertility and focus on differences between disc and ray florets.

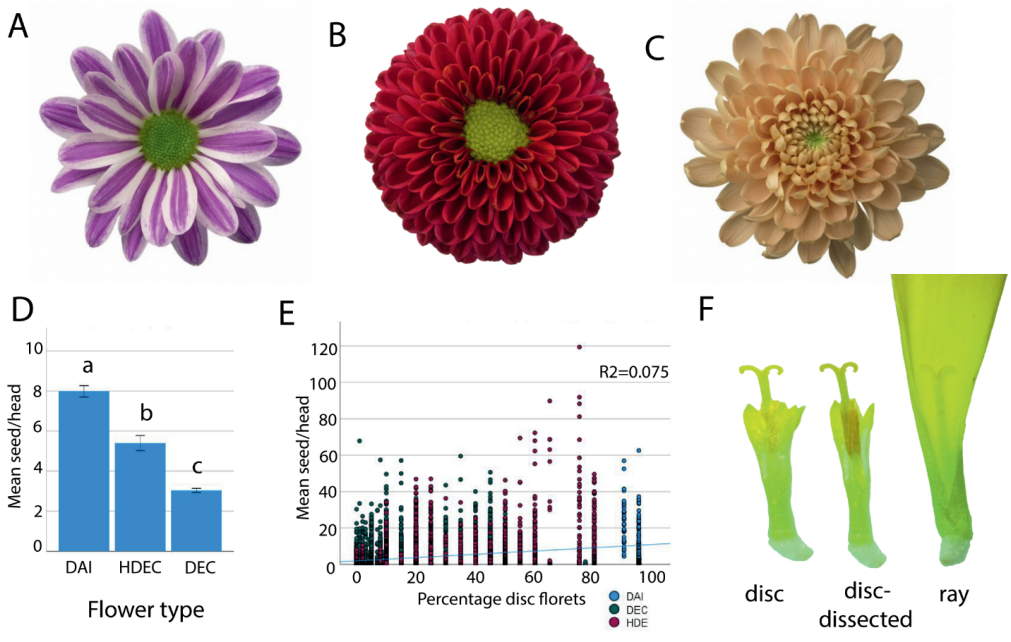

Figure 1. Seed set in different ‘flowertypes’ (inflorescences) with varying amounts of disc and ray florets. (A) Representative example of a daisy-type inflorescence. (B) Representative example of a half-decorative-type inflorescence. (C) Representative example of a decorative-type inflorescence. (D)

Average seed set on a single inflorescence per flower type upon hand pollination. Counts and flower-type abbreviations: daisy (DAI), 7,486 measurements; half-decorative (HDE), 3,086 measurements; decorative (DEC), 17,987 measurements. * Significant difference ANOVA $p < 0.05$. Error bars represent standard error. (E) Scatterplot with average seed set against the percentage of disc florets. The average amount of seed per head was calculated. Per datapoint, the mother flower type is represented in colours, blue is daisy, red is half decorative, and green is decorative type. The linear line estimates the effect of percentage disc florets to seed set over the three flower types. (F) Representative image of a disc and ray floret taken from a daisy type flower. The second disc-floret image has ligules, which are partially removed to display the anthers.

Ray-floret stigmas were generally less developed compared to disc-floret stigmas.

Preliminary observations on the morphology of disc- and ray-floret female reproductive organs revealed a tendency towards underdeveloped stigmas in ray florets. We investigated the morphology of entire pistils, comprising stigma, style and ovary. Although all these reproductive tissues are equally important in successful reproduction, the apparent differences in stigma morphology urged us to investigate this further. There was a high degree of variation in stigma shape in both disc and ray florets (Fig. 2A). Chrysanthemum stigmas are two-lobed, and variations in this stigma shape were mainly observed in the opening of the lobes, which could influence the ability to collect pollen. We investigated the variation in stigma morphology in inflorescences of 189 individuals to determine the difference between disc and ray florets. For each variety, both disc- and ray-floret stigmas were scored in five classes and further categorized as low-, medium- or high-quality stigma (Fig. 2A). This was primarily based on the degree of the opening between the lobes but also on the length and angle of the individual lobes. Some stigmas did not develop sweeping hairs at the tip, and, in the most extreme cases, one or both lobes resembled more a ligule/petal (Fig. 2B). However, most aberrant stigmas displayed reduced opening of the lobes, a phenotype reminiscent of immature stigmas (Fig. 2C-D). The average morphology score of ray-floret stigmas was lower than that of disc-floret stigmas (Fig. 2E), irrespective of variety, showing a link between stigma morphology and floret type. Furthermore, we discovered a link between the percentage of disc florets and the stigma morphology score of disc florets (Fig. 2F). This suggests a connection between the percentage of disc florets, thus flower type, and disc-floret stigma quality that could also cause higher seed set in varieties with more disc florets. Low seed set in varieties with a lower percentage of disc florets could be a result of two factors: low-quality stigmas of

ray florets and/or a generally lower quality of disc-floret stigma in varieties with fewer disc florets.

To further investigate whether the immature-looking stigmas were also underdeveloped for other characteristics, we measured stigma receptivity. The ability of a stigma to germinate pollen (receptivity) is primarily influenced by peroxidase activity on the stigma, which therefore is another measure of stigma functionality (Dupuis and Dumas, 1990; McInnis *et al.*, 2006). We stained the stigmas of mature florets with guaiacol, which detects peroxidase activity as a measure of stigma receptivity. Receptivity was scored in five grades and then categorized as low, medium or high, based on the pattern and degree of staining of the stigma, regardless of stigma shape (Fig. 2G). Also, here, we observed a high degree of variation, indicating that stigma receptivity can differ between florets of different varieties. Contrary to what was seen for morphology, differences in receptivity were not linked to either disc or ray florets (Fig. 2H), nor was there a link with the percentage of disc florets. There was a high correlation between disc and ray floret stigma receptivity within individual capitula (Fig. 2I). This suggests that stigma receptivity is mainly determined by the entire inflorescence structure independent of the floret type. In addition to genetic variation, there is also evidence from other species that stigma receptivity could be influenced by environmental factors such as temperature. Studies showed that heat stress affects pollen fertility and reduces stigma receptivity in peach and cherry (Hedhly, Hormaza and Herrero, 2003, 2005). While environmental conditions might influence stigma quality, the plants used to determine stigma quality were all grown simultaneously, and the measured variation should therefore be mainly attributed to genetic factors. Asynchronous development of stigmatic receptivity has been described for pear flowers; here, immature, receptive and degenerated stigmas co-exist within one flower (Sanzol, Rallo and Herrero, 2003). The authors showed that only the receptive stage could ensure pollen germination, while the other stages lack the necessary hydration or will not allow proper pollen tube growth. This situation is different from the Chrysanthemum, where stigmas from some varieties never become receptive. Moreover, while we measured some variation between different floret stages, the variation within a variety was negligible compared to the variation between varieties, hinting again at a genetic basis for the observed differences.

Subsequently, correlation coefficients were determined based on the entire data set to investigate the extent to which morphology and receptivity are linked to each other and to floret type (Table 1). This analysis further confirmed the correlation between the receptivity of disc and ray florets. However, a low correlation between disc- and ray-floret stigma morphology suggests a limited genetic basis affecting both disc and ray florets for each plant. Additionally,

the correlation between stigma morphology and receptivity was limited for both disc and ray florets.

Taken together, correlation coefficients suggest a limited genetic component that affects stigma morphology and receptivity for both disc and ray florets, complicating the analysis of individual effects. However, there is a strong indication that stigma morphology is more strongly influenced by floret identity, while other factors determine stigma receptivity. The next question is how a difference in morphology affects pollen ingrowth and to what extent this might differ between floret types.

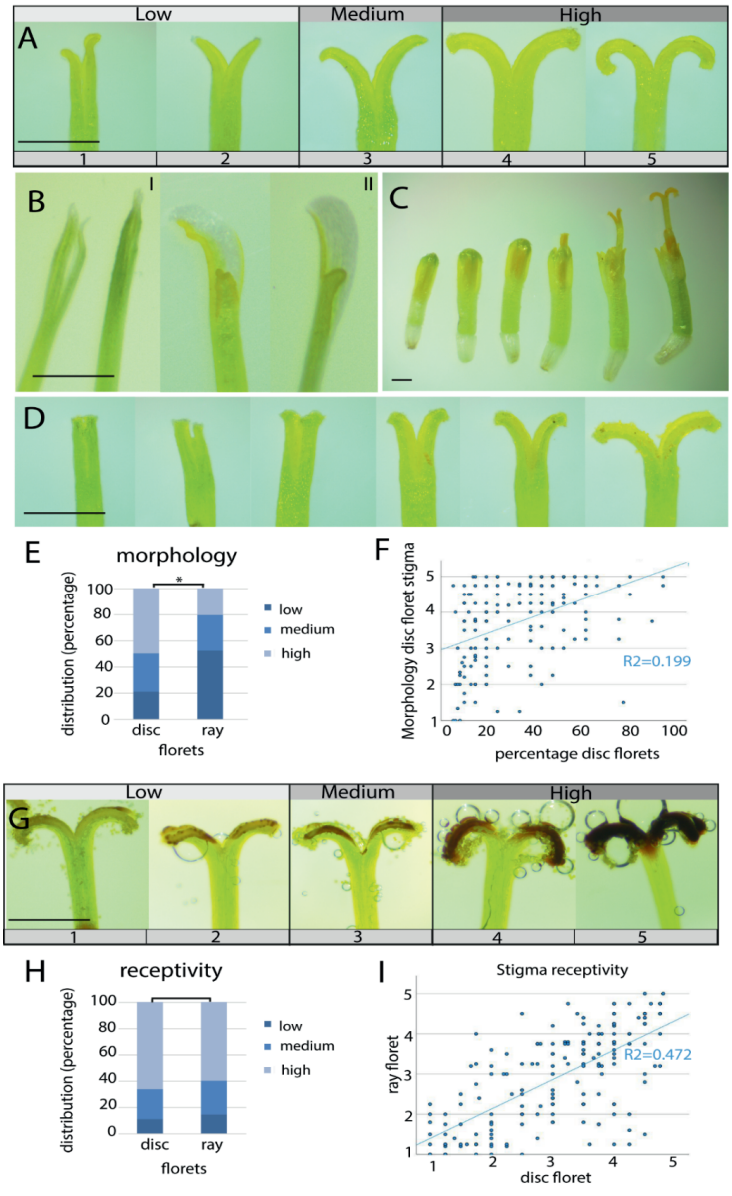

Figure 2. Variation in stigma morphology and receptivity. (A) Stigma morphology is categorized into five defined classes. (B) Stigma with an arm that has partially stigma and partially ligule/petal identity. 'I' is an example of a stigma with bare papillae (yellow tissue), and 'II' are two examples of stigmas with one lobe that seems to exhibit partial ligule identity. (C) Six disc florets were collected from one inflorescence representing various stages of development, with the last being maternally fully matured. (D) These six pistils were dissected from the disc florets in D. (E) Distribution of stigma morphology for disc and ray florets. (F) Distribution of the percentage of disc florets in relation to the stigma

morphology of disc florets. (G) Five grades of stigma receptivity are categorized into three classes (low, medium, high). (H) Distribution of stigma receptivity of disc florets in relation to ray florets. (I) Distribution of stigma receptivity in disc florets in relation to stigma receptivity in ray florets of the same capitulum. Datapoints represent all varieties in the tested panel. (A, B, C, D, G) Bars represent 1mm

Table 1. Correlation coefficients between morphology and receptivity of disc and ray florets and the percentage of disc florets. 2-tailed Pearson correlation * 0.05 and ** 0.01. perc disc= percentage disc florets, disc/ray mp = disc/ray-floret stigma morphology score, disc/ray rcp = disc/ray-floret stigma receptivity score.

	perc disc	disc mp	ray mp	disc rcp	ray rcp
perc disc	1	.446**	-.048	.181*	.137
disc mp	.446**	1	.266**	.373**	.234**
ray mp	-.048	.266**	1	.234**	.219**
disc rcp	.181*	.373**	.234**	1	.687**
ray rcp	.137	.234**	.219**	.687**	1

Pollen ingrowth is affected by stigma quality

To investigate to what extent the high variation in stigma morphology influences seed set, we set out to investigate the effects of differences on pollination and pollen tube germination and found that lower morphology scores negatively impacted pollen tube ingrowth independent of floret types. We expected that varieties with aberrant stigma morphology (a lower degree of opening of the arms) would be less effective in capturing pollen and/or inducing their germination, resulting in less growth into the stigma. We hand-pollinated nine different varieties with varying degrees of stigma morphology scores with a pollen mixture to test this hypothesis. For this purpose, stigmas were rated in three classes as previously (Fig. 2A); low, medium and high quality. We measured the amount of pollen germinating on the stigma 24 hours after pollination and repeated this in total 7-12 times for each variety (Fig. 3A). Pollinations were performed with an equal amount of pollen per inflorescence. Both the amount of pollen still present on the stigma after several washing steps, which indicated pollen interacting with the stigma, and pollen tubes visibly growing into the stigma were measured. The correlation between pollen interacting with the stigma and tubes growing into the stigma was very high (Fig. 3B). If certain factors inhibit pollen tube ingrowth, such as self-incompatibility, these measurements would likely have had a lower correlation. Therefore, both

measurements could be considered reliable for determining the effects of stigma quality on pollination success.

The advantage of analyzing pollen ingrowth compared to seed set is that you can distinguish ingrowth per floret type, which is not feasible for seed set. Overall, the amount of pollen growing into the stigma was constant per variety throughout repeated crosses. The pollen in-growth pattern in relation to stigma morphology showed a positive relationship between high-quality stigma and more pollen ingrowth independent of floret type (Fig. 3C). This is interesting since there is a correlation between stigma quality and floret type (Fig. 2E). Together, the data validate the idea that low seed set in varieties with fewer disc florets results from general lower morphology scores of ray-floret stigma. This small dataset was not extensive enough to distinguish stigma quality and pollen ingrowth effects on seed set due to the complication of a varying percentage of disc florets. Therefore, further research on a larger scale is needed to determine the impact of stigma quality on seed set.

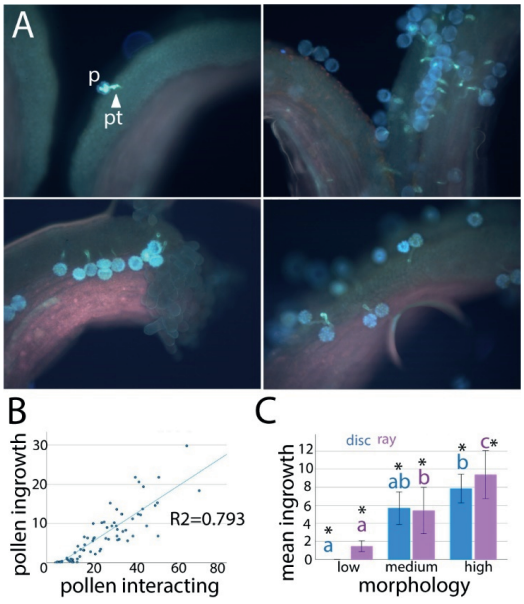

Figure 3. Pollen tube ingrowth is affected by stigma morphology and receptivity. (A) Pollen tube ingrowth in stigmas with aniline blue staining. p= pollen, arrow head with pt=pollen tube. (B) Scatterplot of pollen interacting with stigma vs pollen ingrowth. (C) Average stigma ingrowth for both disc and ray florets per category morphology score. Error bars display the standard error.

The influence of stigma quality on seed set

The previous experiment showed a correlation between stigma quality and pollen tube ingrowth but could not determine the effect of stigma quality on seed set. Therefore, we decided to investigate the impact and interaction of disc floret percentage and stigma quality in a large dataset. The 189 previously phenotyped varieties for percentage disc florets, stigma morphology of both floret types and stigma receptivity were used to determine their influence on seed set in a total number of 8,458 crosses. There was a lot of variation in seed set and this could probably, to a large extent, be explained by environmental conditions during the season. To get an indication of the environmental factors, we checked variation in seed set of the same crosses at different moments during the season, which was 11.2% of the total variance in the entire dataset. This confirmed that, although environmental factors play a significant role in individual seed set, this large dataset is suitable for determining the effect of phenotypical differences caused by genetic differences on seed set.

Disc florets with high-quality scores for stigma morphology had a slightly positive effect on seed set, and as expected, the effect was most pronounced for daisy and half decorative flower types (Fig. 4A) that contain many disc florets. The data for the stigma morphology of ray florets looked very similar, with a slightly positive effect of higher scores on seed set for both daisy and half decorative types (Fig. 4B). This was somewhat unexpected since daisy types contain a limited number of ray florets. decorative type flowers had such low seed set that it was not possible to determine a correlation of stigma morphology of either floret type with seed set. Stigma receptivity exhibited no pronounced effect on seed set (Fig. 4C). These figures suggest that the different stigma parameters we tested have a limited effect on seed set. This is likely due to the high variation caused by other factors affecting seed set.

Some of this variation is caused by crosses not producing seeds. During the analysis, we noticed 23.4% of crosses did not yield any seed and therefore, we focused on this further. Interestingly, the correlation between lower stigma quality and low seed set was apparent for all variables (Fig. 4D-F). The observations for the stigma quality of disc florets on non-producing crosses were as expected: there was a negative correlation between a high stigma morphology score and crosses that did not produce seeds (Fig. 4D). For ray-floret stigma morphology, there were more crosses without seeds in the low-quality category and similar amounts in medium and high (Fig. 4E). While there was no clear effect of the level of receptivity on the variation in seed set (Fig. 4C), there was a pronounced effect in the number of crosses without seeds. Plants scoring low and medium receptivity produced an equal amount of crosses without seeds, but significantly fewer crosses did not produce seeds for mothers with

high receptivity (Fig. 4F). This indicated that the tested variables (stigma morphology and receptivity) might not just influence variation in seed set but could influence whether a cross is successful at all.

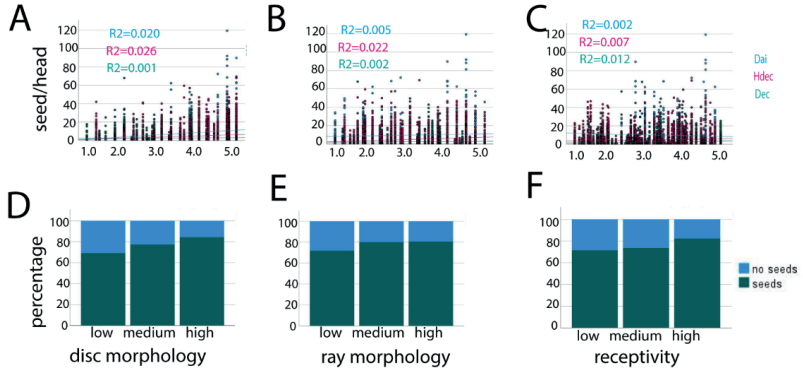

Figure 4. Effect of stigma morphology and receptivity on seed set. (A-C) Scatterplot of stigma quality scores and seed set. Floret type daisy (dai; 55-100% disc florets) N = 721 datapoints, half-deco (hdec; 10-55% disc florets) N = 3770 datapoints, and decorative (dec; 0-10% disc florets) N = 5018 datapoints. (A) Disc-floret morphology, (B) Ray-floret morphology, (C) Receptivity. (D-F) Number of crosses with and without seeds for low, medium or high-quality stigma. Low category 1-2.5, medium category 2.5-3.5 and high is category 3.5-5. (D) Disc-floret morphology, (E) Ray-floret morphology, (F) Receptivity.

The interplay between the percentage of disc florets and stigma quality influences seed set

In order to get a better estimate of the effect of each of our tested factors and the interplay between them, we wanted to test all variables in a model. First, since we discovered that many crosses produced zero seeds, we needed to distinguish which factors may influence the distribution in the number of seeds and which factors determined if crosses were non-productive. Therefore, we tested a zero-inflated model to detect which factors may result in excessive crosses without seeds (Zuur *et al.*, 2009; Bai *et al.*, 2021). The model results are shown in Suppl table 1, with the top (A) showing factors influencing variance in seed set and the bottom (B) showing factors responsible for zero inflation. Interestingly, low stigma receptivity increased the chance of a cross not producing seeds the most. Additionally, low ray-floret stigma quality had a significant effect, while a lower percentage of disc florets had only a limited impact on the occurrence of crosses without seeds (Suppl table 1A). Furthermore, the

remaining variation in seed set was influenced by the percentage of disc florets, disc floret stigma morphology and receptivity (Suppl table 1B). Contrary to what was expected, high stigma receptivity seemed to have a slightly negative effect on seed set, most likely as a consequence of the estimation of the effect on zero inflation. Overall, the analysis indicates a definite overrepresentation of non-producing crosses compared to what was expected based on a normal distribution. The analysis does not consider the interaction between the number of florets and stigma morphology, which is floret dependent.

Thus far, we only observed a limited effect of each variable on seed set (Fig. 1E, 4A-C). However, since these variables are (partly) connected, it is difficult to determine the impact of each without taking the others into account. For instance, a variety with low-quality ray floret stigmas and a high percentage of disc florets with high stigma quality could easily produce many seeds. To test all these variables and correct for interactions they may have, all variables were tested in a model. Both seed set data and residuals were not normally distributed. Residuals represent the deviation from the mean for each data point. When both the data and residuals are not normally distributed, normal statistical models should not be used. Therefore, it was necessary to correct for this by using a model that does not assume a normal distribution. Additionally, since the variance (54.6) of the dataset was higher than the mean (3.9), the data was over-dispersed, and therefore, a negative binomial model was used. The model included the interaction between the percentage of disc florets and both disc- and ray-floret stigma morphology. Notably, it does not consider the over-representation of crosses not producing seeds and uses this as part of the variation.

There was a strong effect of the interaction between these factors in explaining variation in seed set (Table 2). The highest impact contributing to variation was the interaction of the percentage of disc florets with the stigma morphology of ray florets, followed by the interaction with disc-floret stigma morphology. Since a large proportion of the dataset was obtained with varieties with a high number of ray florets, it was not surprising that the interaction with ray-floret stigma morphology had the highest effect on seed set for this analysis. In a different dataset containing more daisy-type flowers, this might be reversed. Additionally, the percentage of disc florets as a sole factor contributed to the variation to a large extent, suggesting other floret-dependent factors might play an additional role. The effect of stigma receptivity in this analysis was significant but less prominent than the other factors.

Overall, the interaction between the number of florets and the stigma morphology of florets further confirms the importance of floret identity on seed set. Additionally, it shows that stigma morphology is an important factor, which is floret dependent and therefore linked to flower

head type (daisy, half-deco or deco). On the other hand, stigma receptivity is not floret dependent and specifically plays a role in crosses not producing seeds. The interplay between these factors is essential in determining reproductive success.

Table 2. General linear model with a negative binomial distribution of floret ratio and stigma quality affecting seed set. This model does not count for zero inflation but shows all observations used in the model and if they influence seed set and to what degree. The dependent Variable is seed per head as an integer. The model includes 'the intercept', 'percentage disc florets', 'disc floret stigma morphology score', 'ray floret stigma morphology score', 'receptivity' and interaction between 'percentage disc florets * disc floret stigma morphology score' and 'percentage disc florets * ray floret stigma morphology score'.

Tests of Model Effects

Source	Type III Wald Chi-Square	Df	Sig.
(Intercept)	155.742	1	.000
percentage_disc	708.470	25	.000
Disc morphology score	96.332	4	.000
Ray morphology score	80.411	4	.000
Receptivity	168.919	8	.000
percentage_disc * Disc morphology score	881.412	25	.000
percentage_disc * Ray morphology score	1215.125	30	.000

Other factors influencing seed set

Although our research pointed toward disc-floret percentage and floret stigma morphology as major reasons for the low seed set in ornamental varieties, it did not explain all variation in seed set observed between varieties. It is also possible that a major problem in crosses with more ray florets is self-incompatibility (Anderson *et al.*, 1988; Wang *et al.*, 2014). Chrysanthemum has been characterized as having sporophytic self-incompatibility (SSI), and this means that fertilization barriers prevent successful self-crosses or crosses with related individuals. As mentioned above, there could be more inbreeding in these varieties. However, Ronald and Ascher discovered in 1975 that garden Chrysanthemum displayed a low degree of pseudo-self-compatibility (PSC). This dominant trait, which is beneficial for reproduction, has probably been fully integrated into the Chrysanthemum germplasm (Ronald and Ascher, 1975). Nonetheless, without active monitoring of the feature, lack of PSC could still be a problem within selective crosses that did not result in seed set. SSI would prevent pollen from growing into the stigma. However, in the nine varieties for which pollen ingrowth was measured, pollen ingrowth was only notably hampered by stigma quality.

We cannot exclude that, in addition to stigma morphology/receptivity, a low seed set could also be a consequence of the poor performance of other reproductive organs, such as the style and ovule. A study that focused on decreasing the generation time of Chrysanthemum found that viable seedling production was increased when embryo rescue was performed (Anderson *et al.*, 1990). This suggests problems with embryo development and could indicate fertility problems related to the development of the ovule and or embryo. Research on early seed abortion of Chrysanthemum interspecific crosses found genes related to programmed cell death and senescence- or death-associated processes involved (Zhang *et al.*, 2014). There are also observations suggesting that ray floret ovules are different compared to disc floret ovules (Cockshull, 2019). While we did not investigate this in detail, there were no obvious differences visible on the outside of the ovules. It might be prudent for varieties with high stigma quality and low seed set to investigate other female reproductive tissues and barriers further.

Breeding against fertility

Several mechanisms could influence stigma development, and there may be a selection on this by breeders because consumers appreciate flowers that remain fresh for an extended period. The quality of Chrysanthemum flowers declines during vase life because of wilting petals and leaves, browning, and disc-floret de-greening (Geest *et al.*, 2016). Many of these processes are related to senescence, which is regulated mainly by ethylene, a hormone that is also very

important in flower maturation and the ability to produce seed (Carbonell-Bejerano *et al.*, 2011; Iqbal *et al.*, 2017). It was found in Carnation that selection against ethylene sensitivity prolonged vase life (Onozaki, Ikeda and Yamaguchi, 2001). Successful selection against ethylene sensitivity in breeding programs could also negatively impact stigma development and quality. It is possible that the selection for these traits is more pronounced in decorative varieties, thereby contributing to the lower fertility of these types.

Recommendations

While other factors may also influence seed set in varieties with more ray florets, we discovered that disc florets produce more seeds than ray florets and that this is at least partially related to their stigma quality. However, there are varieties with more ray florets that form high-quality stigmas and produce more seed. Therefore, it would be very interesting to uncouple these traits to allow breeding for ornamental varieties with better stigma quality. The development of markers for stigma quality could allow Marker Assisted Selection (MAS) to make swift strides. Another possible breeding strategy could be to first focus on breeding for other qualities and introduce a high amount of ray florets at the final stages of breeding. In this scenario, low fertility of these newly created varieties would only be beneficial as it impedes other breeders from taking full advantage of the introduced genetics. Markers for floret identity could be developed to assist in these breeding steps. Focus on stigma receptivity is also important in minimizing the number of crosses that do not produce seed. Additionally, more knowledge on the mechanism that is responsible for the floret ratio could also identify hormones or environmental factors that could temporarily induce more disc florets to get an increased seed set without affecting the genetics of the progeny. Lastly, it would be prudent to test whether ethylene could be used to promote better stigma maturation and facilitate increased seed production.

Material and methods

Dataset for seed set analysis

Varieties were grouped based on flower type regarding the percentage of disc florets. daisy type (DAI) (Fig. 1A); 7,486 daisy and anemone flower type flowers. Both flower types contain mostly disc florets and only limited ray florets on the rim of the capitulum. The difference is the more pronounced elongation of the five fused petals of disc florets in the anemone type (Chapter 1, Fig1C). Half decorative (HDE) (Fig. 1B); 3,086 varieties of half decorative, half pompon and half spider contain an amount of disc and ray florets halfway between the daisy and decorative. The difference between these flower types is in the ray floret ligule, which is more fused in spider varieties and both fused and shorter in pompon types. decorative (DEC) (Fig. 1C); types consist of decorative, pompon, decorative spider types, 17,987 varieties. decorative varieties contain mostly ray florets and limited or no disc florets. The difference in flower types is in the same manner as half-decorative, dependent on the shape of the ray-floret ligule. Since a varying number of flower heads/capitula were used in crosses, data was normalized to seed per head in order to reduce variation.

Stigma morphology and receptivity

Plants were grown for three months in the summer of 2020 in a greenhouse of Dekker Chrysanten in Hensbroek (Netherlands) under short-day conditions. ‘Flowers’ were collected when all florets of the capitulum had open stigmas. The number of disc florets was measured, and 4-5 disc (if present) and ray florets were taken and dissected. This was done for 189 varieties. An image was taken for morphology, and subsequently, florets were incubated with Guaiacol solution (0.1M Guaiacol, 0.1M hydrogen peroxide, 20mM phosphate pH 4.5). After incubation, another image was taken for receptivity. For part of the analysis, stigma morphology and receptivity were divided into low, medium and high quality. Additionally, each stigma was categorized in the five defined classes for both morphology and receptivity, and an average was calculated over the 4-5 stigmas per sample. For the final statistical analysis, an average of disc- and ray-floret stigma receptivity was taken as the receptivity value.

Pollen tube ingrowth

Plants were grown for three months in the fall of 2018 in a greenhouse of Dekker Chrysanten in Hensbroek (Netherlands) under short-day conditions. Pollinations were performed on different days over four weeks with varying plants as father and several times with mixed

pollen samples. For each mother plant pollen ingrowth was determined for 9-12 independent pollinations. The amount of pollen used was always enough to fully cover each inflorescence used with a thin layer of pollen. Crosses were performed between 8:00 and 16:30. Samples were collected 24 hours after pollination and fixed using FAE (Formaldehyde, Acetic acid, Ethanol) solution for 90 minutes, rinsed with water three times and incubated overnight in 1M sodium hydroxide. Samples were rinsed with water again and incubated with aniline blue for 30 minutes. For each cross, several florets were used. The amount of pollen still attached to the stigma after rinsing was counted, as well as the amount that was visibly growing into the stigma. There was a high correlation between the two measurements.

Statistical analysis

IBM SPSS statistics 27 was used for analysis. To distinguish between two groups, a paired T-test was used, while for more groups, ANOVA (Tukey) was used. 'Seed per head' LOG10 transformed data were not normally distributed according to the test of normality (Kolmogorov-Smirnov, default settings), nor were the standardized residuals. Data were skewed. Variance (54.581) was considerably higher than the mean (3.8766). Therefore, a negative binomial distribution was used for the model and not a Poisson distribution. STATS ZEROINFL add-in for SPSS was used working under R package pscl. First ZERO-inflated count models were used to test for factors determining the high number of crosses with zero seeds. Then, the effects' size was estimated using linear regression with negative binomial and default settings. For the model, receptivity of disc and ray floret stigma were pooled together, and the average was calculated per variety because the feature seemed genotype dependent. Number of crosses=10,690 and N-mothers=189. For the analysis, only data with complete sets of phenotypes were used: N=8.458, N mothers=181.

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Supplementary table 1. A general linear regression model with negative binomial distribution testing for zero-inflation and other model effects. General linear model with negative binomial distribution to test for zero inflation in factors influencing seed set in this experiment. Count model coefficients show which factors significantly influence the distribution of the data. Zero-inflation model coefficients explain which factors significantly influence an abundance in crosses without seeds. Negative estimate numbers for percentage of disc- and ray-floret stigma morphology and receptivity define that higher numbers for these values increase the chance of a crossing not producing seeds. Dependent Variable: seed per head as integer.

Count Model Coefficients

	Estimate	Std. Error	z Value	Significance
(Intercept)	.695	.111	6.239	.000
percentage_disc	.011	.001	9.715	.000
Discmorphologyscore	.190	.024	7.995	.000
Raymorphologyscore	.043	.026	1.668	.095
Receptivity	-.078	.026	-3.030	.002
Log(theta)	-.931	.042	-22.090	.000

Zero-Inflation Model Coefficients

	Estimate	Std. Error	z Value	Significance
(Intercept)	2.224	.370	6.019	.000
percentage_disc	-.052	.013	-3.877	.000
Discmorphologyscore	.063	.095	.669	.504
Raymorphologyscore	-.407	.120	-3.392	.001
Receptivity	-.629	.108	-5.838	.000

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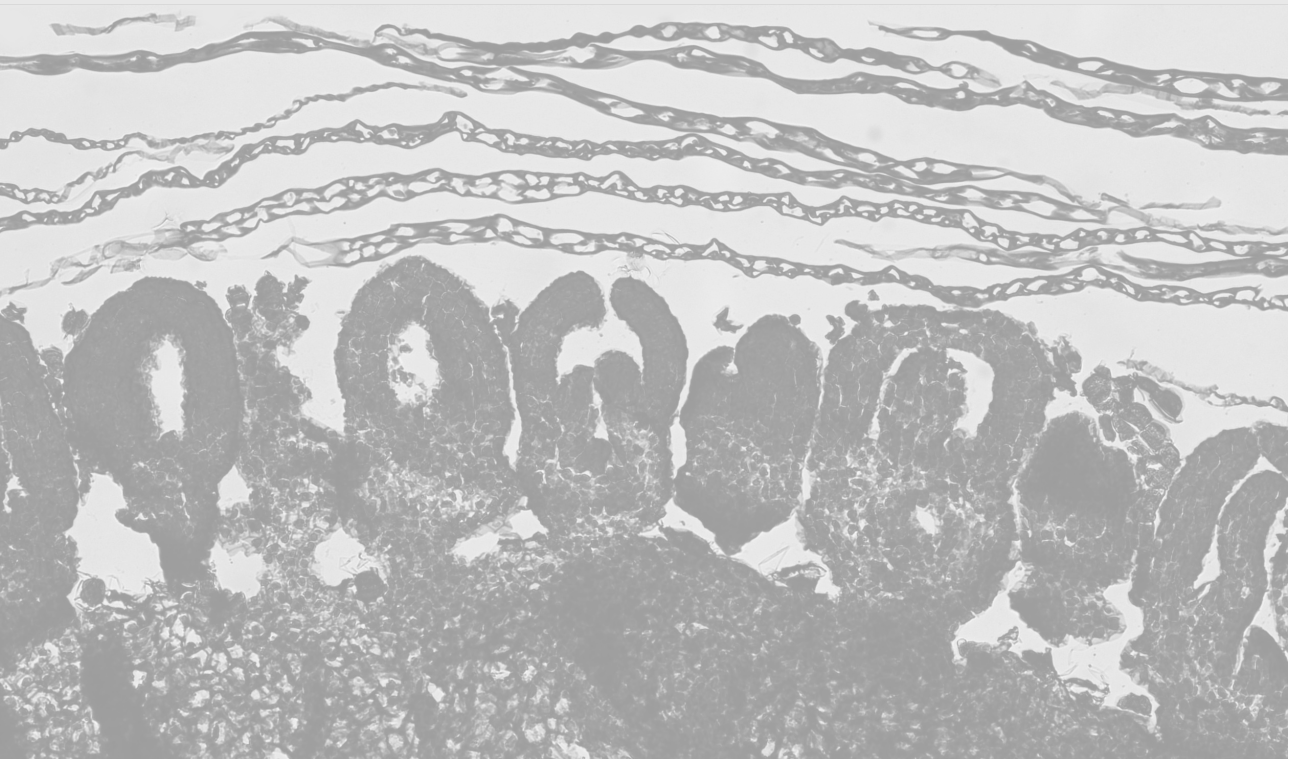

Chapter 3

Identification of genes involved in disc and ray floret identity in *Chrysanthemum morifolium*

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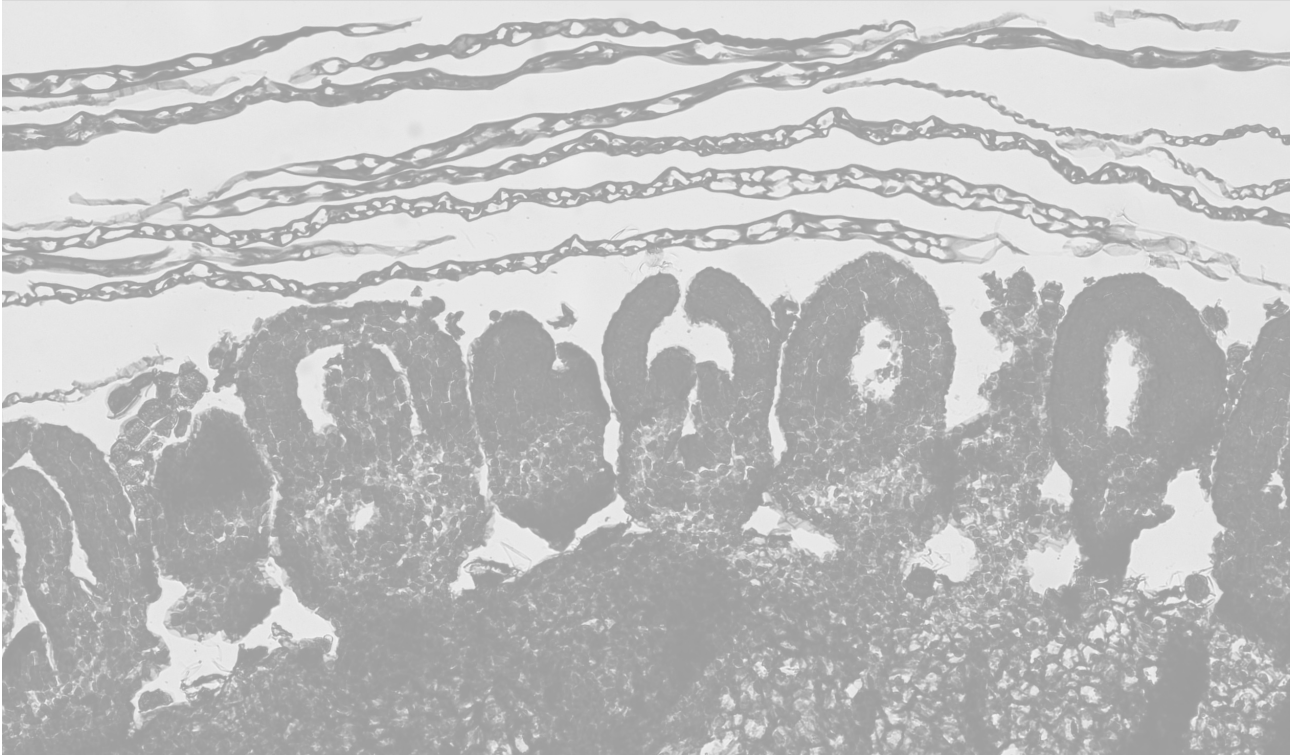


Summary

In *Chrysanthemum morifolium*, the inflorescence phenotype is determined by the ratio and appearance of disc and ray florets. We identified two independent mutants of Chrysanthemum varieties with a substantially increased disc- to ray-floret ratio compared to their wild-type counterparts, indicating a change in floret identity. Microscopic observations and qPCR data indicated the initial stages where floral meristems started differentiating towards disc or ray florets. To comprehensively capture the developmental dynamics and identify genes putatively involved in the regulation of floret identity, the transcriptomes of flower buds in the two stages before differentiation and the two subsequent stages, in which differentiation becomes visible, were analyzed. When datasets for both mutants were combined, 145 differentially expressed genes (DEGs) were upregulated, and 245 DEGs were downregulated in both mutants in at least one of the stages analysed. Although gene ontology (GO) analysis did not reveal differentially regulated pathways that may be linked to floret identity specification, various interesting single DEGs were identified. The sampled later stages contained most DEGs and confirmed several genes expected based on previous research that are most likely involved in downstream processes. For candidate gene selection, we focused on the first two developmental stages. Interestingly, both mutants contained several DEGs related to Brassinosteroid (BR) signalling and several homeodomain transcription factors. 24 DEGs could be confirmed in new samples of the first two stages in at least one of the mutants. We hypothesise that BR signalling, homeodomain transcription factors, and other genes discovered in this analysis are important regulators of floret identity in Chrysanthemum.

Introduction

The mechanism of how Chrysanthemum and other Asteraceae species initiate and specify floret identity is key in elucidating the beauty of these flowers but is not yet well understood. Chrysanthemum, the second most important cut flower in the world, appeals to many people because it has a diverse range of flower shapes and colours, thanks to its composite flowers that are characteristic of the Asteraceae family. A composite flower head resembles a typical flower but is actually an inflorescence structure where many different florets are packed together on a capitulum surrounded by involucral bracts (Harris, 1995; Gillies, 2002). Depending on the species, the Asteraceae capitulum may contain only disc florets, ray florets, or both. Disc florets are hermaphroditic actinomorphic florets that first produce pollen and later develop a functioning pistil. Ray florets are zygomorphic florets with showy petals and generally lack stamen and pollen. Originally, Chrysanthemum varieties had flowers with many

CYC and *RAD* expression (Maria Pereira Garcês, R Spencer and Kim, 2016). Chrysanthemum *CYC2* seems to play a different role, and overexpression of two *CYC2* genes, *CYC2c* and *CYC2d,* only has minor effects on the outgrowth of organs (Huang *et al.*, 2016; Chen *et al.*, 2018). *CYC2c* has a positive effect on ligule length in both disc and ray florets (Huang *et al.*, 2016), and *CYC2d* has a negative effect on ray-floret length (Chen *et al.*, 2018). Nevertheless, the CYC2 proteins in Chrysanthemum form heterodimers with the A-class gene products ClAP1 and ClAP2, indicating their role in the identity of flower development (Wen *et al.*, 2019). However, recent studies comparing Chrysanthemum and *Ajania* species showed that malfunction of *CYC2g* in *Ajania* led to the disruption of outgrowth of ligules in ray florets, while stamen abortion was maintained, indicating that *CYC2g* alone is insufficient to change the identity to disc floret completely (Shen *et al.*, 2021). Thus, *CYC2c*, *CYC2d,* and *CYC2g* alone are not sufficient to specify the difference in identity between disc and ray floret. Furthermore, it is still unclear whether other *CYC* genes, like *RAD* or *DIV,* function in the specification of Chrysanthemum floret identity. Therefore, we propose there are still other unknown factors involved in floret identity.

To investigate the mechanisms responsible for floret identity, we have identified two mutants of different varieties with more disc florets than the corresponding normal variety. We collected RNA samples of the flower buds in early developmental stages and performed RNAseq analysis to identify differentially expressed genes (DEGs) between variety and mutant. We then selected DEGs that showed a similar trend in both mutants. As a result, several interesting candidate genes were identified, including many genes related to Brassinosteroid (BR) signaling and several homeodomain transcription factors. Interestingly, *CYC*, *RAD* and *DIV* were only differentially expressed in later developmental stages. The identified differentially expressed genes are important candidates to elucidate the mechanism of floret identity specification in Chrysanthemum.

Results

Identification of flower mutants with increased disc- to ray floret ratio.

Two mutants with an increased disc- to ray-floret ratio were identified, one as a spontaneous mutant and the second from a mutagenesis screen as a result of radiation. These mutants contain more disc florets than their corresponding genetic background varieties. Variety 1 (V1; Fig. 1A) has a white decorative flower with mostly showy ray florets and only a limited number of disc florets in the middle of the capitulum (Fig. 1C). Its spontaneous mutant (M1; Fig. 1B) is

yellow and has considerably fewer ray florets and more disc florets (Fig. 1D). Variety 2 (V2; Fig. 1E) has green flowers and, like V1, has a decorative flower type with many ray florets (Fig. 1G). Mutant 2 (M2) was obtained in a mutant screen after x-ray treatment of cuttings of V2 with 17.5 Gy and also displayed fewer ray florets and more disc florets than the normal variety (Fig. 1F, 1H). To calculate the ratio disc:ray florets, capitula of seven plants for each variety and mutant were phenotyped (Table 1). The disc-floret percentage increased from 22.9 in V1 to 59.5 in M1, changing the disc/ray floret ratio from 0.3 (V1) to 1.47 (M1). For V2, the percentage of disc florets was 6.4, increasing to 33.4 in M2 with a disc/ray-floret ratio of 0.07 in V2 and 0.5 in M2. While the percentual difference between V2 and M2 was lower, the times it was increased (5.2x) was considerably higher than for M1 vs V1 (2.6x). The total number of florets was also different for the varieties and mutants (Fig. 1J). V1 had an average of 377.9 florets, M1 403.4, V2 315.0 and M2 had an average of 357.1 total florets. This is a slight increase, which is significant for M2. However, the significance of the ratio difference is much higher, confirming that the ratio difference is more likely a result of identity change. The slight increase in the number of florets in M2 could be because disc florets are smaller than ray florets and require less space on the capitulum. Altogether, the data show that the flower mutants had a substantial increase in the number of disc florets and a decrease in the number of ray florets suggesting a change of identity for a portion of the capitulum.

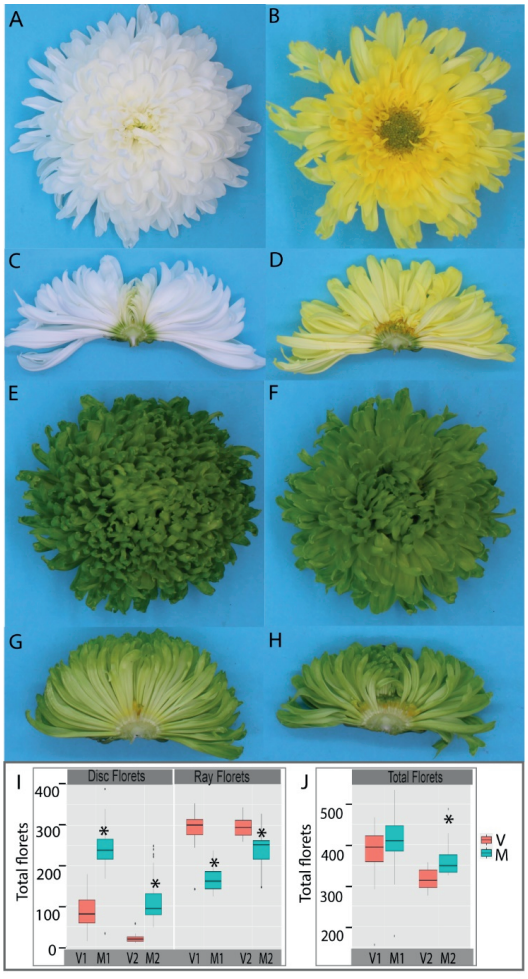


Figure 1. Phenotypic characterisation of the selected varieties and corresponding mutants. Images of V1, M1, V2 and M2 inflorescences and cross sections of the entire inflorescences. (A, B, E and F) Inflorescences pictures. (C, D, G and H) Cross sections of inflorescences. (A and C) V1 inflorescence with many ray florets with white ligules. (B and D) M1 with fewer ray florets with yellow ligules. (E and G) V2 with many ray florets with green ligules. (F and H) M2 with fewer ray florets with green ligules. (I) Boxplots showing differences in number of disc and ray florets between varieties and corresponding mutants. (J) Boxplots showing the total amount of florets for both mutants and varieties.

Table 1. Average number of disc and ray florets for V1, M1, V2 and M2. The percentage disc florets and ratio of disc to ray florets was calculated in the last columns.

	Disc florets	Ray florets	total florets	% Disc florets	Ratio disc/ray
V1	86.7	291.2	377.9	22.9	0.30
M1	239.9	163.5	403.4	59.5	1.47
V2	20.1	294.9	315	6.4	0.07
M2	119.3	237.9	357.1	33.4	0.50

Ray- and disc-floret primordia start to differentiate from developmental stage 2 onwards. To get insight into the mechanism responsible for the determination of floret identity, we first investigated the stage at which floret identity is established, using both microscopic observations and, at a molecular level, using qPCR. Developing flower buds of different developmental stages were dissected under the microscope and imaged (Fig. 2A). At stage 0, the inflorescence meristem (IM) was visible, and the meristem was smooth (Fig. 2B). At stage 1, part of the IM was replaced by developing floret primordia at the outer rim of the capitulum (Fig. 2C). At stage 2, the IM was wholly consumed by floret primordia (Fig. 2D). At this stage, the floret primordia were differentiating, and a difference between ray and disc florets became visible, as seen in the enlargement in (Fig. 2H). At stage 3, floret identity became more apparent, but ray florets seemed to be differentiating slower than disc florets even though these are located more at the center (Fig. 2E and Fig. 2I). At stage 4, all florets had developed further, and florets were easily distinguishable (Fig. 2F). Finally, at stage 5, the inflorescence was almost fully developed, and the ligules started to colour (Fig. 2G).

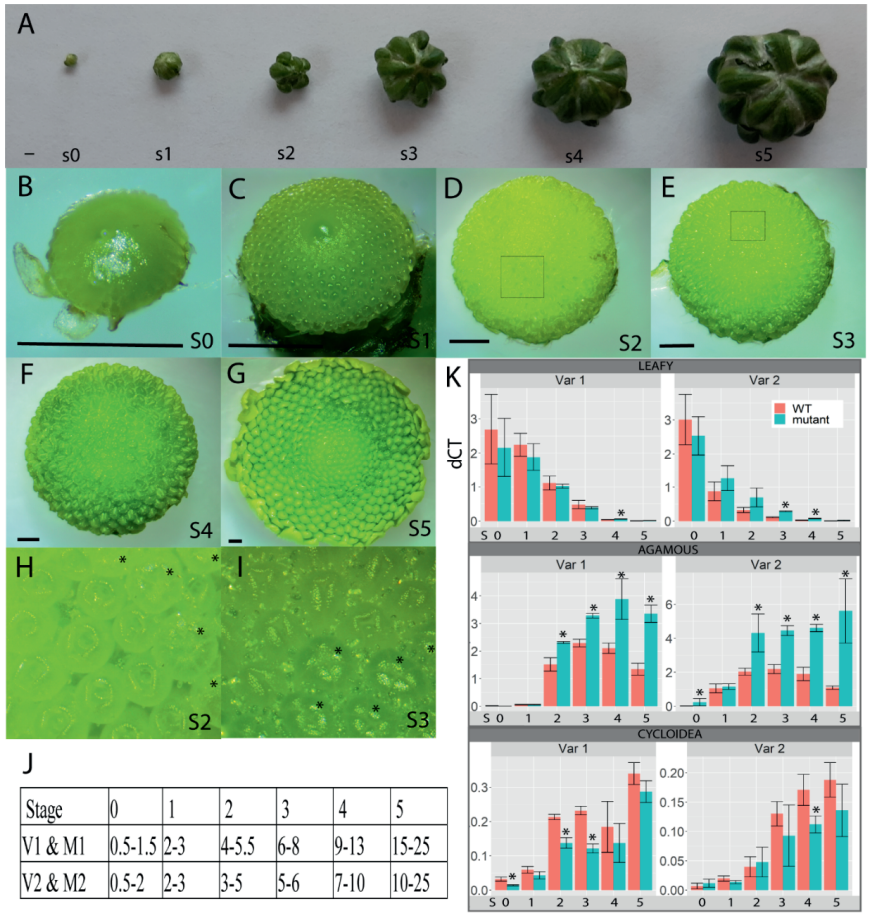
We used qPCR with three flower-related markers to validate the developmental stages at a molecular level. *LEAFY* (*LFY*) is an essential gene involved in inflorescence development that is expressed during early floral developmental stages when the floral meristem has not differentiated yet. In Chrysanthemum, *LFY* was lowly expressed in vegetative meristem tissue, and expression increased upon floral initiation (Liu *et al.*, 2015). Research in *Gerbera* showed that *LFY* is the master regulator of flower meristem identity and is primarily expressed in the undifferentiated inflorescence meristem dome. However, it is expressed in both involucral bracts and emerging floret primordia (Zhao *et al.*, 2016). As expected, *LFY* was highest expressed at stage 0, and expression decreased during development in both varieties and mutants (Fig. 2K). However, there was a significantly higher expression in both mutants at later stages; this is contradictory to the data from *Gerbera* since the mutants have fewer ray

florets. Nevertheless, because of the high *LFY* expression at earlier stages, it does confirm that the earlier stages represent inflorescence with developing floral meristems.

AGAMOUS (*AG*) is a gene encoding a C-class MADS-box transcription factor responsible for stamen and carpel development and starts to be expressed at the onset of reproductive organ primordia formation in the model species Arabidopsis (Shchennikova *et al.*, 2004). It is expressed higher in disc florets because these contain both anthers and carpels, whereas ray florets only develop a carpel (suppl fig 1A) (Aida *et al.*, 2008). As expected, qPCR confirmed that the mutants, which develop more disc florets, showed higher *AG* expression. In V1/M1, there was no detectable expression at stages 0 and 1. The expression was high in stages 2-5, with a significantly higher expression in M1 for all these stages. In V2/M2, *AG* is expressed earlier in development. The expression could weakly be detected at stage 0 of M2 and increased for both M2 and V2 during development, with a higher expression for M2 in all stages, except for stage 1. *AG* expression is differential from stage 2 onward, indicating that the florets are developing differently from this stage onwards. The high expression at stage 2 further indicates that floral organ primordia start to initiate (see Fig. 2D, H).

CYCLOIDEA (*CYC*) is a TCP transcription factor responsible for the outgrowth of tissues. Studies in Chrysanthemum have shown that *CYC2c* expression is higher in ray florets because it is particularly important for ligule growth (Huang *et al.*, 2016; Yang *et al.*, 2019). We tested the *CYC2a-f* variants mentioned in these papers and found similar results (Suppl fig B-G) and decided to use *CYC2c* as an indicator. As expected, *CYC2c* had higher expression in the later stages of the non-mutated varieties, as these developed more ray florets. In both varieties and mutants, the expression of *CYC2c* was still low in developmental stages 0 and 1. The expression increased from stage 2 onwards in V1/M1 and stage 3 in V2/M2. Although *CYC2c* expression seemed higher in both non-mutated varieties compared to their mutants, this difference was only significant for M1 stages 0, 2, and 4 and M2 stage 4. Apart from M1 stage 0, expression was only differential at later stages when floret organ primordia are already becoming visible, confirming the role in the outgrowth of ligules. Similar to *AG*, expression increased from stage 2 onwards, further confirming this stage as the first at which disc and ray florets develop differentially.

In conclusion, the microscopic observations and qPCR data of *AG* and *CYC2c* showed that disc and ray florets started to develop differently from stage 2 onwards.



Stage	0	1	2	3	4	5
V1 & M1	0.5-1.5	2-3	4-5.5	6-8	9-13	15-25
V2 & M2	0.5-2	2-3	3-5	5-6	7-10	10-25

Figure 2. Developing flower buds of V1 showing differentiation of florets starting at stage 2 (S2). (A) Flower buds from all six stages. (B) Dissected stage 0 inflorescence without clear development of separate floret primordia. (C) Dissected stage 1 inflorescence with capitula partly covered with developing floret primordia. (D) Dissected stage 2 inflorescence with the capitula completely covered with floret primordia, some of which are showing distinct morphological traits related to disc or ray florets. (E) Dissected stage 3 inflorescence with disc and ray florets developing more distinctly. (F) Dissected stage 4 inflorescence with clearly distinguishable disc and ray florets for the entire capitulum. (G) Dissected stage 5 inflorescence that is ready to flower, ligules of the outermost ray florets have increased in size. (H) Enlarged section of (D) stage 2 inflorescence. (I) Enlarged section of (E) stage 3 inflorescence. Asterisks in (I) and (H) indicate disc florets, while ray florets are not marked. Disc florets are radially symmetrical, while ray florets are zygomorphically symmetrical. (A-G) Size bar is 1 mm. (J) Table of size distribution per stage of samples collected for analysis. (K) QPCR results of *LFY*, *AG* and *CYC2c* in 6 flower developmental stages of both varieties and mutants. Significance, T-test $p<0.05$ is indicated with an asterisk.

Generation of a high-depth early-stage inflorescence-specific transcriptome for mapping and quantification.

For transcriptome analysis, flower buds of developing inflorescence structures from the first four stages of development were used from both the normal varieties and their mutants. We decided to use stages 0 and 1 because these represent developmental stages before visible floret differentiation starts and most likely express the gene(s) responsible for floret identity differentiation. Furthermore, we included stages 2 and 3 since these contain differentiating florets and thus most likely include many downstream processes that could help to elucidate the processes involved.

The reference transcriptome was developed based on all sequence reads from V1 using Trinity de novo transcriptome assembly. This transcriptome assembly resulted in a reference collection of 746,130 isoforms, representing 251,805 putative genes used for further analysis. Blast was used to annotate the Chrysanthemum sequences based on their homology to Arabidopsis and sunflower genes. Arabidopsis was used because it has the best-annotated genome and sunflower since it is one of the closest relatives of Chrysanthemum for which a genome sequence is available. We were able to annotate 62 percent of the genes expressed in our samples. Analysis of several individual sequences for which homologs could not be identified in the initial analysis were identified through individual blast against all sequences in the NCBI database. For the most part, the remaining unidentified sequences were shorter sequences and most likely incomplete sequences or sequences that were not assembled correctly. To validate if low abundance transcripts were represented in the reference, we selected several important flower meristem genes, which are only expressed in specific cells for a fleeting time and could potentially be important to our study. We discovered a Chrysanthemum homolog in the reference transcriptome for *WUSCHEL, BRC1/TCP18, CLAVATA 3*, *LFY*, *SPL8* and *JAGGED*. Of the selected genes, only *NUBBIN (NUB)* could not be identified, which indicates that some weakly expressed genes may still be lacking in the reference transcriptome. However, it is also possible that *NUB* is not present in the Chrysanthemum genome or that the *NUB* orthologs lack the required sequence homology level. Although the generated transcriptome reference contains more than the expected number of genes, it is very suitable for this analysis because of the tissue specificity, meaning that it also contains most of the expected low abundance genes. A part of the transcriptome reference contains alleles rather than separate genes.

Differential expression analysis.

After establishing the reference transcriptome, the reads from all individual samples were mapped. Read mapping and quantification were performed using RSEM and Bowtie2 with an average mapping percentage of 70.2%, resulting in an average of 29.3 million mapped read pairs per sample. Differential expression (DE) analysis was performed separately for both mutants and all stages. First, genes differentially expressed between M1 and V1 with an adjusted P-value <0.01 were identified for each stage. Then, the same process was repeated for M2 compared to V2. This resulted in many differentially expressed (DE) genes for each stage of development (Table 2).

For M1 versus V1, a total of 641 differentially expressed genes (DEGs) were identified that showed higher expression in the mutant and 1616 genes with a lower expression. M2 versus V2 revealed more DEGs, of which 5066 had a higher expression in the mutant and 7065 a lower expression. The high number of DEGs reflects the nature of both mutants, with the genome of M2 likely containing numerous mutations as a result of gamma radiation. The quality of RNAseq data and the analysis were validated by qPCR. Seven DEGs were selected, with a variance in expression level and all seven genes showed the same expression pattern, confirming the reliability of the transcriptome analysis.

While the fact that in the M1 mutant ligule colour changed from white to yellow is not of direct interest to this study, we noticed in our transcriptome analysis that *CAROTENOID CLEAVAGE DIOXYGENASE 4a* (*CCD4a*), a gene known to be responsible for conversion of white Chrysanthemum to yellow (Ohmiya, Sumitomo and Aida, 2009), is accordingly differentially expressed. This further confirmed the reliability of the analysis.

Both mutants show many differentially expressed genes, most of which are unrelated to the floret ratio. Genes overlapping in both mutants are more likely to be involved in the floret ratio (Table 2). For the combined datasets, 145 DEGs were expressed higher, and 245 were expressed lower in both mutants for at least one stage (Table 2). Gene ontology (GO) analysis of DEGs was used to get insight into pathways that were differentially regulated in the mutants compared to the normal variety using the Arabidopsis gene identifiers. It was not possible to perform a GO analysis for overlapping DEGs per stage because of their limited number. Therefore, the analysis was performed for all stages together (Fig. 3C). Overall, the GO analysis revealed the category ‘Metabolic process’ to be most prevalent. Furthermore, reproductive development GO terms were enriched. Further inspection of the data indicated this reproductive development was only enriched in stages 2 and 3 but lacking in stages 0 and

1. This gave only limited additional insight into the mechanisms that might be responsible for floret identity.

Interestingly, most DEGs were differential in more than one stage (Fig. 3A, 3B). Three DEGs were expressed higher, and ten were expressed lower in all mutant stages. Only a few DEGs were unique for stage 0, and stage 2 had the highest number of unique DEGs. The number of DEGs increased from stage 2 onwards, which is consistent with the phenotypic observations that disc- and ray florets develop differently from stage 2 onwards.

Based on previous studies about floret identity in Chrysanthemum and other Asteraceae species, we expected to find *CYC2c* or its partners *RAY* and *DIV* to be differentially expressed in the mutants. *CYC* is suggested to play a key role in Chrysanthemum ray-floret development, and its expression is indeed higher in ray florets (Huang *et al.*, 2016). *CYC2c* qPCR of stages 0 to 5 confirmed that this gene had lower expression in mutants' stages 0, 2, and 3 for M1 and stage 4 for M2 (Fig. 2K). Since the RNAseq data is based on the de novo assembled transcriptome reference and annotated based on Arabidopsis and sunflower, related genes can be combined and named differently depending on blast parameters. As a result, the RNAseq showed only significant downregulation of *CYC2d* in stage 2 of M2 (Fig. 3D). In addition, two variants of the CYC target *RADIALIS* (*RAD*) showed lower expression in M2, one in stage 2 and the other in stage 3 (Fig. 3D). *DIV* was also downregulated, specifically in M2 at stage 2. This was unexpected because *DIV* is known to counteract *CYC* and *RAD* in *Antirrhinum* and *Senecio* (Corley *et al.*, 2005; Garcês, Spencer and Kim, 2016). According to the transcriptome analysis, none of these genes was significantly differentially expressed in M1. Taken together, these data show that *CYC*, *RAD* and *DIV* are only differentially expressed in later stages of M2, suggesting that these genes are involved in floret organ development rather than floret identity.

In the absence of a clear indication for certain enriched developmental or hormonal pathways and with a *CYC2* variant only differential in later stages of the RNAseq samples, we decided to take a closer look at the DEG list.

Table 2. Summary of differentially expressed higher and lower expressed genes per stage. *M = mutant, V = variety and vs =versus. Higher = higher in the mutant.

comparison	direction	Stage 0	Stage 1	Stage 2	Stage 3	total
M1 vs V1*	Higher	210	153	348	161	641
M1 vs V1*	Lower	677	879	975	820	1616
M2 vs V2*	Higher	589	1295	2638	3015	5066
M2 vs V2*	Lower	1270	2182	4524	4413	7065
overlap	Higher	16	14	105	47	145
overlap	Lower	29	52	165	116	245

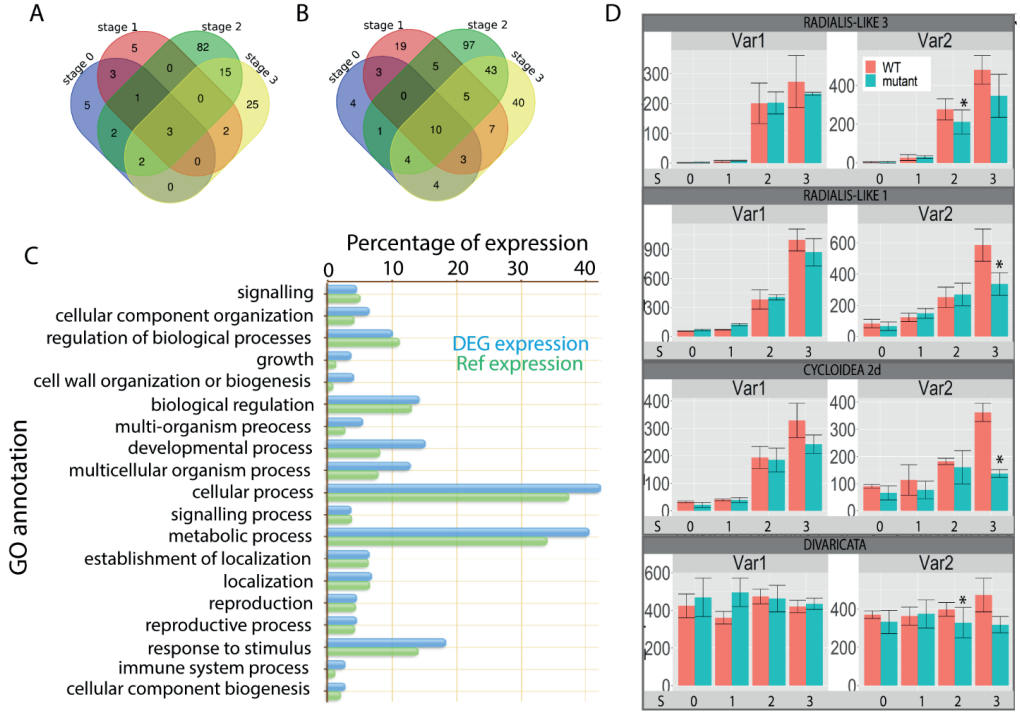


Figure 3. Differential expression analysis. Venn diagram of DEGs upregulated (A) and downregulated (B) for both V1 versus M1 and V2 versus M2 per developmental stage. (C) GO-term analysis of biological functions that were significantly enriched. Figure based on DEGs in common between both M1 versus V1 and M2 versus V2. Blue represents DEGs and green represents the customised reference based on all expressed genes in this specific dataset. (D) Read counts (RPKM) of *RAD, CYC* and *DIV* genes from the four stages. 'S' with numbers at the bottom of each graph represent the stages. Red

indicates both V1 and V2, and blue indicates both M1 and M2. Asterisks show significance between mutant and variety per stage, RNAseq Padj <0.01.

Candidate selection reveals many interesting genes that could be responsible for floret identity.

From the 390 overlapping DEGs for both mutants, a selection was made for further investigation. 268 were annotated based on Arabidopsis, and an additional 14 annotations could be added using sunflower. We hypothesised that genes responsible for floret identity were expressed in stages 0 and 1 and that stages 2 and 3 are more indicative of the downstream processes involved. Therefore, we focused on genes differentially expressed in both mutants in stages 0 and 1 (Table 3, category 1). Several genes were selected based on their clear differential expression pattern in both mutants. These genes were differential in at least stage 0 or 1 and had a minimum average read count of 100 (read count) and a minimum sequence length of 600 base pairs. Several of these genes were not identified through blast analysis with Arabidopsis or sunflower; however, a homolog could be identified directly through the NCBI database for most genes. The NCBI database search included sequence information from various plant species.

In addition, homologs of Arabidopsis genes that are known to be involved in flower development or tissue specification were selected for further analysis, also if they were only differential in one of the mutants or in later stages. In the composed list, we focused on transcription factors and genes involved in hormonal responses as these are more likely to influence the development and thereby to regulate floret identity (Table 3 category 2). One of the problems encountered during the analysis was that several Chrysanthemum DEGs shared the same closest homolog in Arabidopsis. Thus, the annotation was the same, but the underlying Chrysanthemum gene seemed different in both mutants. Therefore, we cannot exclude the possibility that we are dealing with different alleles of the same gene rather than different genes being co-orthologs of one Arabidopsis gene. To tackle this problem, the selection of candidate genes present in both DEG lists was based on the closest homolog in Arabidopsis instead of on the Chrysanthemum gene identifier (Table 3, category 3). In addition to *ANL2* and *GIR1* (adaptor protein), which were present in the overlapping list, several homeodomain transcription factors of class IV (HD-ZIP IV) were specifically differential in M1. Furthermore, although most were specifically differential in one mutant, there appeared to be a high number of DEGs related to Brassinosteroid (BR) signalling for both mutants, especially for M2. Thus, both HD-ZIP IV and BR could be distinct factors that induce the same

phenotypic changes in floret ratio for both mutants in a different manner. Therefore, several of these genes were included for further analysis even if they were not differentially expressed in both mutants. Forty-two genes were selected for further analysis to determine if any of these are consistently differentially expressed between the mutants and their variety (Table 3). These selected genes were further investigated by qPCR on new independent tissue samples.

Table 3. RNAseq data of DEGs selected for further investigation. The table is sorted based on the Arabidopsis reference code (column 2). Positive numbers (green/yellow) represent lower expression in the mutant, and negative numbers (red) represent higher expression in the mutant than in the wild-type variety. The selection column shows the category based on which DEGs were selected. Category 1 is based on expression pattern, category 2 is based on what is known from literature, mainly hormone-related or transcription factor-encoding genes, and category 3 contains differentially expressed transcripts which occur only in one mutant, but are very similar to another transcript that is differentially expressed in the other mutant, and are interesting based on literature and expression pattern. M1 = mutant 1, M2 = mutant 2.

Transcript	Gene identifier	Gene	Selection	LOG2foldchange							
				Stage 0		Stage 1		Stage 2		Stage 3	
				M1	M2	M1	M2	M1	M2	M1	M2
57215_c1_g2_i15	AT1G06740.1	MUG3	3	1.9		1.6		1.7		1.9	
11285_c0_g1_i1	AT1G06740.1	MUG3	3		6.0		4.6		5.0		7.0
47283_c0_g1_i1	AT1G1444.2	HB31	2					-0.7	-1.0		
60262_c0_g2_i1	AT1G1536.2	SHINE1	2					0.6	0.5	0.9	
38328_c0_g4_i1	AT1G1788.1	BTF3	2			0.7		0.4	0.5		
43844_c0_g1_i3	AT1G2558.1	SOG1	2	-2.8	-2.2						
54326_c0_g2_i3	AT1G2737.6	SPL1	2			0.9		1.3	0.8	0.8	
51002_c0_g2_i3	AT1G6371.1	CYP86A	2					0.7	0.5	0.6	0.3
52512_c0_g2_i1	AT1G6545.1	GLC	2			0.6		0.8	0.4	1.0	0.4
40035_c2_g3_i4	AT1G6848.1	JAGGED	2					-0.9	-0.7	-1.1	-0.6
41145_c0_g1_i1	AT1G7297.1	HOTHEAD	2					0.5	0.5		0.5
52540_c2_g2_i2	AT1G7524.1	HB33	2					-0.5	-0.7		
44279_c1_g2_i1	AT1G779.1	HTB1	2		1.0	0.5	0.6		0.6		0.3
61085_c8_g1_i2	AT2G2166.2	GRP7	2				0.4	0.4	0.4		0.4
36484_c0_g1_i2	AT2G2266.1	GRDP1	1	1.1		0.9	1.3	0.8		0.6	
57996_c2_g1_i5	AT2G2298.2	SCPL13	1	0.7	1.3		0.7	1.0	0.7	0.9	0.8
41694_c0_g1_i2	AT2G27385.2	Pollen Ole e	1	1.1	1.1	1.0	0.7	1.3	0.8	1.2	0.6
45086_c0_g4_i1	AT2G45400.1	BEN1c	3	-0.8							-0.4
45086_c0_g1_i7	AT2G45400.1	BEN1b	3	2.2		2.1	-0.5	2.5		2.8	

42938_c3_g1_i1	AT2G45400.3	BEN1a	3			-0.9		-0.6			
61248_c4_g6_i1	AT3G05850.1	MUG7	2	-1.0	-0.9	-0.9	-0.7	-1.1	-1.6	-1.2	-1.5
34877_c0_g1_i1	AT3G1171.1	ATKRS-1	1	-1.3	-2.0	-1.4	-1.8	-0.8	-1.5	-1.1	-1.5
39380_c0_g2_i1	AT3G1223.2	SCL17	1	0.6	1.5		0.9	0.8		0.9	0.8
96938_c0_g1_i1	AT3G19820.3	DWF1a	3	5.4		5.8					
45082_c0_g2_i4	AT3G19820.3	DWF1b	3						0.9		0.7
52920_c0_g1_i4	AT3G46290.1	HERK1	3	3.8		3.3		1.3			
68032_c0_g1_i2	AT3G46290.1	HERK1	3		4.0		5.5		4.2		4.4
52295_c0_g2_i1	AT3G5767.1	WIP2	2			0.8		0.7	0.8		0.7
62803_c1_g6_i1	AT3G61150	HDG1	2	0.7		0.7		0.8		0.9	
49251_c0_g2_i2	AT3G622.1	ATL4	1	1.8		1.8	0.9	2.0	1.5		1.1
65126_c1_g2_i2	AT4G04890.2	PDF2	3	1.1		1.2		1.1		1.0	
65126_c1_g1_i5	AT4G04890.2	PDF2	3								0.4
32648_c0_g1_i1	AT4G04890.2	PDF2	3				0.6				
65147_c1_g4_i2	AT4G13195.1	CLE44	2				0.8	-0.6	-1.0		
63588_c1_g2_i11	AT4G2999.1	LRR kinase	1	-1.0	-1.2		-1.4	-0.8	-1.4	-1.2	
56697_c1_g10_i1	AT4G315.1	CYP83	2					0.6	0.7	0.7	0.7
62574_c2_g1_i3	AT4G3781.1	ATEPFL2	2					-0.6	-0.6		-0.6
62380_c1_g1_i6	AT5G04700	ank rep fam	1	1.2		1.3	0.8	1.5	0.6	1.2	0.8
50834_c0_g1_i2	AT5G1159.1	TINY2	2				0.7	0.7	1.0	0.8	1.1
63627_c1_g1_i1	AT5G1699.1	unknown	1	1.8		1.9		1.3	0.4	1.1	
48265_c2_g4_i1	AT5G2561.1	RD22	1	1.0		1.0	0.4	1.2	0.6	1.1	0.6
66874_c6_g7_i1	AT5G39.1	MEDOS2	2	6.3		5.5		4.9	6.3	6.7	6.2
66874_c5_g1_i1	AT5G6135.1	ERULUS/ CAP1	2	1.3	6.6	1.5	7.1	1.7	7.5	1.5	7.0
33154_c0_g1_i1	AT5G627.2	GIR1	2	1.8	0.8	2.1		2.4	0.5	2.5	
49301_c0_g1_i1	AT5G6900.1	CYP93D1	1	-0.7		-0.6	-0.5		-0.4		-0.5
37849_c0_g2_i5			1			1.3	0.7	1.7		1.4	0.8
45805_c1_g1_i1			1		0.9	1.6	1.3		0.5	1.4	0.8
49884_c0_g1_i4			1		0.7	2.2	1.2	2.4		2.2	1.1
57336_c4_g1_i2			1	1.8		1.7	3.1	1.6	2.6	1.5	3.0
58927_c2_g1_i1			1	2.7		3.0	0.7	2.3		2.2	0.7
60393_c1_g2_i4			1		0.9	0.6	1.2	0.7	1.1		1.0
67307_c3_g1_i12			1	1.4	0.7	1.5	0.7	1.1	-0.5	1.3	
67771_c2_g1_i4	XP_21995786	RICESLEEPER	2	2.2		1.7	0.6	1.5		1.8	0.4

The majority of DEGs could be validated in new samples.

The expression of the selected candidate genes was tested on new biological samples of stages 0 and 1 of both mutants and varieties. Overall, the differential expression is not very extreme, as was expected because of the quantitative difference in phenotype (Fig. 3). Differential expression of *GL2-INTERACTING REPRESSOR* (*GIR1*), *HOTHEAD (HTH)* and transcript 45805 could be validated in both mutants. Twenty of the selected genes could be partially validated because they were significantly differentially expressed in one of the mutants. Most genes that could not be confirmed were not significantly expressed in qPCR data, or an opposite trend in at least one of the mutants was visible. These genes were therefore excluded from further analysis. As expected, most genes had lower expression in the mutants. For *HERK1*, *HTH*, *MUG7* and *PDF2,* several different transcripts seem to represent a single gene; this is common with de novo transcriptome assemblies but makes analysis harder. For these cases, a particular transcript had to be chosen to represent the RNAseq data in the figure, while the qPCR data probably represent the cumulative expression of all transcripts of the gene. This difference causes extra variation between RNAseq and qPCR data for these genes.

The HD-ZIP IV genes that could be confirmed in the qPCR analysis were *HDG1* and *PDF2,* albeit *PDF2* only in M1. Also GIR1, acting in Arabidopsis together with the HD-ZIP IV GL2, was confirmed. This increases the likelihood that HD-ZIP IV homeodomain factors are involved in the regulation of floret identity.

While several BR-related genes were differentially expressed, mainly in M2, only a few were differential in both mutants. To further confirm the involvement of BR, it was possible to validate the differential expression of *BRL2* (M2), *TINY2* (M2), and *HERK1* (M1). We included *BRL2* despite it not being differential in M1. However, lower expression of M2 was consistent between transcriptome and qPCR data. We did not test other BR-related genes because most were only differential in one of the two mutants. However, the amount of BR-related DEGs in both mutants and confirmation of *BRL2*, *TINY2* and *HERK* further indicated the likelihood of BR involvement.

Given that genes in Figure 3 are often significantly differentially expressed in both mutants in the transcriptome data and at least in one mutant in the new qPCR data, they are likely candidates to be involved in floret type specification.


RNAseq
qPCR
RNAseq
qPCR
ANKYRIN REPEAT FAM PROTEIN
MUG7
ATKRS-1
PDF2
ATL4
RSL2
BRL2
SCL17
CYP83
SCPL13
CYP93D1
TINY2
V1
V2
read count
dCT
S 0 1 2 3
0 1 2 3
0 1
0 1

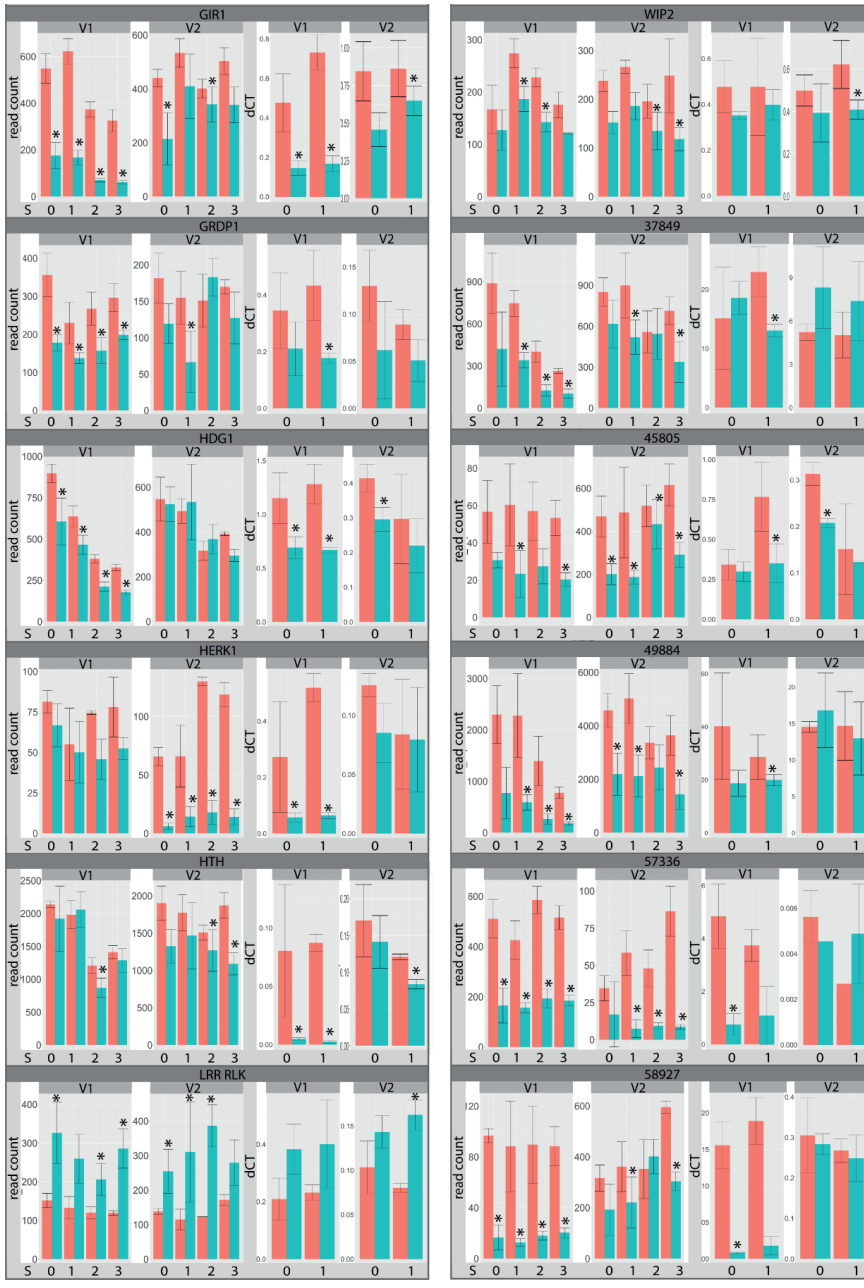


Figure 3. Transcriptome and qPCR results of genes selected based on function. RNAseq/transcriptome data include four stages, and qPCR results only include the first two stages of new, independent biological samples. 'V' specifies which variety and corresponding mutant are depicted, and 'S' indicates the various stages. Red indicates the normal variety for both V1 and V2, and blue indicates their corresponding mutants. Asterisks show significance between mutant and variety per stage,

RNAseq Padj (P-adjusted value) <0.01, and qPCR P-value <0.05. Reference gene *PGK* was used for the normalisation of qPCR data.

Discussion

In this study, we analysed the transcriptome data of two Chrysanthemum flower mutants with more disc florets compared to their respected varieties. Differential expression analysis revealed that 390 genes were differentially expressed in both mutants, and several of these differentially expressed genes (DEGs) could be responsible for floret identity. Moreover, the results suggest that Brassinosteroid-related genes and genes encoding homeodomain transcription factors IV could play an important role in floret identity.

In this study, we used two unrelated mutants of Chrysanthemum that both show the same trend in phenotype, strengthening the likelihood of identifying genes involved in floret identity. Another important aspect is that from the four samples taken at different developmental stages, the first two were taken before any phenotypic distinction could be made between disc or ray florets. This contrasts with other studies in which they collected developing disc or ray florets when they could already be distinguished (Laitinen *et al.*, 2005; Kuang *et al.*, 2013; Liu *et al.*, 2016). To obtain sufficient RNA for transcriptome analysis, we collected complete flower buds/inflorescence structures for both the normal varieties and the mutants. The downside of taking the entire flower bud is that each sample contains hundreds of florets that are not completely synchronised in development. As a consequence of collecting the entire developing inflorescence, including both disc and ray florets, the differences in gene expression between variety and mutant were quantitative and not absolute because both types of florets were present in all samples, albeit in a different ratio.

The transcriptome analysis was challenging due to the absence of a fully sequenced and annotated Chrysanthemum genome at that time. During this study, a difficulty we encountered was that some of the DEGs were represented more than once. This was expected because the reference sequence was constructed using de novo transcriptome assembly of the short sequence reads from the transcriptome data, resulting in a reference transcriptome containing 251,805 sequences. This number is several times higher than the expected total number of genes in a species, suggesting that many genes were represented by more than one transcript. In addition, several of these extra variants of genes could also represent different alleles since Chrysanthemum is hexaploid and highly heterozygous. Despite this inconvenience, the composed reference transcriptome was suitable for the analysis. The fact that we were able to

validate low abundance genes such as *CLV3* in the reference convinced us that we should be able to identify genes responsible for the floret identity phenotypes.

Surprisingly, the transcriptome analysis revealed far more DEGs for M2 than M1. This could be because M2 was acquired using x-ray treatment, which induces mutations at a high frequency and thus affects many genes (Broertjes, 1966; Yamaguchi, 2018). These mutated genes and their downstream targets could be differentially expressed without having any relation to the identity of florets. Our approach circumvents this, investigating genes that were differentially expressed similarly in both mutants.

Several papers focusing on floret identity have suggested that *CYC* is responsible for ray-floret identity. This was seen in sunflower, where overexpression induced an identity switch of all disc florets to ray florets, and a similar phenomenon has been observed in other Asteraceae species (Chapman *et al.*, 2012; Tahtiharju *et al.*, 2012). However, this was not confirmed in Chrysanthemum yet, and *CYC* overexpression of the transformed variants seems to influence ligule length only (Huang *et al.*, 2016; Yang *et al.*, 2019). Therefore, we expected to find differential expression of either a *CYC* gene or some co-regulator or repressor genes in our data. However, significant differences were only detected in later stages, suggesting they might not be responsible for floret identity per se but may act as downstream targets playing a role in the outgrowth of ligules. Because we did not find genes like *CYC*, we first analysed other mechanisms and pathways that could be responsible. The highest enriched GO category was 'involved in metabolic processes,' but this most likely reflects the differences in metabolites needed to develop the different floral organs and was unlikely to be involved in the specification of floret morphology. However, when inspecting the list of DEGs for both mutants, we discovered many genes involved in BR synthesis and signalling. This result was intriguing because BR hormones have never been connected with Asteraceae floret identity until recently when they were discovered to play a role in controlling organ boundaries in ray florets in Chrysanthemum (Cheng *et al.*, 2020). A study in Gerbera showed that exogenous BR treatment could increase ligule elongation in ray florets (Huang *et al.*, 2017). Another study in the monocot *Setaria* also found that BR could influence floret identity, further confirming the potential role of this hormone in the formation of different floret types (Yang *et al.*, 2018).

Most BR-related genes discovered in this study were not differentially regulated in both mutants but were unique for one. This could indicate that the mutation(s) underlying the two mutants are different for each mutant but that the same downstream processes are affected. There were far more differentially expressed BR-related genes in the M2 mutant, suggesting a more extensive role of BR in its phenotype. This is consistent with some additional phenotypic

observations of the M2 plants, which were shorter in length than V2. and its disc florets seemed to produce less pollen; both phenotypes are consistent with lower BR levels in other plant species (Li and He, 2020). Three BR-related genes could be validated in new samples; both *TINY2* and *HERK1* were expressed lower in M1 and M2, while *BRL2* was only lower expressed in M2. *DWARF1* (DWF1) and *BRI1-5 ENHANCED1* (*BEN1)* were differentially expressed in both mutants, but these genes could not be validated with qPCR. *TINY2* showed significantly lower expression in M2 , as well as in later stages of M1 and could be confirmed with qPCR in M2. *TINY2* is an AP2-ERF transcription factor that was in Arabidopsis shown to play a major role in regulating BR signalling and downstream processes under stress conditions (Xie *et al.*, 2019). As such, it could have a function in regulating floret identity, which may explain the observed variation of disc/ray–floret ratio we observed in response to stress. *BRL2* expression was lower in M2 than V2 in both RNAseq and qPCR data. *BRL2* is a receptor kinase closely related to *BRI*, which is essential for BR signalling. Although *BRL2* is not activated by or even able to bind BRASSINOLIDE (BL) in Arabidopsis, it seems to have a similar function in stress responses and vascular growth by activating the same processes (Ceserani *et al.*, 2009). *HERK1* is a receptor-like kinase involved in BR-regulated growth and showed downregulation in mutants for all stages depending on the specific RNAseq transcript, even though the expression was low. The lower expression could be confirmed with qPCR for M1. This lower expression of receptors is accompanied by the downregulation of several BR biosynthesis genes in both mutants. This is consistent with a study by Cheng et al. 2020, which suggested that higher expression of *BES1*, a positive regulator of BR, negatively impacts the number of disc florets. Based on the lower expression of *BRL2* in M2, *TINY2* and *HERK1* in both mutants and several other BR-related genes that were differentially expressed in the RNAseq data set, we hypothesise that the higher ratio of disc/ray florets in the mutants is caused by lower BR concentrations.

Several HD-ZIP IV transcription factor genes had lower expression in the mutants with a more notable effect in M1. This could be significant because HD-ZIP IV transcription factors are involved in meristem determinacy and the development of specific tissues. In Arabidopsis, *GIR1* is an adapter protein that, together with *GIR2,* interacts with co-repressors *GLABRA2* (*GL2*) and *TOPLESS* (*TPL*) to promote histone deacetylation of target chromatin to regulate root hair development (Wu and Citovsky, 2017a, 2017b). It was also shown in cotton that *GIR1* regulates fuzz formation that grows from the ovule, together with the more valuable lint, which is used in the cotton industry (Feng *et al.*, 2019). The ability to determine the outgrowth of root hair and fuzz tissues may be comparable to the outgrowth of anthers in disc florets or the ligule

of ray florets. *HDG1* and *PDF2* belong to the class IV homeodomain-leucine zipper (HD-ZIP IV) gene family and together appear to be involved in the regulation of floral organ development in Arabidopsis (Kamata *et al.*, 2014). *PDF2* is important for regulating the number of stamens, while a double *hdg* and *pdf* mutant showed homeotic conversions of petals and stamen. This is because several of these class-IV homeodomain-leucine zipper transcription factors (*PDF2*, *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*), *HDG1* and *HDG2*) can regulate the MADS-box genes *APETALA3 (AP3)* and to a lesser extent *PISTILLATA (PI),* which are responsible for petal and stamen identity specification (Kamata *et al.*, 2013). In Arabidopsis, *PDF2* and *ATML1* are close homologs with a redundant function in embryo development (Ogawa *et al.*, 2014), and the identified Chrysanthemum homolog is equally related to both. Many of these HD-ZIP IV genes have also been implicated in the development of trichomes (Pan *et al.*, 2015). This may explain why trichome development was enriched in the GO analysis. *GIR1*, *HDG1* and *PDF2* show a consistently lower expression in the mutants, suggesting that they could be acting together in the regulation of floret identity, similar to their combined role in regulating trichome identity. There is even a connection between BR and HD-ZIP IV gene *GL2* as *BRI-1* can positively regulate *WEREWOL*F (*WER*), *ENHANCER OF GLABRA3* (*EGL3)* and *TRANSPARENT TESTA* GLABRA (*TTG1*), which regulate *GL2* and repress root hair formation (Kuppusamy, Chen and Nemhauser, 2009; Wei and Li, 2016).

Several other interesting genes that could be involved in floret identity were identified, and differential expression was confirmed by qPCR on new samples. We will discuss here why these genes, *MUG7*, *RSL2*, *SHINE2* and *WIP2,* may be involved in the specification of floret identity. *MUG3* and *MUG7* are part of the *MUSTANG* family of domesticated transposons, which have gained essential functions in plant development, including the development of reproductive organs. Loss-of-function double mutants of *MUG7* and *MUG8* show signs of premature senescence, and anthers do not develop normally nor contain pollen (Joly-Lopez *et al.*, 2012). Because *MUG7* seems important for stamen development, it is expected that this gene would be expressed in disc florets, which is consistent with the higher expression of *MUG7* in both mutants. *RICESLEEPER* in rice, an ortholog of the Arabidopsis *DAYSLEEPER*, is also a domesticated transposable element family member (Knip, de Pater and Hooykaas, 2012). Overexpression of *DAYSLEEPER* genes results in a severe reduction of growth, sepal and petal development, and disruption of the margins, and both knockout or overexpression plants show severely impaired growth (Bundock and Hooykaas, 2005; Knip, de Pater and Hooykaas, 2012). *SHINE2* is one of three *SHINE* transcription factor clade members that shape

surface and flower morphology in Arabidopsis (Shi *et al.*, 2011). Triple *SHINE* mutants show severe defects in flower development and are sterile. The expression of *SHINE2* is only lower in M1 and not in M2, suggesting a different mechanism might be involved in M2. *WIP DOMAIN PROTEIN2* (*WIP2*)/*NO TRANSMITTING TRACT* (*NTT*) is a zinc-finger transcription factor that is important for replum development by activating homeobox protein BREVIPEDICELLUS (*BP*) (Marsch-Martínez *et al.*, 2014). It also interacts with *REPLUMLESS* (*RPL*), *SHOOT MERISTEMLESS* (*STM*), *FRUITFULL* (*FUL*), *SHATTERPROOF 1* (*SHP1*), and *SHATTERPROOF 2* (*SHP2*), all of which have important functions in Arabidopsis carpel development. Expression of *WIP2* is slightly lower in both mutants in several stages of the transcriptome analysis but is not verified in new samples. The pattern, however, is consistent in all samples and stages, which is more important since the phenotypic difference of the mutants is quantitative. Therefore, minor differences in expression, especially of a transcription factor, could reflect shifts in floret identity.

In conclusion, the transcriptome analysis revealed several novel candidate genes that may be involved in the regulation of floret identity in *Chrysanthemum morifolium*. Among these candidates are genes involved in Brassinosteroid signalling and genes encoding HD-ZIP IV transcription factors. These findings could be used in further studies concerning both Asteraceae floret identity and flower development.

Material and methods

Samples

Plants were grown in the greenhouse of Dekker Chrysanten B.V. in Hensbroek the Netherlands. Cuttings were planted on 30-11-2017, moved to the greenhouse on 13-12-2017, and transitioned to short day on 26-12-2017. V1 and M1 samples for transcriptome analysis were collected between 12-1-2018 and 06-02-2018 at around 3 hours after the light was turned on. Samples for V2 and M2 were collected on the 10th of January using the different capitula (flower buds) stages present at several plants. This was not possible for V1 and M1 due to the browning of younger flower bud stages present on the plants. Therefore, V1 and M1 samples were collected between January 12th and February 6th at various stages of development. New samples for qPCR were grown and collected at the beginning of 2019 in the same way as described previously. Plants were grown from December 2018 to February 2019, and V2 and M2 were sampled on 06-02-2019 and for V1 and M1 between 19-01-2019 and 06-02-2019. The samples were meticulously sorted in specific sizes to ensure the stage was always the same. Diameter sizes per stage for V1 and M1; stage 0, 0.5–2 mm; stage 1, 2-3 mm; stage 2, 4–5.5 mm; stage 3, 6–8 mm; stage 4, 9–13 mm; and stage 5, 15–25 mm. V2 and M2; stage 0, 0.5–2 mm; stage 1, 2-3 mm; stage 2, 3–5 mm; stage 3, 5–6 mm; stage 4, 7–10 mm; and stage 5, 10–25 mm. All samples were taken as biological triplicates, and at least 2 to 5 flower buds from different plants were used per biological sample. All samples were immediately frozen in liquid nitrogen and stored at -80 degrees Celsius. Samples were ground using a mortar and pestle while kept frozen using liquid nitrogen. RNA was isolated using the innuPREP Plant RNA Kit from analytic-Jena.

Phenotyping

Phenotyping of the number of disc and ray florets of the two varieties and their mutants was performed several times in 2018 and 2019. Most phenotypic measurements were done using a slice method. For the slice method, the fully developed capitulum is sliced in half, exposing the middle section of the florets on the flower head. The number of disc and ray florets were counted for the slice and the complete capitulum in the summer of 2018. In total, 86 capitula were counted in their entirety, 19 (V1), 17 (M1), 26 (V2), 24 (M2) and a total of 252 capitula that were counted by using the slice method.

Stage determination using microscopy

Flower buds of several sizes per stage were collected and dissected for each variety and mutant. These were analysed, and images were taken using a stereo microscope with magnification ranging from 7 to 45 times. A ruler was used to determine the size in millimeters for each magnification. For several dissected inflorescences, a series of images was taken focusing on different layers, and these were compiled using Photoshop to get a sharp image of the entire inflorescence.

Library prep

RNAseq library preparation was done using Illumina TruSeq® Stranded mRNA sample preparation kit according to the manufacturer's protocol. The low sample protocol was used with a sample input of 2,5 µg RNA per sample. The Elution 2 - Frag - Prime (94°C for 8 minutes, 4°C hold) to elute, fragment, and prime the RNA was done for 3 minutes instead of 8, to preserve larger fragments for sequencing. The enrichment PCR was set to 13 cycles instead of 15 cycles to decrease PCR bias in samples. Library sample quantity and quality were determined by Qubit and fragment analyser. The average fragment size per sample was 311.4bp, the shortest 296 and the longest 366bp. 125 bp paired-end sequencing was performed using four lanes on Illumina HiSeq2500. Data de-multiplexing and adapter trimming were done using bcl2fastq v2.20.0.422 with default settings. The average number of sequenced read pairs was 41,866,204 per sample, with the lowest number of 24,061,638 and the highest number of 65,622,549. This resulted in a total of 2,009,577,786 read pairs sequenced.

Analysis of RNAseq data

Reference sequences used for mapping the reads were constructed using Trinity de novo assembly of all read pairs of V1 using default settings. The isoforms for each gene were reduced to one by selecting the most abundantly expressed isoform. Mapping and quantification were done using RSEM and Bowtie2 as a mapper. Mapping of samples for V1 and M1 was performed using default settings, and for V2 and M2 bowtie2 sensitivity settings were adjusted to 'very_sensitive' to adjust for a higher variance compared to the reference sequence used. Differential expression analysis was done in R using DESeq2 with normalised data, performing the nbinomWaldTest and only focusing on genes with a maximum 0.01 padj value but not taking any minimum fold change into account. Sequences were compared to Arabidopsis database Araport11_genes.201606.pep using blastx with various e-value settings to identify

homology. This was also done for the proteome sequences downloaded from NCBI for the more related species *Lactuca* and *Helianthus*. GO-term analysis was done using AgriGo with default settings based on Arabidopsis homologs. The GO-term analysis compared DEGs to a customised reference using all genes expressed in the tissue. Selection criteria for candidate genes were: differentially expressed in at least stage 0 or 1 and one other stage, minimal average expression of 100 RPKM, minimal fragment length of 600 base pairs. Some sequences were excluded based on annotation if they seemed unlikely to play a role in floret identity.

Expression analysis with qPCR

Flower buds of various stages were fixed in liquid nitrogen and stored at -80^{0}C. RNA was isolated using the innuPREP Plant RNA Kit from analytic-jena, and cDNA synthesis was done using Invitrogen superscript® IV reverse transcriptase and random hexamer primers according to the manufacturer's instructions. Real-time PCR was performed using Power^{TM}SYBR® green master mix. *CmSAND* and *CmPGK* (Qi *et al.*, 2016) were both used as reference genes. For disc- and ray-floret tissues, qPCRs for *AG* and *CYC2* variants, *CmEF-1a* (Qi *et al.*, 2016), was used as a reference. PCR and measurements were done using the CFX maestro Bio-rad real-time PCR machine with 384 well-plates. The significance of potential expression differences was calculated using the student-t test. *AG* is *CAG1* (Aida *et al.*, 2008), *LFY* is *CmLFY* (Ma *et al.*, 2020), and *CYC2* versions are from Huang et al 2016 (Huang *et al.*, 2016).

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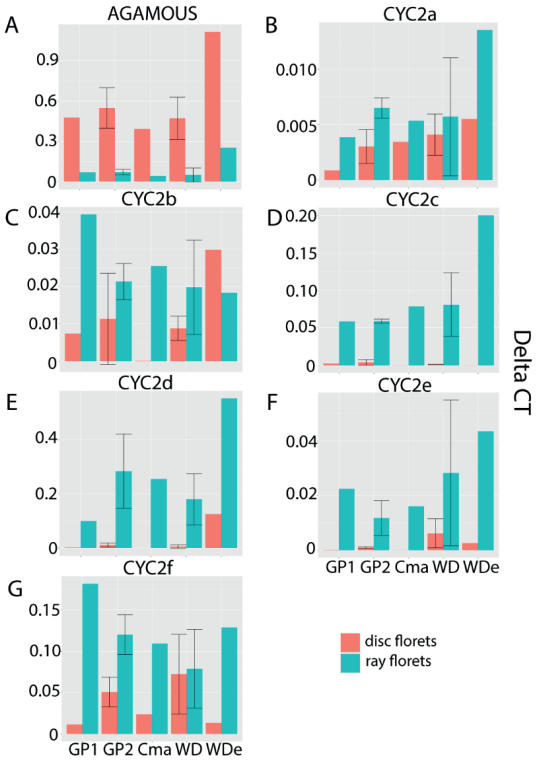

Suppl fig 1, floret specific expression of *AG* and *CYC2* variants in disc and ray florets. Expression of (A) *AG*, (B) *CYC2a*, (C) *CYC2b*, (D) *CYC2c*, (E) *CYC2d*, (F) *CYC2e* and (G) *CYC2f*. Red is disc, and blue is ray florets. Samples; GP1=green pompon variety 1, GP2=green pompon variety 2, Cma=*C. makinoi*, WD=white daisy, WDe=white decorative.

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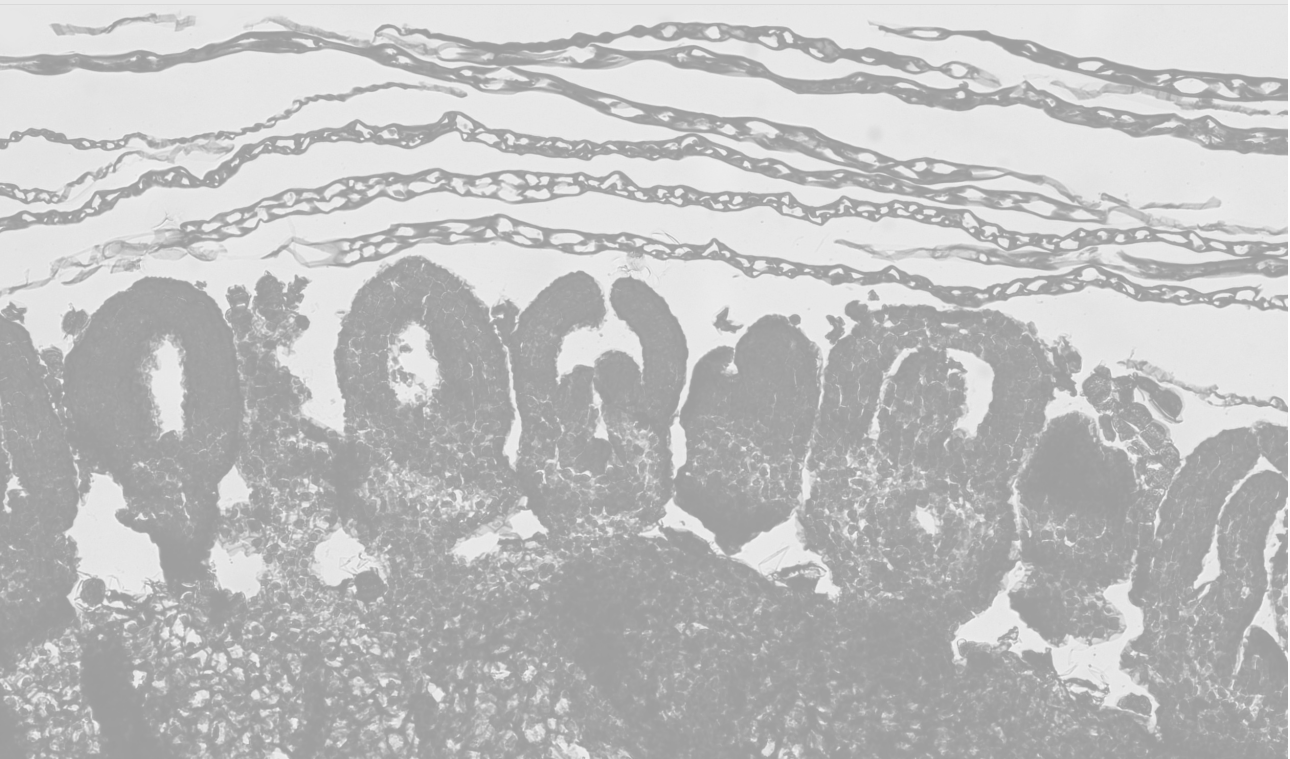

Chapter 4

Identification of loci involved in Chrysanthemum inflorescence morphology traits using a genetics approach

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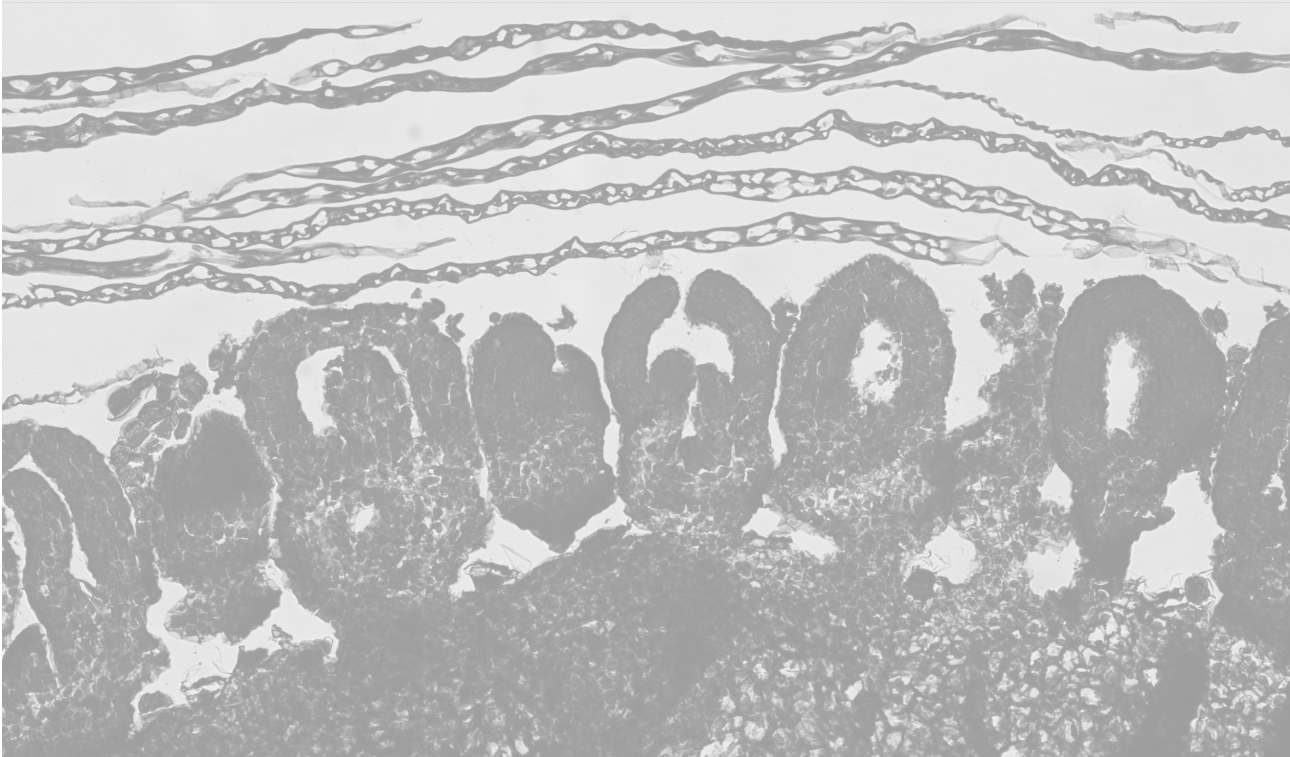

Summary

One of the main factors influencing the phenotype of Chrysanthemum flowers is the ratio between disc and ray florets. decorative varieties with mostly ray florets are beautiful but produce a limited number of seeds. The underlying genetic mechanisms for this floral trait are poorly understood. In this study, we performed a quantitative trait loci (QTL) analysis in a biparental population of a cross between a pink daisy (mainly disc florets) and a green pompon (mainly ray florets) type Chrysanthemum. We recorded the percentage of disc florets over two seasons and identified two QTLs on linkage group 1 (LG1) and LG4. We also phenotyped morphology and receptivity of both floret types and discovered a QTL for disc floret stigma receptivity on LG3. This QTL coincided with green flower colour, and these traits seem to be linked. Additionally, we performed a genome-wide association study (GWAS) on a large set of individual plants segregating for disc/ray-floret ratio. The GWAS analysis confirmed both QTLs on the corresponding chromosomes. Furthermore, we used the GWAS analysis to reduce the size of the target regions aiming at the identification of potential causal genes. Interestingly, the region on chromosome 4 included variants of *CYCLOIDEA 2*, a TCP transcription factor widely suggested to be involved in determining ray-floret identity in several Asteraceae species. Additionally, further analysis of differentially expressed genes in Chrysanthemum mutants with an increased percentage of disc florets identified several interesting candidate genes, including homeodomain transcription factor *PDF2*, located in the QTL region on chromosome 1. These findings give insight into the genetic basis of how disc/ray floret ratio is determined and give leads to improve seed set in varieties with more ray florets.

Introduction

Chrysanthemum is an important ornamental species with an extended breeding history that has produced a high diversity of flower shapes and colours. However, some reproductive limitations combat progress in the breeding of highly decorative types. As part of the Asteraceae family, Chrysanthemum flowers are composite flowers. Many flowers, also named florets, are packed together to resemble a single flowerhead. Chrysanthemum has two floret types, the showy female ray florets at the rim of the inflorescence structure and the hermaphroditic disc florets at the center.

Breeding varieties with a large amount of ray florets, which are considered to have high ornamental value, generally produce fewer seeds than daisy-type flowers with mostly disc florets. In a previous study (Chapter 2), we established that in addition to lower amounts of available pollen due to the limited amount of disc florets, the low fertility of these varieties is

also linked to reduced performance of the female reproductive organs. Ray florets generally had reduced opening of the stigma lobes and appeared to cease development before being fully mature. However, there is substantial variation in this trait for both disc- and ray-floret stigmas. Furthermore, stigma receptivity also played a role in reproductive success. Still, the most important factor associated with a low seed set was an increased fraction of ray florets, thereby limiting breeding success for these flower types. This suggests that seed set is somehow linked with the flower type, although the molecular connection is unclear. To address this problem, more knowledge on the genetics underlying the disc/ray-floret ratio and possibly stigma quality could help improve seed set.

Two commonly used tools in breeding to obtain more insight into the genetics underlying a particular trait are quantitative trait locus (QTL) analysis and genome-wide association study (GWAS). However, both tools have been highly challenging for Chrysanthemums due to its hexaploid nature. This largely complicated the development of integrative linkage maps, because polyploid mapping tools were not available until recently. Another limitation was the production of a high number of markers needed to distinguish all homeologs for each chromosome. Genetic analyses are far more complicated for species that show polysomic inheritance (when all homologous chromosomes can pair with each other). Although it has been unclear for a long time whether Chrysanthemum exhibits polysomic or disomic inheritance, recent papers all reported polysomic inheritance, and van Geest *et al.* claimed they had conclusive evidence that Chrysanthemum has hexasomic inheritance (van Geest, Voorrips, *et al.*, 2017; Sumitomo *et al.*, 2019). They found that their population exhibited polysomic inheritance and postulated that this mode of inheritance is most prevalent, albeit that there are likely chromosomal segments that exhibit preferential pairing (van Geest, Voorrips, *et al.*, 2017). Although the polysomic inheritance complicates QTL analysis and GWAS for Chrysanthemum, several tools have become available for genetic mapping in polyploid species in recent years (Bourke, Voorrips, *et al.*, 2018; Su *et al.*, 2019; Bourke *et al.*, 2021). So, we decided to perform QTL and GWAS analyses to address the seed set problem in ornamental Chrysanthemum varieties.

QTL analysis generally identifies a large genomic region, and it is difficult to narrow down the region without extensive further research such as fine mapping (Akond *et al.*, 2019; Thérèse Navarro *et al.*, 2022). Furthermore, the choice of the population is critical since the analysis can only identify variation that is present in the population. Additionally, markers developed based on specific genotypic information may not be as informative in the broader germplasm. These markers could be coupled to other non-advantageous alleles. The region

identified using QTL analysis may differ in size depending on the population size, quality of the phenotypical observations and the number and distribution of markers used. Still, there can be thousands of genes in the identified region, and breeders often have to work with a functional marker without knowing the causal gene. With GWAS, it is possible to identify smaller regions due to a higher number of informative markers and an increased number of recombination events as a result of more genetic distance between varieties (Rosyara *et al.*, 2016). Additionally, unlike in a QTL population study, where data are limited to variation available within the population, in a GWAS study, many varieties/accessions are being used, increasing the chance of identifying relevant loci. However, due to the massive variation available in the association population, it can be difficult to identify relevant loci that control only a small proportion of individuals used in the dataset (Korte and Farlow, 2013). Although QTL regions identified with GWAS might be smaller, they usually still contain hundreds of genes. Without other analysis methods to help narrow down the number of candidate genes, identifying the causal gene would still be too difficult. Getting closer to the causative gene can be achieved by fine mapping and developing more markers in the identified region (Jaganathan *et al.*, 2020). Another option is combining the GWAS results with other data, such as transcriptome data, to identify possible candidate genes.

Thus far, only a few genetic studies have been performed aiming at identifying regions responsible for floret type identity, and some have been able to narrow down QTL regions small enough to identify candidate genes (Zhang *et al.*, 2011; van Geest, Bourke, *et al.*, 2017; Fan *et al.*, 2020). No reports are available describing loci that influence the traits specified for female reproductive organs, such as stigma quality and receptivity. Most research in Chrysanthemum that focuses on the mechanism that influences floret identity is based on findings in other Asteraceae species. Several studies have shown that *CYCLOIDEA* 2 (*CYC2*), encoding a TCP transcription factor, is involved in ray floret development in Asteraceae species, including sunflower, *Gerbera*, and *Senecio* (Chapman *et al.*, 2012; Juntheikki-Palovaara *et al.*, 2014; Garcês, Spencer and Kim, 2016; Chen *et al.*, 2018). However, research in Chrysanthemum has been hindered by the presence of several different *CYC2* variants, of which the expression patterns do suggest involvement, as most have increased expression in ray florets compared to disc florets (Huang *et al.*, 2016; Wen *et al.*, 2019). Overexpression of *CYC2c* increased ligule length in ray florets and resulted in conversion of disc florets to trans-like florets (slightly elongated ligule) but did not affect stamen identity (Huang *et al.*, 2016). Another study found that *CYC2CL* is alternatively spliced at the flower differentiation phase (Liu *et al.*, 2021). *CYC2CL-1* is more highly available during early stages, while *CYC2CL-2* is

Results

Phenotyping and correlation analysis of flower traits related to seed set

In this project, we aimed to identify loci that control disc/ray-floret ratio and stigma quality using QTL analysis. We first had to determine if these traits were segregating in the QTL population we intended to use. This population of 89 progeny descended from a mother plant with small, daisy-type flowers containing only a single row of ray florets with pink ligules (Fig. 1A) and a paternal plant with green pompon-type flowers with mostly ray florets and only a few disc florets (Fig. 1B). Figure 1C shows the wide segregation of flower types in the segregating population. The mother had 86,4 and the father 8.8 per cent disc florets. As the parents were extremes for this trait, the percentage of disc florets in the population showed a high variation (Fig. 1D), which skewed more towards high disc floret percentages, which are more consistent with the maternal phenotype. Measurements were performed in both spring and autumn to account for environmental effects, and there is a slight difference in these measurements.

Additionally, disc- and ray floret stigma morphology and receptivity were measured and categorised based on the scale developed in Chapter 2 (Ch2 Fig. 2A, 2G). Also, these traits were segregating in the population, although there was much less variation between both parents than for the disc floret ratio (Fig. 1E). Stigma receptivity slightly differed between both parents, with a score of 4 for the disc florets of the maternal parent and 3 for the paternal parent. The progeny showed variation in stigma morphology and receptivity, as was expected for most quantitative traits.

The possibility of correlation between traits is essential to consider in order to understand the QTL results. Interestingly, there was a positive correlation between the percentage of disc florets and disc-floret stigma morphology, in agreement with the findings in Chapter 2 (Suppl. Table 1). All stigma phenotypes had some correlation, which was highest between disc- and ray-floret stigma receptivity. Additionally, green flower colour showed a correlation with all stigma quality scores, the highest being ray-floret stigma morphology. Overall, the population segregated for our traits of interest, which is essential for QTL analysis.

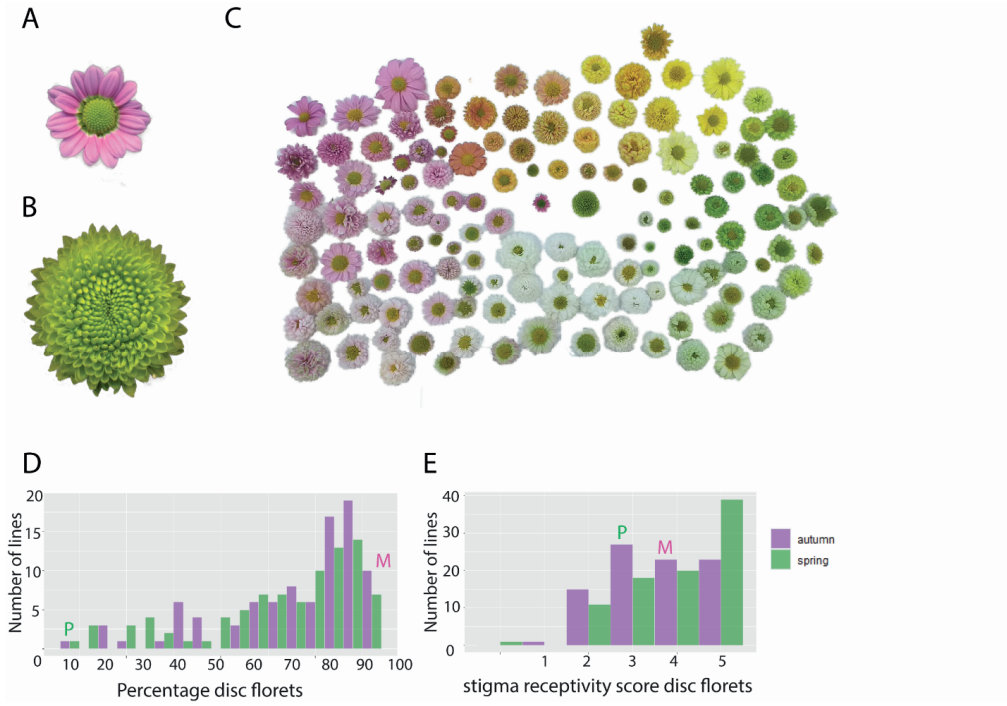

Figure 1. Population for QTL analysis. (A) Flower of the maternal plant, pink daisy type. (B) Flower of the paternal plant, green pompon type. (C) Flowers of the population used for this study show segregation in disc/ray-floret ratio, size and colour. (D) Histogram of the percentage of disc florets distributed over this population. (E) Histogram of disc-floret receptivity score distributed over this population. (D, E) Green is spring, purple is autumn, M is the maternal parent, and P is the paternal parent.

Analysis of percentage disc florets shows significant QTLs on LG1 and LG4.

QTL analysis was performed for each phenotyped trait for spring and autumn data sets using a previously generated genetic map. Significant loci were identified for the percentage of disc florets and disc-floret stigma receptivity, despite a logarithm of the odds (LOD) threshold of 8.4, calculated with a thousand permutations. Significant QTLs were detected for the percentage of disc florets on Linkage Group 1 (LG1) and LG4 (Fig. 2A).

The QTL on LG1 was most prominent and significant in both spring (12.8) and autumn (10.3), while the QTL on LG4 was 10.8 in autumn and less pronounced, just below the significance threshold in spring (8.3), suggesting that it is more dependent on environmental factors. This also suggests that the QTL on LG1 will likely be more effective in breeding for other locations in the world and for different production locations (Bourke, Gitonga, *et al.*,

2018). We further analysed the QTLs in more detail using identity-by-descent (IBD) probabilities for predicting haplotype-specific effects of the QTL. Haplotypes 1 to 6 belong to the daisy-type parent with many disc flowers and haplotypes 7 (h7) to h12 to the pompon-type parent. The model for LG1 suggests that a high percentage of disc flowers is negatively affected by one haplotype of the maternal plant (h6) and one of the paternal plant (h8) and positively affected by one haplotype of the paternal plant (h10) (Fig. 2B). Further inspection of the markers in the area with the highest LOD score revealed duplex markers for haplotypes 6 and 8, which could indicate a common ancestry in this region. However, if either allele is not coupled to the trait, the analysis could not discriminate between both haplotypes in that region and could thus result in a false association effect. It might be more likely that the negative effect originates from haplotype 8 alone since this is from the parent with fewer disc florets, which would mean that the pompon-type parent has both an allele that represses disc floret identity (haplotype 8) and an allele that can promote it (haplotype 10). On LG4, the model predicts a negative effect from haplotypes 5, 6 and 12 and a positive effect from haplotype 1 (Fig. 2C). The negative effect from haplotype 12 is an artefact from the software in response to missing data from that haplotype and should therefore be disregarded. It was surprising that there would be a causal negative effect from haplotypes 5 and 6 since these are from the daisy-type parent. The positive effect from haplotype 1 matches better with the phenotype and can potentially counter adverse effects from haplotypes 5 and 6. However, many haplotypes have a limited impact, and it is challenging to distinguish or detect interaction with other loci in this small population.

For stigma quality, there was only one significant QTL with a LOD score of 11.3 for the receptivity of disc floret stigma in spring on LG3 (Suppl. Table 2, Fig. 2D). Interestingly, there was a QTL with a LOD score of 45.2 in the same region for green flower colour (Suppl. Table 2, Fig. 2D). This was not entirely unexpected based on the correlation between the traits both in this population and in the varieties reported in Chapter 2 (Suppl. Table 2; Chapter 2). Upon further examination of the haplotypes, there was a distinct dominant effect of haplotype 12 (pompon-type flower), which negatively affects disc-floret stigma receptivity and positively affects green flower colour (Fig. 2E, 2F).

With respect to seed set, these two QTLs, for percentage disc florets and for disc-floret stigma receptivity, were highly interesting but they still spanned a substantial portion of the linkage groups. Therefore, narrowing down the regions is essential to identify potential candidate genes.

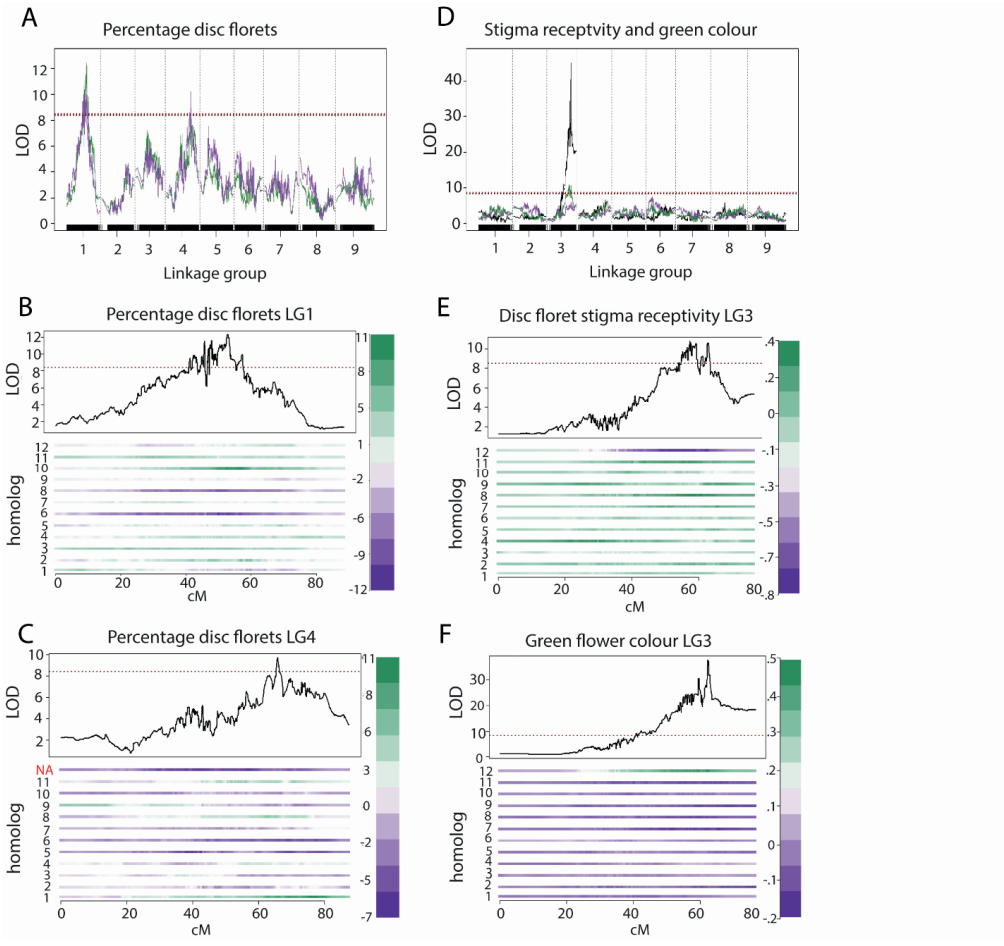

Figure 2. QTL analysis results of flower-related phenotypes for seed set in Chrysanthemum. (A) Percentage of disc florets. Haplotype effects of QTL for percentage disc florets on LG1 (B) and LG4 (C). (D) QTL results for disc-floret stigma receptivity and green colour. Haplotype effects of QTL for disc-floret stigma receptivity (E) and green ligules (F) on LG3. (A-F) The red-dotted horizontal line is the threshold determining significance. (A, D) Green = spring, purple = autumn, black = green flower. (B, C, E, F) Green displays a positive effect, and purple has a negative effect on the trait. H1-6 homeologs from the mother (daisy type parent), H7-12 homeologs from the father (green pompon type parent). (C) There were only five homeologs attributed to the father for LG4; therefore, H12 is missing, and the purple effect is an artifact.

GWAS analysis confirmed loci on chromosomes 1 and 4 as important for the disc/ray-floret ratio.

To find support for the identified QTLs and narrow down the QTL region further and/or to identify additional loci, we performed a Genome-Wide Association Study (GWAS). The GWAS consisted of 958 individuals, using 69,475 markers for the analysis. Because the cultivars in the GWAS population had not been phenotyped for stigma receptivity, we only focused on the disc floret percentage, which is linked to the flower type. Therefore, GWAS analysis was performed with the percentage of disc florets scored as a qualitative trait using the flower type daisy (high percentage of disc florets) versus all other flower types (more ornamental, fewer disc florets). Approximately a third of the individuals on the array were daisy-type. The other flower types include decorative flowers and other flower types that contain more ray florets. The markers used in the GWAS were mapped onto the *Chrysanthemum makinoi* (diploid) genome sequence (van Lieshout *et al.*, 2021). The result of the GWAS yielded several significant markers that together explained 66.49 percent of the observed variance (Fig. 3A). Several markers were scattered and most likely false positives or errors due to flawed genome reconstruction. However, there was a significant peak or string of markers in the middle of chromosome 4. These markers had significant log(P) values and were located close to one another. Additionally, some non-significant strings of markers were on the middle of chromosomes 1 and 8 and at the end of chromosome 9. The strings on chromosomes 1 and 4 are exciting because they coincide with the QTLs found for disc-floret percentage (Fig. 2A), supporting those data.

Identification of candidate genes in the identified regions

The regions on chromosomes 1 and 4 that we selected based on the high-scoring GWAS markers were much smaller than the regions identified in the QTL analysis for the same chromosomes. The region we selected on chromosome 1 spans 7,742,518 bp and is located between positions 139,537,628 and 147,280,146. This region contains 253 predicted genes, of which 167 genes could be annotated based on the Arabidopsis database. 71 of these genes were also differentially expressed (DEGs) in at least one of the mutants of Chapter 3 (Fig. 3B). This number of DEGs seemed high, except that a random set of 167 genes produced a similar overlap. Therefore, there is no indication that a large region is mutated in one of the mutants. Several interesting genes encoding known regulators, located in the region, and differentially expressed in the mutants described in Chapter 3 are *PROTODERMAL FACTOR 2* (*PDF2*), *GROWTH FACTOR 14* (*GF14)* (interacts with *BZR1*), and *CONSTANS LIKE 5* (*COL5)*.

The region we identified on Chromosome 4 spans 18,901,178 bp between positions 177,302,554 and 196,203,732 on that chromosome. This region contains 499 predicted genes, of which 310 could be annotated based on Arabidopsis. Similarly, as for chromosome 1, a substantial proportion of genes, namely 111, were also differentially expressed in the mutants of Chapter 3 (Fig. 3C). Notable genes in the region include *MUSTANG 7* (*MUG7*), *SEPALLATA3* (*SEP3*), *TCP12*, *DEMETER*, *ARGONAUTE 4* (*AGO4*), *FERONIA* and *BEL1-LIKE HOMEODOMAIN 2*. Interestingly, although not differentially expressed in early stages of the mutants (Chapter 3), this region contains all Chrysanthemum *CYC2-A* to *-F* gene variants previously identified as important for floret differentiation in *Asteraceae* species (Juntheikki-Palovaara *et al.*, 2014; Chen *et al.*, 2018). In Chrysanthemum, this role of the *CYC*2 genes has not been confirmed yet, possibly due to the existence of several copies, of which only two have been further investigated (Liu *et al.*, 2021; Shen *et al.*, 2021). Their presence in this region makes them more likely to be involved in the variation in disc/ray-floret ratio seen in the varieties used for the GWAS and in the QTL population. Additionally, some differentially regulated genes in these two regions (Suppl. Table 3) are candidates that could play a crucial role in determining floret identity in Chrysanthemum. Therefore, it will be highly valuable to investigate the candidate genes in these regions further.

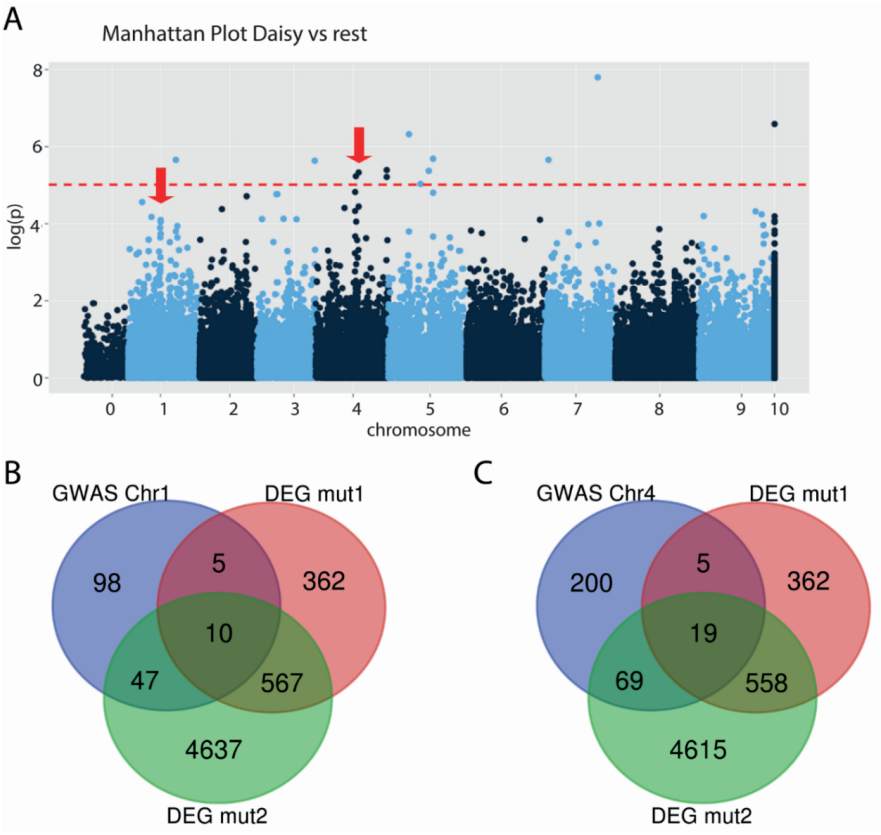

Figure 3. GWAS analysis and candidate genes. (A) Manhattan plot of GWAS of flower type daisy compared to other flower types. The marker strings located in regions that overlap with the identified QTLs are indicated with a red arrow. Chromosome 0 is composed of several contigs that could not be placed during the construction of the *C. makinoi* genome sequence, and chromosome 10 contains probes based on transcriptome data that could not be placed on the genome sequence. (B) Venn diagram of genes in the GWAS region of chromosome 1, DEGs of mutant 1 and DEGs of mutant 2 from Chapter 3. (C) Venn diagram of genes in the GWAS region of chromosome 4, DEGs of mutant 1 and DEGs of mutant 2.

Discussion

This chapter aimed to identify genomic regions responsible for traits related to seed set in Chrysanthemum. To this end, we phenotyped a population descending from a cross between a pink daisy-type parent and a green pompon-type parent that segregated several flower-related

traits. We identified QTLs for both the percentage of disc florets and stigma receptivity. These traits were identified as important for seed set in Chapter 2.

The QTL for disc-floret stigma receptivity was located at LG3 (Fig. 2D). Analysis of the effect from the haplotypes indicated that only one haplotype (12) from the pompon parent negatively affected stigma receptivity at this locus (Fig. 2E). Interestingly, a QTL for green flower colour was located in the same region and affected by the same haplotype (Fig. 2F). This was not fully unexpected since a correlation between these traits was already found in Chapter 2. It could mean that the two traits are coupled by proximity at the same haplotype, or both regulated by the same mechanism, although for the latter option, any suggestion for a common mechanism remains highly speculative. Because the region was rather large and we did not have any other leads for stigma receptivity, we further focused on the disc/ray-floret ratio.

The two parents used to generate the population were derived from unrelated breeding programs and were therefore genetically distinct. As such, there is a risk of identifying regions that might be important for the variation of these traits but that are not relevant to the general breeding program. For example, the identified variation responsible for more ray florets might only be present in these green pompon varieties. Introducing this into different genetic backgrounds could introduce undesirable coupled traits or present the trait in a different manner as a result of interactions with another genetic background. However, it is not likely that the identified QTLs on LG 1 and 4 are specific to green pompons since the GWAS results identified the same regions, while the genotypes used for GWAS were more diverse and included many different daisy types and decorative type varieties. Additional confirmation for these QTLs came from a QTL analysis in a different bi-parental population by Van Geest *et al.* For the number of ray florets in a daisy-type population, a significant region on chromosome 4 was found and there even appears to be a non-significant peak on chromosome 1 (van Geest, Bourke, *et al.*, 2017). Thus, there is increasing evidence that both chromosomal regions are involved in specifying floret identity. Two other studies reported QTLs for disc/ray-floret ratio. However, we could not compare the locations because the linkage group numbers do not correspond to the identifier used in this genetic map (Fan *et al.*, 2020; Song *et al.*, 2020).

The GWAS analysis showed a clear string of markers with high log(P) values in a distinct region on chromosome 4 and a non-significant string on chromosome 1. The peaks on chromosomes 1 and 4 were very low and might have been impacted by the population structure correction. Correcting for population structure is essential for reliable results but can also severely hamper the detection of certain traits (Korte and Farlow, 2013; Ott, Wang and Leal,

2015; Zhao *et al.*, 2018). daisy and decorative flowers are mostly contained to separate breeding programs and, as a result, were divided into different populations according to the second principal component in the analysis. The software corrects for this population structure, and it is hard to detect to what extent the breeding programs for daisy and decorative flower types overlap; therefore, QTLs could still be distinguished for these flower type differences. However, there were small peaks at the same location as the bi-parental QTLs. Consequently, the reliability of the results was greatly enhanced by the QTL results. The regions identified by GWAS were much smaller than those identified by the QTL analysis. Therefore, it was feasible to look at the genes in these regions to identify candidate genes. There were several hundred genes in these regions, and many of these corresponded to genes identified in the differential expression analysis of Chapter 3 (Fig. 3B). This analysis was based on mutants with more disc florets, which is the same type of phenotype as focused on here. The high number of genes overlapping between the GWAS regions and differentially expressed genes (DEGs) could suggest that part of the mutants' chromosome is deleted or that the epigenetic state could be affected. Deletion of a larger part of a chromosome is usually not the case for viable mutants because it results in lethality. However, it is common in a polyploid species with more chromosomes to overcome lethality, and most DEGs have lower but not abolished expression in the mutants (Broertjes, 1966; Ohmiya, Sumitomo and Aida, 2009). Several deletions would be likely, specifically in the second mutant, which was generated using radiation. Nevertheless, if part of a chromosome is deleted for either mutant, we would see a high overlap specific to either chromosome and not in both. Additionally, a random set of genes had a similar overlap with the DEGs, which does not support the occurrence of large deletions in the GWAS regions.

To get an idea about candidate genes in GWAS regions 1 and 4 that could potentially regulate floret identity, we looked at the annotations of those genes overlapping with the DEGs. The most interesting DEG of chromosome 1 is *ARABIDOPSIS THALIANA MERISTEM LAYER 1 (ATML1)/ PROTODERMAL FACTOR 2* (*PDF2*), a *HOMEODOMAIN ZIPPER IV* transcription factor (HD-ZIP IV). HD-ZIP IV proteins are involved in meristem determinacy in several tissues and organs, including root hairs, floral organs, trichomes and embryos. *PDF2* is known to regulate the Arabidopsis *APETALA 3* (*AP3*) gene, a homeotic gene that specifies the identity of petals and stamens (Kamata *et al.*, 2013, 2014; Ogawa *et al.*, 2014). With the genetic distance between Arabidopsis and Chrysanthemum, specific HD-ZIP IV proteins could have evolved in a regulator determining floret determinacy in Asteraceae.

Although not differentially regulated in early stages of the two mutants studied, the presence of Chrysanthemum *CYC2-like* genes a-f in the identified region on chromosome 4 is

also highly interesting. Many papers suggest a role for one of the *CmCYC2* variants in floret type formation based on what has been found in other Asteraceae species (Chapman *et al.*, 2012; Tähtiharju *et al.*, 2012; Garcês, Spencer and Kim, 2016; Chen *et al.*, 2018). There is proof of *CYC2* involvement in ligule outgrowth in Chrysanthemum. However, it is still unclear whether it is affecting floret identity (Huang *et al.*, 2016; Wen *et al.*, 2019; Yang *et al.*, 2019; Liu *et al.*, 2021; Shen *et al.*, 2021). Among the DEGs found in Chapter 3, we did find a *CYC2* (d) homolog and two *RADIALIS-LIKE* (*RAD*) and one *DIVARICATA* (*DIV*) homolog but only at later developmental stages. *RAD* is a co-regulator and *DIV* an inhibitor of *CYC* (Clark and Coen, 2002; Galego and Almeida, 2002; Garcês, Spencer and Kim, 2016). Additionally, we tested *CmCYC2c* using qPCR and found lower expression in the mutants from stage 2 onwards (Chapter 3). This was the stage at which the first floret differentiation was visible, and the disc and ray florets could be distinguished. We hypothesised that the expression of genes responsible for floret identity would be differential in a stage before visible differentiation. If *CYC2* is responsible for ray-floret development, this later differential expression would suggest a role in floret outgrowth and differentiation instead of identity. None of the six *CYC2* variants was differentially regulated in the early stages of floret identity specification. However, it should be noted that a mutation in the coding region of one of these CYC2 genes without an effect on its expression could be causal for the mutant phenotype, affecting floret identity in the early stages.

Further research into the loci and the potential candidate genes is still valuable. For example, candidate genes could be sequenced to develop haplotype-specific SNP markers. These markers could be used to verify the haplotypes that influence the phenotype. Sequence data could also be used to predict the effect of polymorphisms on expression or protein function, in particular in the two mutants of Chapter 3. However, candidate genes still need to be validated by functional analysis because markers could simply be coupled by proximity. Independently, markers could be developed based on these findings to assist breeding programs in selecting parental combinations that will produce enough seeds. So, focusing on breeding values rather than genetic values. Selecting varieties with relatively high amounts of disc florets will produce enough seeds while carrying the genetics to produce flowers with increased numbers of ray florets. Additionally, GWAS results suggest other loci that also influence this disc/ray-floret ratio, further research on these loci will also be helpful for optimizing the understanding of the trait.

In conclusion, the obtained results are very interesting, since both the GWAS and QTL analyses point to the same loci on chromosomes 1 and 4, which is confirmed especially for

chromosome 4 by the analysis of van Geest et al. (van Geest, Bourke, *et al.*, 2017). These three diverse sources combined suggest that the identified loci are major players influencing current variation in disc/ray-floret ratio in the *Chrysanthemum morifolium* germplasm.

Material and methods

Plant material

The QTL population was generated by Dekker Chrysanten in 2011. It resulted from a bi-parental cross between daisy x pompon; the mother was pollinated in quarantine to ensure that no external pollen contaminated the cross. 89 progeny plants were used to develop a genetic map for this QTL study. The mother is a pink daisy type and the father a green pompon type. Plants were grown in pots in the greenhouse in Hensbroek (the Netherlands). The plants used for the GWAS were chosen from representative varieties, 276 had a daisy flower type, and 660 had a different flower type.

Phenotypes

Measurements of the QTL population were taken in spring and autumn 2019 for both parents and the progeny. Three plants were grown from cuttings for each genotype, and at least two were used for phenotyping. Four inflorescences from each plant were measured for the number of disc and ray florets. The inflorescences were sliced in half, and all florets on the slice were counted separately, after which the percentage of disc florets was calculated. Additionally, in autumn, all florets of the second ripe inflorescence for each plant were counted completely to assure that measurements from only the slice were representative of florets on the entire inflorescence. These data had an extremely high correlation, thus validating the method of counting floret numbers in the slice. In spring, the diameter of the entire inflorescence, the diameter of the involucral bracts, and the diameter and height of the receptacle of the main flower (the first to ripen) were measured. The colour of the ligules of ray florets of ripe flowers was noted when three inflorescences were considered fully opened. 4-5 stigmas were collected from florets of various parts of the inflorescence when the entire inflorescence had fully opened. For morphology measurements, pictures were taken using a microscope with 20x enlargement. Receptivity assays were performed based on an assay used in *Senecio* stigma receptivity research (McInnis *et al.*, 2006). Stigma was incubated for 10 minutes in guaiacol solution before taking pictures for receptivity. For morphology and receptivity, five categories

were selected based on the range for all stigmas, and each stigma was appointed to a category, and the average was calculated.

Genetic map

The genetic map was made available by Dekker Chrysanten. Transcriptome data from the parents of this population and several other varieties had been used to detect SNPs and design an Affymetrix SNP array. Dosages were scored using fitPoly, and construction of the genetic map was performed using polymapR using the same method as described by Bourke *et al.* and van Geest *et al.* (van Geest, Bourke, *et al.*, 2017; Bourke, van Geest, *et al.*, 2018). In addition, the marker set published by van Geest et al. (2017) was included in the array and was used to assign LGs to coincide with the map in that article (van Geest, Bourke, *et al.*, 2017). This article named their linkage groups based on the lettuce genome, and the genome sequence of *Chrysanthemum makinoi* consequently also uses this order.

QTL analysis

QTL analyses were performed using package polyqtlR in RStudio.

We used a 'fast.permute' function to check potential QTLs. Calculated IBD probabilities and performed QTL analysis based on IBD values using function 'QTLpoly' with permutations set to 1000. Function 'visualiseQTLeffects' to visualise and function 'exploreQTL' to determine individual haplotype effects of QTL loci.

GWAS analysis

GWAS was performed using the R package GWASpoly.

Genotypical data were from an SNP array. Two SNP arrays with the same markers equally divided the 960 varieties. Default settings were used in addition to a minor allele frequency (MAF) of 0.01 and false discovery rate (FDR) of 0.05 leaving 69475 markers. A minimum threshold of 80 per cent of missing values per sample was set, leaving 936 varieties. 276 varieties had daisy flower type and 660 varieties other.

Acknowledgements

We are grateful to Paul Arens for reading the manuscript. Additionally, we are grateful to our interns, Marantha Kaagman and Milou Bruin, for their help in phenotyping the QTL population.

Supplementary table 1. Correlations between several population phenotypes for percentage disc florets and stigma morphology/receptivity. * P <0.05, ** P <0.01, *** P <0.001.

	percentage disc florets	disc morphology	disc receptivity	ray morphology	ray receptivity	green colour
percentage disc florets	1	.336**	0.197	0.108	-0.003	-0.14
disc morphology	.336**	1	.511**	.410**	.272**	-.236*
disc receptivity	0.108	.410**	.417**	1	.666**	-.525**
ray morphology	0.197	.511**	1	.417**	.544**	-.387**
ray receptivity	-0.003	.272**	.544**	.666**	1	-.425**
green colour	-0.14	-.236*	-.387**	-.525**	-.425**	1

Supplementary table 2. Table with QTL results for all phenotypical traits that were found in this population. NA# (not applicable) is not measured in this season. '-'= there is no significant QTL found associated with this phenotype. The numbers indicate the LG for which a significant QTL was detected. Data from the head flower were separated from that of the other flowers, because head flowers generally have a phenotype distinct from all other flowers

Phenotype	Spring	Autumn
Percentage disc florets	1	1,4
Disc floret stigma receptivity	3	-
Ray floret stigma receptivity	-	-
Disc floret stigma morphology	-	-
Ray floret stigma morphology	-	-
Green colour	3	3

Supplementary Table 3. Table of interesting genes overlapping between GWAS regions and DEGs. Selection was made based on known regulatory function in Arabidopsis and mainly transcription factors and flower development related genes were selected.

GWAS	ara code short	aracode	gene_name
chr1	AT1G35160	AT1G35160.1	GF14 PROTEIN PHI CHAIN (GF14 PHI)
chr1	AT2G03480	AT2G03480.3	QUASIMODO2 LIKE2
chr1	AT4G21750	AT4G21750.4	PDF2
chr1	AT5G13010	AT5G13010.1	EMBRYO DEFECTIVE 3011 (EMB3011)
chr1	AT5G43290	AT5G43290.1	WRKY DNA-BINDING PROTEIN 49 (WRKY49)
chr1	AT5G57660	AT5G57660.1	CONSTANS-LIKE 5 (COL5)
chr1	AT5G65360	AT5G65360.1	HISTONE 3.1 (H3.1)
chr4	AT1G06740	AT1G06740.1	MUSTANG 3 (MUG3)
chr4	AT1G47790	AT1G47790.1	Putative F-box protein
chr4	AT1G68800	AT1G68800.1	TCP12
chr4	AT2G27040	AT2G27040.2	ARGONAUTE 4 (AGO4)
chr4	AT2G36490	AT2G36490.1	DEMETER-LIKE 1 (DML1)
chr4	AT3G02110	AT3G02110.1	SERINE CARBOXYPEPTIDASE-LIKE 25 (scpl25)
chr4	AT3G05850	AT3G05850.1	MUSTANG 7 (MUG7)
chr4	AT3G25140	AT3G25140.1	QUASIMODO 1 (QUA1)
chr4	AT3G51550	AT3G51550.1	FERONIA (FER)
chr4	AT4G34210	AT4G34210.1	SKP1-LIKE 11 (SK11)
chr4	AT4G37050	AT4G37050.1	PATATIN-LIKE PROTEIN 4 (PLP4)
chr4	AT5G15600	AT5G15600.2	SPIRAL1-like 4
chr4	AT5G15900	AT5G15900.1	TRICHOME BIREFRINGENCE-LIKE 19 (TBL19)
chr4	AT5G28680	AT5G28680.2	ANX2
chr4	AT5G44800	AT5G44800.1	CHROMATIN REMODELING 4 (CHR4)
chr4	AT5G66940	AT5G66940.1	(ATDOF5.8)
chr4	AT1G24260	AT1G24260.3	SEPALLATA3 (SEP3)
chr4	AT4G36870	AT4G36870.4	BLH2

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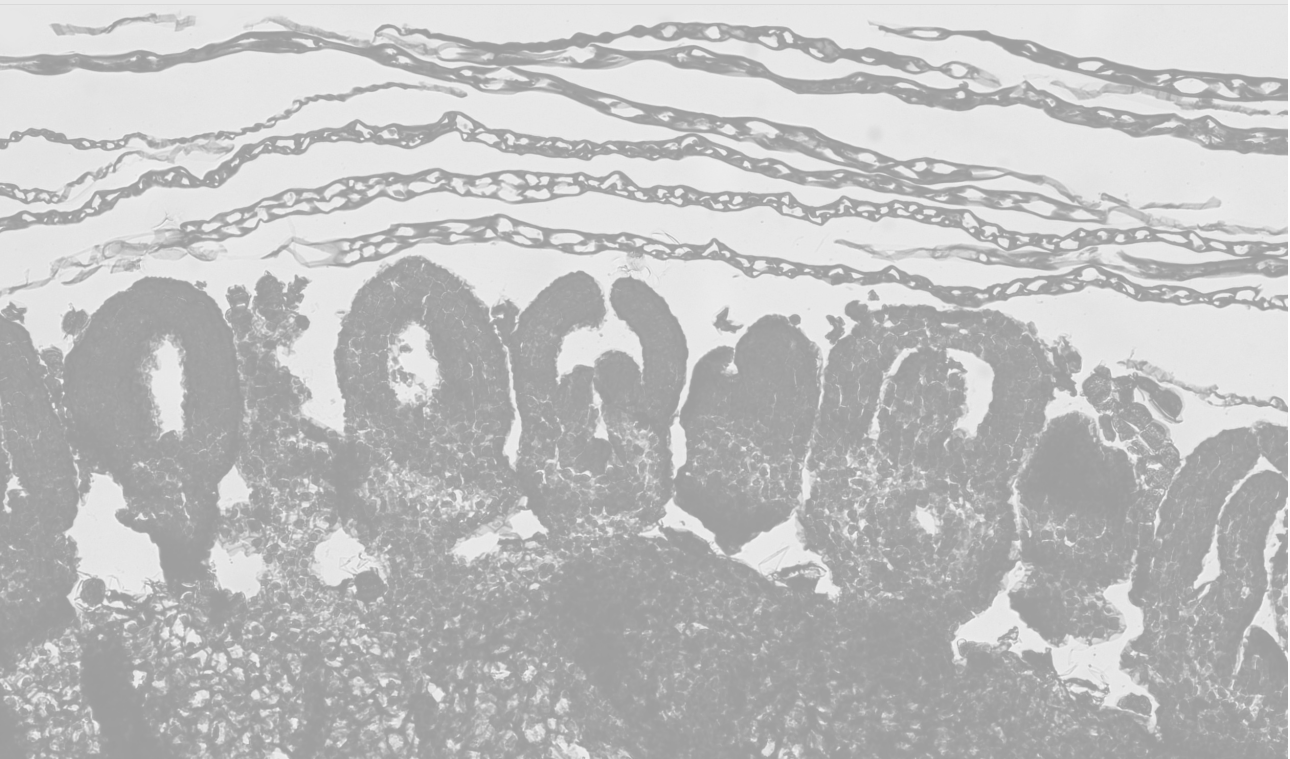

Chapter 5

Characterization of candidate genes that may regulate the disc/ray floret ratio reveals a role for Brassinosteroid signaling

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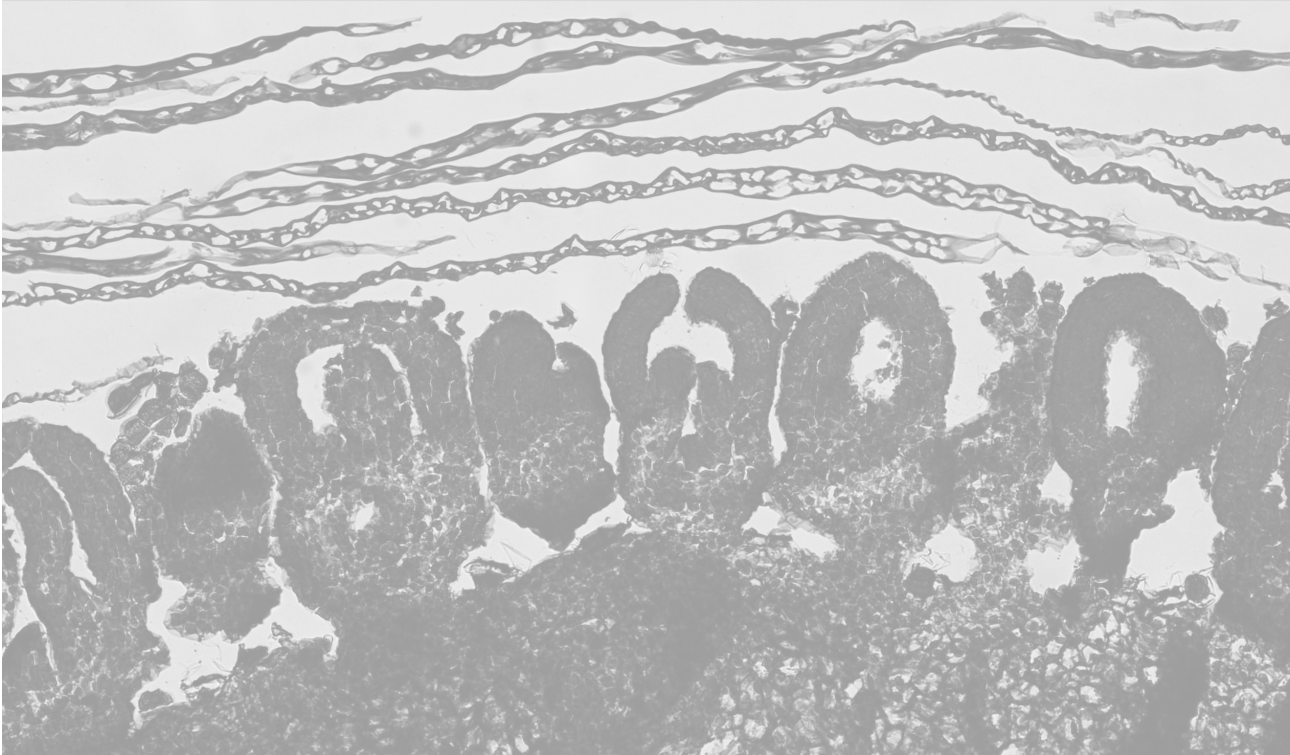

Summary

Chrysanthemum has a composite flower and produces female showy ray florets and hermaphroditic disc florets on one inflorescence structure, packed together to mimic a single flower. Previously, we showed that the ratio between disc and ray florets is not only determining ornamental value but also the number of seeds produced. Varieties with more ray florets produce fewer seeds and are therefore more difficult to breed. Transcriptome analysis of two mutants with an increased percentage of disc florets resulted in the identification of candidate genes that could be responsible for floret identity and thereby determined the disc/ray floret ratio. Here, We tried to validate the function of the identified Chrysanthemum homologs of *GIR1, RSL2, WIP2, HTH, TINY2, DWF1* and *PDF2* using knock-down and/or overexpression approaches. For five of these candidate genes, we could not validate a relationship between their expression levels and the disc/ray floret ratio, either because the downregulation/overexpression approach was not successful *(RSL2, WIP2*) or because there was no significant change in the disc/ray floret ratio observed (*GIR1*, *HTH*, *TINY2*). Interestingly, downregulation of a *PDF2* homolog, a homeodomain-leucine zipper (HD-ZIP IV) transcription factor, which was both identified by the previously performed transcriptomics and QTL approaches, did significantly increase the percentage of disc florets for three independent lines. Possibly, this *PDF2* homolog can influence the difference in the outgrowth of the stamen and ligules of disc and ray florets, similar to its Arabidopsis homolog that regulates the outgrowth of organs through the L1 layer. Since the expression of *PDF2* was not tested for all obtained transgenic lines, this data is not conclusive, and further research should still be performed. The other candidate gene for which downregulation yielded interesting results was *DWF1*, an early Brassinosteroid (BR) biosynthesis gene. Four RNAi lines were generated with decreased expression and an increase in disc floret percentage. Treatments with brassinazole (BZ), which inhibits BR, produced similar results. Little is known of the role of BR signalling in the specification or development of the floral organs, but since BR is essential for the outgrowth of several tissues, it could have an important function in the outgrowth of ray floret ligules. Together, our data suggest that BR influences the disc/ray floret ratio in *Chrysanthemum morifolium*.

Introduction

Chrysanthemum is one of the most important cut flowers globally, and breeding for new varieties with improved traits is essential for breeding companies. However, there is a significant problem with low seed set, and we confirmed in Chapter 2 that this is more

pronounced in varieties with ornamental flower types. In the case of Asteraceae family members such as Chrysanthemum, these flowers are actually inflorescence structures composed of many small flowers referred to as florets. Chrysanthemum has showy female ray florets and hermaphrodite disc florets. The ratio of disc/ray florets highly affects flower phenotype, and analysis in Chapter 2 revealed that flower types with more ray florets produce fewer seeds. This is partly due to a generally lower ray floret stigma quality. Therefore, we investigated what mechanisms may be responsible for an altered disc/ray ratio and identified several candidate genes that were differentially regulated in two mutants with more disc florets compared to their corresponding varieties (Chapter 3). In this chapter, we describe the functional analysis of several candidate genes identified in Chapter 3 by altering their expression levels and determination of its effect on the floret ratio.

The most valuable technique for functional analysis is gene knockout using transgenic techniques such as CRISPR-Cas9. However, a knockout is very difficult in Chrysanthemum because it is a hexaploid, and therefore six potential alleles need to be successfully targeted and analyzed for a single gene. Suppose the gene has been duplicated in the past, which is likely since Chrysanthemum has undergone several duplication events, it is essential that other homologs will be targeted as well (Won *et al.*, 2017). A recent study knocking out the *CmDMC1* gene using a TALEN approach reported that they successfully affected all six alleles in five out of 23 transgenic lines produced of one variety and two out of 126 produced transgenic lines of another variety (Shinoyama *et al.*, 2020). However, only very few studies have reported knocking out all alleles in polyploid crops and depending on the gene, this might prove very difficult. In addition, obtaining homozygous lines is not feasible in Chrysanthemums because of the outcrossing nature. Because of this, transgenic approaches such as RNA interference (RNAi), virus-induced gene silencing (VIGS) and overexpression are more often used for functional gene characterization in polyploid crops. RNAi is a technique where a DNA template is provided with a sense and antisense fragment of the gene of interest, such that upon transcription, a double-stranded RNA will be produced, which induces the plant defense system against viruses or transposons. The enzyme Dicer cleaves this dsRNA in ~21bp small interfering RNAs (siRNAs). These are then used as guides for targeting complementary messenger RNA (mRNA) by Argonaute 2 and cleaved by the catalytic component of the RISC complex to break down target mRNA, resulting in transcriptional silencing (Fire *et al.*, 1998; Chen *et al.*, 2010).

In Chrysanthemum, RNAi and overexpression are applied with varying results, but RNAi generally results in lines with expression ranging between 100% and 30% of wild-type levels

(Zhang *et al.*, 2013; Yang *et al.*, 2014; Xu *et al.*, 2020). Despite the varying expression levels of the reported RNAi transgenic lines, aberrant phenotypes were noticeable, suggesting that RNAi-knockdown is a useful method. This approach is also preferential for essential genes to prevent lethality that could result from the total knockout of the target gene. The choice of the promotor to drive the RNAi and overexpression constructs in Chrysanthemum is crucial for successful knock-down/overexpression. The 35S promoter from the Cauliflower mosaic virus is most commonly used to achieve high expression levels, but this promotor seems to produce only weak expression in Chrysanthemum (Sherman, Moyer and Daub, 1998; Takatsu, Hayashi and Sakuma, 2000a, 2000b). Several studies have reported that the *Petroselinum crispum UBIQUITIN* promotor (PcUbi) was more effective in driving high expression in Chrysanthemum (Fauser, Schiml and Puchta, 2014; Kishi-Kaboshi, Aida and Sasaki, 2017).

The mutant transcriptome analysis indicated several interesting genes for functional analysis, which could be validated with qPCR in independent samples (Chapter 3). These interesting genes were selected based on their homology to Arabidopsis genes, of which the function might be linked to floret identity or floral organ outgrowth. These included Chrysanthemum homologs of *PROTODERMAL FACTOR2* (*PDF2*), *GLABRA2-INTERACTING REPRESSOR* (*GIR1*), *DWARF1* (*DWF1*), *TINY2* (*TNY2*), *HOTHEAD* (*HTH*), *RICESLEEPER2* (*RSL2*) and *WIP DOMAIN PROTEIN2* (*WIP2*). In addition, many DEGs appeared involved in Brassinosteroid (BR) synthesis or signalling, which was an additional reason to select *DWF1* and *TINY2* as candidate genes. All of the selected genes had lower expression levels in the mutants, and we, therefore, chose, in most cases, a downregulation approach to simulate the same effect.

PDF2 belongs to the class IV homeodomain-leucine zipper (HD-ZIP IV) family, a family of 16 proteins in Arabidopsis that have essential functions in layer 1 (L1) epidermal patterning (Kamata *et al.*, 2014; Ogawa *et al.*, 2014). While several of these HD-ZIP IV transcription factors (TF), such as *GLABRA 2 (GL2),* have functions in root hair development or trichome development. *PDF2* has redundant functions in embryo and floral organ development together with *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*) (Kamata *et al.*, 2013; Ogawa *et al.*, 2014). Furthermore, double mutants with other homeodomain genes in Arabidopsis resulted in conversions of petals into sepaloid organs and stamens into infertile and/or carpelloid organs, indicating homeotic involvement in petal and stamen identity (Kamata *et al.*, 2013). A gene possibly related to *PDF2* is *GIR1*. GIR1 is an adaptor protein that, together with GIR2*,* assists GL2 and possibly binds TOPLESS (TPL) to repress root hair differentiation (Wu and Citovsky, 2017a, 2017b). *GaFzl*, a *GIR* homolog in

cotton, was discovered by fine mapping and transcriptomics to be the most likely candidate for a fuzzless phenotype. Fuzz is one of two types of cotton fibres, and fuzz inhibits seed germination (Feng *et al.*, 2019). Possibly, Chrysanthemum *GIR1* could assist *PDF2* or other HD-ZIP IV genes in the repression of stamen development in ray florets.

An interesting transcription factor is *WIP2*/*NO TRANSMITTING TRACT* (*NTT*), which is a C2H2/C2HC zinc finger transcription factor (Marsch-Martínez *et al.*, 2014). This gene was selected based on the expression pattern of a close homolog that was exclusively detected in female samples of the Asteraceae family member Ambrosia (Mátyás *et al.*, 2019). *WIP2* function seemed diverse over different species. It is involved in transmitting tract development and expression during pollen tube growth in Arabidopsis (Marsch-Martínez *et al.*, 2014; Crawford *et al*., 2007) and is more highly expressed in Gerbera stamen (Ren *et al.*, 2018). Overexpression in both Arabidopsis and Gerbera resulted in a reduction of length in several organs, including disc and ray floret petals. This last paper suggests that *GhWIP2* is a transcriptional repressor involved in organ morphogenesis by modulating crosstalk between GA, ABA, and auxin (Ren et al., 2018), thereby making it a plausible candidate for floret differentiation.

Two other DEGs, mainly selected based on the expression patterns, were *RSL2* and *HTH*. *RSL2* is a domesticated transposable element (TE) from the hAT-superfamily, which evolved a role in Arabidopsis development (Jiao and Deng, 2007; Knip, de Pater and Hooykaas, 2012). Overexpression caused reduced sepal and petal growth, with the margins of the sepals often appearing petaloid (Bundock and Hooykaas, 2005). Knock-out resulted in severely distorted plant growth and plants in which no floral organs were initiated. While the function remains largely unknown, *DAYSLEEPER*, another *SLEEPER* homolog in Arabidopsis, is most highly expressed in developing flower buds and specifically in the developing carpel and anthers, suggesting a role in the reproductive tissues (Knip *et al.*, 2013). Depending on the insertion location of the TE, the *RSL2* homolog could have evolved different functions in the Asteraceae family and therefore be responsible for the evolution of flower type. Similar to *PDF2*, *HTH* is an L1-specific gene in Arabidopsis, which is important for normal shoot development by producing long-chain fatty acids that prevent inappropriate organ fusion (Krolikowski *et al.*, 2003). In Rice, *HTH1*/*ONION*3 (*ONI3*) is involved in cutin biosynthesis and has a role in anther development and pollen fertility (Akiba *et al.*, 2014; Xu *et al.*, 2017). In Chrysanthemum, *HTH* may be involved in stamen development in disc florets, and lower expression in ray florets could result in the lack of outgrowth of these organs.

Because several genes related to BR-signaling were identified in the set of DEGs, we set out to investigate whether BR could regulate floret identity. Brassinosteroid is a relatively recently discovered steroid phytohormone with several functions in plant development (Li and He, 2020). While BR is essential for many functions, the most notable phenotype of plants with ineffective BR biosynthesis is dwarfism because BR plays an important role in cell elongation. In maize, BR is essential for male fertility, and BR mutants displayed impaired anther and pollen development (Hartwig *et al.*, 2011). Moreover, BR seems to play an important role in female flower development in cucumber. Exogenous, BR increased and accelerated female flower organogenesis, possibly by crosstalk between ethylene and BR (Papadopoulou and Grumet, 2019). *DWARF1* (*DWF1*) is an early enzyme in Brassinosteroid biosynthesis, converting 24-Methylenecholesterol to Campesterol (Choe *et al.*, 1999). Arabidopsis *dwf1* mutants exhibit typical symptoms associated with BR-deficiency, such as abnormal root and leaf development, reduced stem elongation (dwarfism), delayed senescence, failed stamen formation, and reduced silique and seed formation. In addition, *dwf1* mutants displayed a defect in stamen development, which could be rescued by exogenous castasterone (CS) and brassinolide (BL) treatments (Youn *et al.*, 2018).

Another differentially expressed gene associated with BR is *TINY2*, which encodes a member of the DREB subfamily A-4 of the ERF/AP2 transcription factor family with important functions in stress responses (Sun *et al.*, 2008). Expression is greatly induced by drought, cold, ethylene, and slightly by methyl jasmonate. Overexpression of *TINY* gives a dwarfism phenotype (Sun *et al.*, 2008). An Arabidopsis *TINY* mutant had shorter stigmas and anthers, barely dehiscent pollen, and appeared to be female sterile (Wilson *et al.*, 1996). Furthermore, *TINY* has an antagonistic interaction with *BRASSINOSTERIOID INSENSITIVE1-ETHYL METHANESULFONATE SUPPRESSOR* 1 (*BES1*) and inhibits BR-regulated growth while promoting drought responses (Xie *et al.*, 2019).

To the best of our knowledge, no functional analysis has ever been performed in Chrysanthemum or any other Asteraceae member for the selected genes. Because composite flower types are unique to the Asteraceae family, it is likely that genes we know from research in model species like Arabidopsis have evolved novel functions that are unique to Asteraceae. Functional analysis of these genes could elucidate the mechanism that has made this unique inflorescence structure possible. In this Chapter, we have generated RNAi lines for *PDF2*, *GIR1*, *DWF1*, *HTH* and *RSL2* and overexpression lines for *GIR1*, *TINY2* and *WIP2*. For *DWF1* and *PDF2,* we found that downregulation caused an increase of disc florets in transgenic lines, in line with their downregulation in mutants with more disc florets. In addition to this effect of

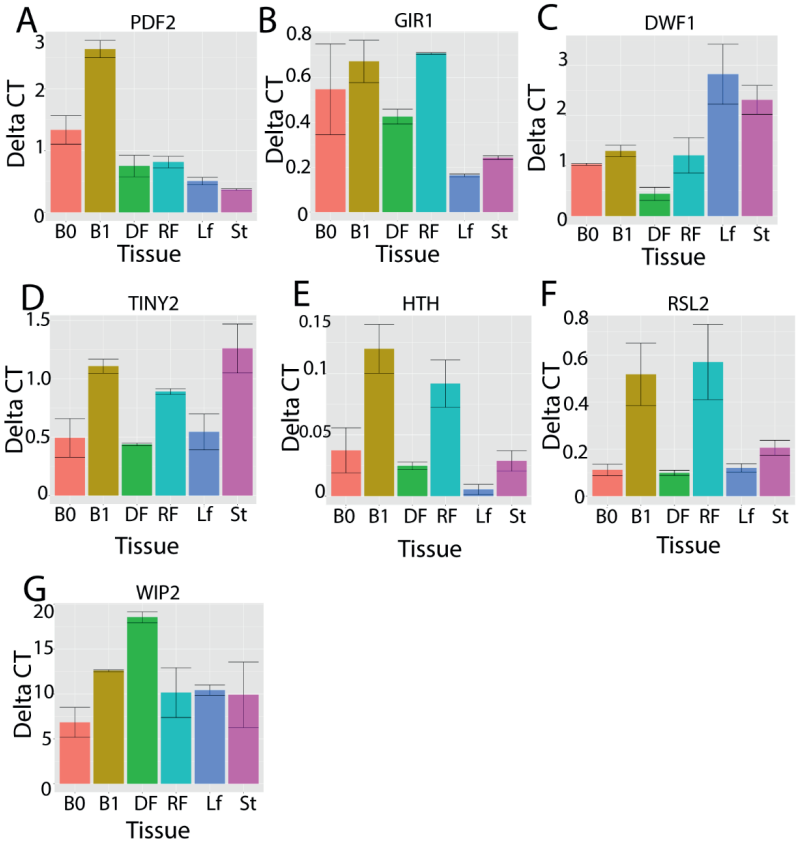

Figure 1. Expression of candidate genes in different tissues. DeltaCT is calculated relative to *PHOSPHOGLYCERATE KINASE* (*PGK*) expression. B0=bud at stage 0, B1=bud at stage 1, DF=disc floret, RF= ray floret, Lf=leaf and St= stem. Expression patterns for (A) *PDF2*, (B) *GIR1*, (C) *DWF1*, (D) *TINY2*, (E) *HTH*, (F) *RSL2* and (G) *WIP2*.

No evidence that altered *GIR1*, *RSL2*, *WIP2*, *HTH* or *TINY2* expression influences the disc/ray floret ratio.

To characterize the selected genes further, RNAi downregulation lines were generated in Chrysanthemum morifolium cultivar '1581' for the *GIR1, HTH, RSL2, PDF2* and *DWF1* homologs. If the reduced expression of these genes in the M1/M2 mutants (Chapter 3) is at least partial causal to the increased disc/ray floret ratio in these mutants, transgenic lines with sufficient downregulation were expected to mimic the M1/M2 phenotypes and to exhibit more disc florets. An alternative approach was taken for *WIP2* and *TINY2*, for which overexpression lines were generated under the control of the PcUbi promoter, which should result in an opposite phenotype. For *GIR1,* we developed both RNAi and overexpression lines. Due to

limitations in growth chamber space, only one individual from each transformed line was grown, which limited the initial sampling of flower buds for detecting expression levels and phenotyping. However, we took cuttings in early growth for part of the transformations, thereby gaining new biological duplicate plants.

For *GIR1*, twelve RNAi lines were generated, of which the majority seemed to have a lower expression (Fig. 2A), while most of the eleven overexpression lines seemed to have higher expression than the controls (Fig. 2B). Although four RNAi lines with an apparent lower expression level have more disc florets (g3, g15, g7 and g21), there is no clear correlation between expression level and phenotype. For example, g20 had a lower expression level but significantly fewer disc florets. The overexpression lines did not show a correlation between expression level and phenotype either, and we, therefore, concluded that GIR1 does probably not regulate the disc/ray floret ratio.

For *RSL2* downregulation, we developed ten lines (Fig. 2C), and none of them seemed to have lower expression in the stage 0 or 1 flower buds, possibly because the endogenous expression of *RSL2* was already relatively low. It was therefore not possible to draw conclusions about the putative involvement of *RSL2* in the regulation of disc/ray floret ratio. A similar problem was experienced with the 17 generated *WIP2* overexpression lines, of which only one appeared to have a somewhat higher expression than the wild-type control (Fig. 2D). This may have been a technical problem because there were difficulties in confirming that the *WIP2* lines contained the overexpression construct. The only line with higher expression, W40, did not exhibit a significantly different disc floret number.

For *HTH* downregulation, we produced 26 lines, most of which seemed to have a slightly lower expression (Fig. 2E). However, also for these lines, no significant correlation could be found between the expression level and a decrease in the percentage of disc florets. Finally, 20 lines were produced with the *TINY2* overexpression construct (Fig. 2F). Most of these lines exhibited increased expression, but the expected decrease in disc florets number was not observed.

Although we can preliminary conclude that *GIR1*, *HTH,* and *TINY2* homologs do not contribute to the regulation of the disc/ray floret ratio in Chrysanthemum, several aspects make it difficult to draw a conclusion. First, we observed considerable phenotypic variation for this quantitative trait also in the control plants that were not infiltrated with Agrobacterium and which were included for each transformation (Fig. 2F). This large variation may be because of tissue culture and/or repeated cuttings. Second, the expression data for each line was, in most cases, based on a single sample of a few tiny stage 0/1 buds, and the lack of replicates make

the expression data less reliable. Third, RNAi for *GIR1* and *HTH* and overexpression for *GIR1* and *TINY2* did not lead to a drastic reduction or increase of expression, respectively, and we can therefore not rule out that complete knock-out or more severe overexpression would give the expected phenotypes. For *RSL2* and *WIP2,* conclusions cannot be drawn based on the failure to modify the expression of these genes in the transgenic lines. Fortunately, the results were much more convincing for *PDF2* and *DWF1*, which are discussed in the next paragraph.

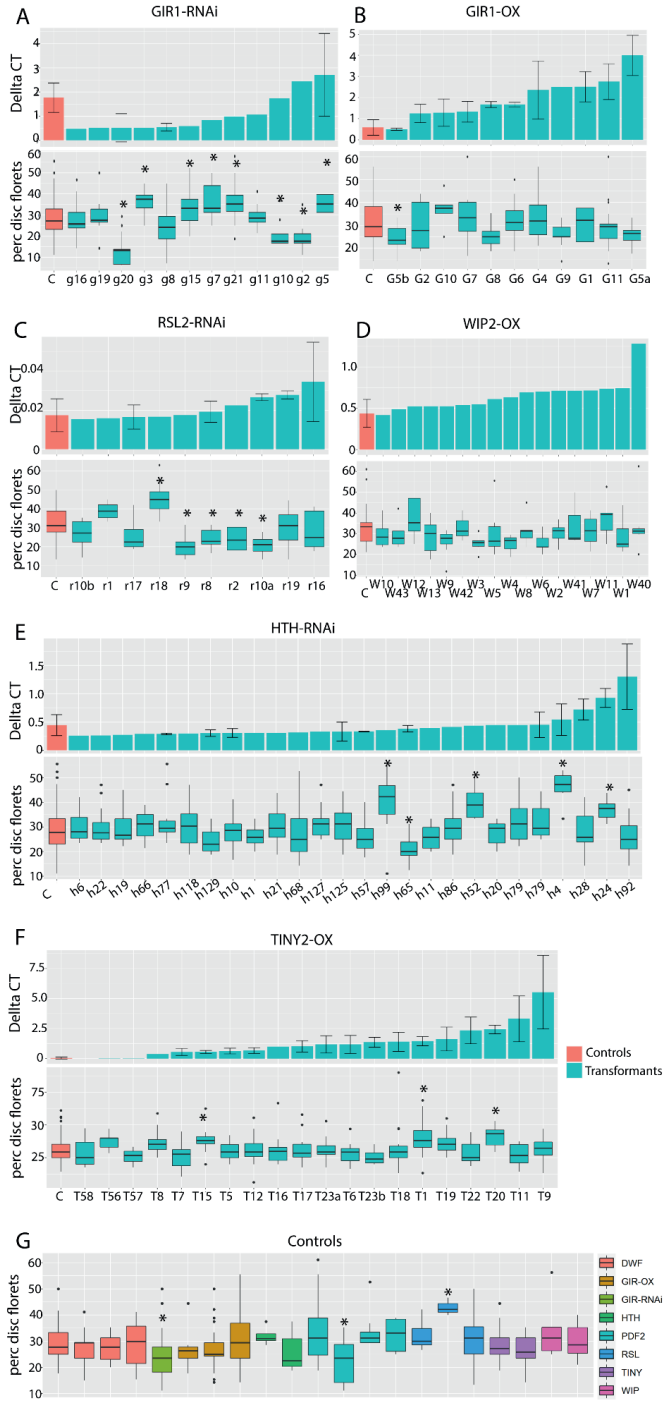

Figure 2. Analysis of the transgenic lines: expression levels and phenotypes. (A) *GIR1* RNAi, (B) *GIR1* overexpression, (C) *RSL2* RNAi, (D) *WIP2* overexpression, (E) *HTH* RNAi and (F) *TINY2* overexpression. For each gene, the top graph shows expression as DeltaCT compared to the reference gene expression, and the bottom graph shows the percentage of disc florets. The pink bar shows a combination of the controls, and blue represents each transformant in the same order in both figures. Samples with capital letters represent overexpression lines, and small letters represent RNAi lines. Samples are ordered based on expression level. Error bars in qPCR data are based on two biological replicates. (G) percentage disc florets of control plants that were grown in tissue culture along with the agro-infiltrated transgenic explants.

Downregulation of *PDF2* increased the disc/ray floret ratio in the majority of lines

The *PDF2* gene was of special interest because, in addition to its significant differential expression in the M1 mutant with more disc florets (Chapter 3), it is also located in the GWAS region identified in Chapter 4, suggesting that it is a key factor influencing the ratio of disc/ray florets. Transformation with the *PDF* RNAi construct resulted in 32 transgenic lines. However, due to time constraints, these were first phenotyped, and four lines showing the most pronounced increase in the percentage of disc florets were propagated and further molecularly characterized. Significantly more disc florets were present in lines: p11, p12, p46 and p18. In these lines, the increase in the percentage of disc florets measured at the capitulum slice was significant, and this was even more pronounced when we investigated the floret numbers in the entire capitulum (Fig. 3A and 3B). No other noticeable phenotypical differences between the transformed lines and controls were observed. Subsequently, the relationship between *PDF2* expression level and disc/ray floret ratio was investigated by measuring the *PDF2* expression levels in bud and leaf tissues. Leaf tissue was used in addition to buds because multiple replicates could be easily sampled. Significantly lower expression was detected in lines p11, p12, and p46, which coincided with their increase in the percentage of disc florets counted on the slice and for the entire flower (Fig. 3A). While p18 did not have a significantly lower expression, this line did produce a higher percentage of disc florets. Further investigations into the total number of florets on a flower indicated that flowers of this line also had significantly fewer florets (143) compared to the average (188). This lower number of florets suggests other problems in flower development of this line, which could explain the effect on the floret ratio. To investigate whether the decrease in expression affected floret development during normal differentiation or during later developmental stages, we performed a morphological analysis during the normal differentiation stages determined in chapter 3. This showed an increased amount of disc florets distinguishable at stages s2 and s3 (Suppl fig 1), suggesting that transgenics were affected during the normal floret differentiation stage. So, to conclude, the correlation between lower expression and the increased percentage of disc florets for three independent lines suggest *PDF2* could be involved in floret identity. However, due to time constraints, not all obtained transgenic lines could be analysed by qRT-PCR, and therefore the analysis is not conclusive.

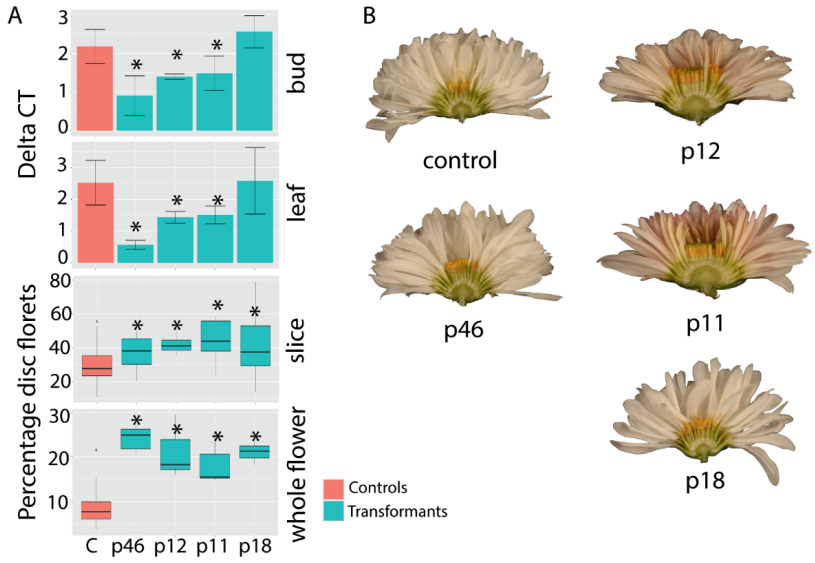

Figure 3. *PDF2* expression and phenotype of four lines with increased percentage disc florets. (A) Expression of *PDF2* in flower bud and leaf and the phenotype for the slice and entire flower for controls and four selected lines. The qPCR experiments for buds and leaves were performed with two and three biological replicates, respectively. Significance was tested using a student t-test over the difference in delta-ct values between gene and reference gene. (B) Image of the slice for one control and the four selected lines.

Lower levels of BR increase the percentage of disc florets.

Several BR-related genes were identified as differentially expressed in the transcriptome analysis of Chapter 3, and most of these are proposed to result in a lower level of BR in the mutants that developed more disc florets. Therefore, we hypothesized that reduction of BR would support disc floret development. We decided to develop an RNAi construct for *DWF1*, an early BR biosynthesis gene involved in converting 24-Methylenecholesterol to Campesterol. This gene also had a decreased expression during early flower bud stages of Mutant 1 (M1) and later stages of M2 of the RNAseq (Chapter 3 Table 2). For *DWF1* RNAi, we developed 18 lines, measured expression in bud and leaves, and phenotyped the percentage of disc florets at the slice (Suppl fig 2A and 2B). Four lines, d2, d7, d33 and d35, showed downregulation of *DWF1* in leaf tissue, and all of these developed more disc florets than the controls (Fig. 4A). For d7, d33 and d35, significant downregulation was also observed in the floral buds, while for d2, there was no significant difference in *DWF1* expression. While the decreased expression was more pronounced than that detected in the *PDF2* RNAi lines, the

phenotypical difference with the control lines was less pronounced. All four lines have an increase in disc florets, measured at the slice and for the complete flower, as seen in the slice's images (Fig. 4B). Interestingly, other classical phenotypes known for lower BR, such as dwarfism, were not noticed. Similarly to *PDF2*, floret differentiation appeared to increase in d35 at s2 and s3 (suppl fig 1), suggesting differentiation is affected during the typical floret differentiation stage. Although the correlation between expression level and phenotype was, in general, what was expected, there were a few outlier lines which exhibited decreased *DWF1* expression levels but rather showed a somewhat lower disc/ray floret ratio instead of a higher one (suppl fig 2). However, given the considerable number of lines in which phenotype and expression level were correlated, there is a good chance that the outliers were due to tissue culture or cutting effects.

Since BR is a hormone, we also decided to perform further functional analysis by applying exogenous Brassinolide (BL), a synthetic variant of BR, and Brassinazole (BZ), a BR inhibitor. Two experiments were performed, the first with both BL and BZ in two concentrations and the second with only 50 µM BZ for a larger number of plants. We applied both hormones three times a week from the first visual indication of developing buds until the flowers were mature. While BL did increase ray floret ligule length, it did not affect the percentage of disc florets (suppl fig 2C). The ligule length was increased for both 1581 (the variety used for transformations) and V1 (variety 1 used in Chapter 3). Unexpectedly, ligule length was decreased for V2 (variety 2 used in Chapter 3). The percentage of disc florets increased for V2 and appeared to increase for 1581, but the difference was not significant, probably due to limited numbers.

In the second experiment with only BZ treatments, BZ increased the percentage of disc florets of 1581 (Fig. 4C). For V1 and V2, an increased percentage of disc florets was not observed in the entire flowers (one measured per plant) nor in the flower slices (several flowers measured per plant) (Suppl fig 2D). The BZ treatment seemed to be performed under physiologically relevant conditions since there was an effect on the length of the treated plants, which were shorter at two weeks after the treatment was initiated. This difference in length disappeared later during development. Overall, both the *DWF1* downregulation and the BZ treatments should decrease BR levels in developing flowers, and both increased the percentage of disc florets in 1581, suggesting BR's involvement in the determination of disc/ray floret ratio.

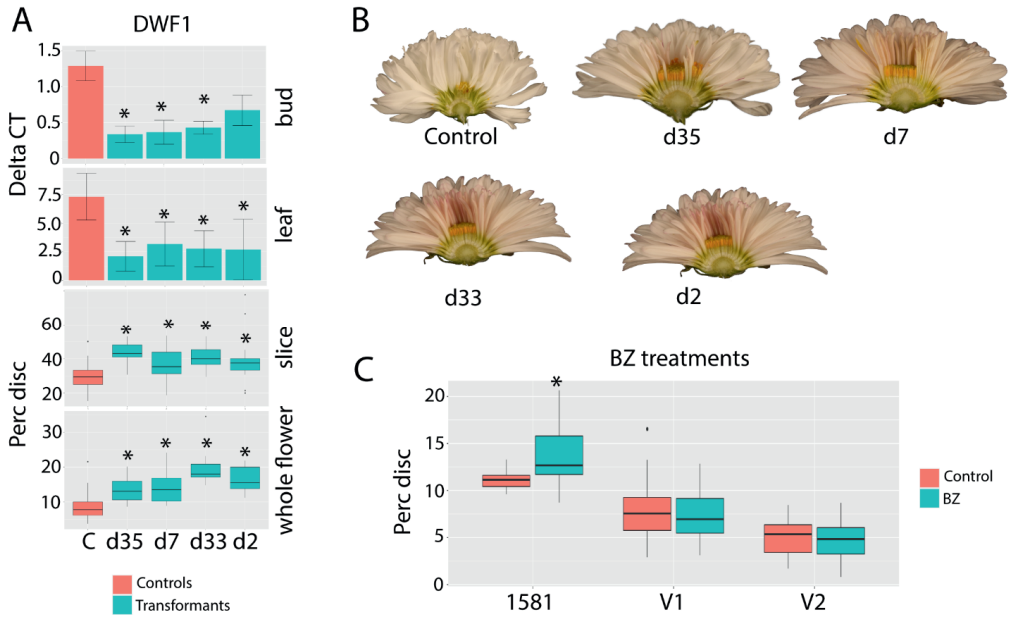

Figure 4. The effect of BR on the percentage of disc florets. (A) Expression levels of *DWF1* in flower bud and leaf tissue of selected *DWF1* RNAi lines. Significance was tested using a student t-test over the difference in delta-ct values between gene and reference gene, including a graph showing the percentage of disc florets at the slice and for the entire flower of selected *DWF1* RNAi lines. (B) Representative images of the flower slice of control and *DWF1* RNAi lines. (C) The percentage of disc florets measured for the entire flower for both control and BZ-treated plants of 1581.

Discussion

In this study, several DEGs identified in Chapter 3 were further characterized to determine if changes in their expression levels affected the disc/ray floret ratio. Various transformations did not produce the desired phenotype, and this could either be the result of an ineffective transformation or show that the candidate gene is not involved in the regulation of the disc/ray floret ratio. Here, these scenarios are discussed for the different genes.

The majority of the *RSL2* and *WIP2* lines did not have differences in the expression level of the targeted genes, suggesting that the transformations were not successful. Therefore, these genes could still be involved in regulating floret identity. Especially, the expression pattern of *RSL2*, with higher expression in ray than in disc florets and particularly high expression in flower bud stage 1 (Fig. 1F), remains interesting. For *HTH*, RNAi lines with

decreased expression were obtained, but this decrease was generally mild, and a phenotypic correlation with the expression level was not observed (Fig. 2E). Because of the mild effect of the RNAi construct, conclusions are difficult to draw here, though it seems unlikely that *HTH* is very important for the regulation of the disc/ray floret ratio. Given its expression, which is much higher in ray florets than in disc florets, and its putative function in the fusion of organ tissue (Krolikowski *et al.*, 2003; Xu *et al.*, 2017), *HTH* may rather function in the fusion of outgrowing ligules, which are more pronounced in ray florets. An aberrant ligule phenotype was not observed in the transgenic lines, however. Therefore, it is more likely that *HTH* is regulated by transcription factors involved in floret development (hence its differential expression in the mutants with more disc florets), but is itself not essential for floret specification or development.

GIR1 was selected because it may be involved in the outgrowth of organs by acting as an adaptor protein for *GL2* and possibly other HD-ZIP IV transcription factors (Wu and Citovsky, 2017a, 2017b). Several HD-ZIP IV genes were differentially expressed in M1/M2, but *GIR1* expression showed the most promising pattern in both mutants (Chapter 3), and this gene is predominantly expressed in reproductive tissues. Therefore, *GIR1* was chosen for both RNAi and overexpression. Several RNAi lines showed considerable downregulation, but a correlation between flower phenotype and the extent of downregulation was not observed. This indicates that *GIR1* is not required for the regulation of disc/ray floret identity, although it cannot be excluded that the gene would act redundantly with a yet unidentified factor or that the extent of down-regulation was not sufficient to exert an effect. The overexpression lines had some increase in expression. However, the only line that showed the expected decrease in the percentage of disc florets was the one not exhibiting any difference in expression (Fig. 2B). This supports the idea that *GIR1* does not have an important function in the regulation of floret identity, though the lack of an effect could also indicate that overexpression of *GIR1* alone is not sufficient, given the fact that it is an adapter protein. In conclusion, for *GIR1,* we cannot exclude the possibility that it is involved in determining floret identity, but the RNAi results suggest that it is certainly not a key regulator.

Finally, *TINY2* expression was high in stage 1 buds, ray florets and stem, suggesting a more diverse role of *TINY2* (Fig. 1D). This was expected based on the literature since it is an integrator regulating stress and BR-induced growth. There seemed to be a high variation in the *TINY2* overexpression lines, varying from a limited increase to an enormous increase in expression (Fig. 2F). However, none of them had the expected negative effect on the percentage

of disc florets. Thus, it can be concluded that *TINY2* does not influence the percentage of disc florets.

Unfortunately, we observed also a high phenotypic variation in the control lines, which had gone through tissue culture but were not infiltrated with Agrobacterium. A possible explanation for this is that random mutations occurred during the tissue culture process. This is a common effect in tissue culture cultivation, specifically when plants go through a single cell phase, which can induce genotypical and epigenetic modifications compared to the starting material (Larkin and Scowcroft, 1981; Filipecki and Malepszy, 2006; Wang *et al.*, 2019). Early research in sugarcane even noticed chromosome number differences because of going through a callus phase, with a difference in mutation rate between populations (Heinz and Mee, 1971). Such modifications may also have occurred during the Chrysanthemum tissue culture in our studies. Of the 21 controls, two had a lower percentage, and one had a significantly higher percentage of disc florets (Fig. 2G). This clearly shows that the phenotype of transformed plants can be changed during tissue culture independent of the presence of a transgene. This effect may also explain some of the outliers among the transgenic lines, such as in the case of p18 (*PDF2* RNAi line), which had no *PDF2* downregulation, but an increase in the disc/ray floret ratio (discussed in the next paragraph). In some cases, transformants appeared very weak or looked overall aberrant, possibly also due to a tissue-culture effect. These plants were excluded from the analyses. Thus, single outliers may be possible in the analysis of the transgenic lines, requiring an overall correlation between phenotype and expression level for a larger number of confirmed transgenics to conclude that a gene is involved in the determination of floret identity. This correlation was not identified for the above-mentioned genes, but for *PDF2* and *DWF1*, inspiring results were obtained, and these genes are therefore further discussed below.

***PDF2* effect on disc/ray ratio**

PDF2 is interesting because it was identified in both the differential expression analysis of Chapter 3 and the genetic analysis of chapter 4. Interestingly, three lines consistently showed downregulation in leaf and bud tissue and showed the expected increase in the percentage of disc florets (Fig. 3A and 3B). However, we were only able to sufficiently characterize four lines and did not manage to determine the expression of other lines. Hence, evidence that this gene is involved in regulating disc/ray floret ratio is not conclusive. Analysis of *PDF2* expression in tissues indicated high expression in both bud stages, which decreased in the florets (Fig. 1A). This suggests an involvement in the specification of floret identity rather than

in its outgrowth. Since more of these HD-ZIP IV were differentially expressed, they could also be involved, as they are known to work together and form dimers (Kamata *et al.*, 2013). Because Arabidopsis and Chrysanthemum are only distantly related, it was not completely clear whether the gene we found was more similar to *PDF2* or to *ATML1*. *ATML1*, a close homolog of *PDF2*, is known to regulate organ identity. In Arabidopsis, *PDF2* and *ATML1* play an essential role in regulating shoot organs from the L1 layer during embryo development (Abe *et al.*, 2003; Ogawa *et al.*, 2014). Research in Chrysanthemum using a CpYGFP reporter, which was specifically transformed in the L1 layer, showed that the epidermal layer of receptacle, petal, anther, filament, style, stigma, and ovule tissues was developed from L1 (Aida *et al.*, 2020). In Arabidopsis, *PDF2* and *ATML1* had a redundant function, and both needed to be knocked out. Using a heat-activated silencing construct, it was also noticed that *PDF2*/*ATML1* had an important function in flower development (Ogawa *et al.*, 2014). Other research has revealed that *PDF2* can regulate B function MADS-box gene *AP3* and, to a lesser extent, *PISTILLATA* (*PI*) (Kamata *et al.*, 2013, 2014). In that experiment, knocking down of *PDF2* did not influence *AP3* expression in young primordia, but it reduced *AP3* expression during the development of the petals and stamens, thereby affecting the outgrowth. This is remarkably similar to the development of ray florets in Asteraceae species such as Gerbera, where stamen primordia are formed but do not develop (Kotilainen *et al.*, 2000; Laitinen *et al.*, 2006; Stuessy and Urtubey, 2006). Therefore, we consider *PDF2* and possibly other HD-ZIP IV factors good candidates to regulate *AP3* differently in both types of florets[47], but future research will have to clarify the functions of *PDF2*-like genes in Chrysanthemum further.

The effect of brassinosteroid on disc/ray floret ratio

In this Chapter, both downregulation of a BR biosynthesis gene using RNAi and BZ treatments resulted in an increased percentage of disc florets. This was consistent with the RNAseq in Chapter 3, which discovered that many BR-related DEGs were more lowly expressed in mutants with more disc florets.

We decided to perform RNAi on *DWF1* because it encodes an early BR biosynthesis gene (Choe *et al.*, 1999). Since it is an early biosynthesis gene, it was likely that this would affect BR levels in all tissues, including the developing floral meristems (FM). In four lines, we had significantly lower expression of *DWF1* in leaves. For buds, expression was also lower. However, for line d2, this could not be confirmed (Fig. 4A, B). The phenotypes of all lines were as expected, increasing the percentage of disc florets. Unexpectedly, we did not see any classical phenotypes associated with lower BR, such as dwarfism (Peres *et al.*, 2019). The

plants were not noticeably shorter, nor did there appear to be a difference in the length of inflorescence stems. This was unexpected since *DWF1* was most highly expressed in stem and leaf (Fig. 1C). These tissues may have more active mechanisms that compensate for reduced BR biosynthesis since it is essential for normal plant growth. Alternatively, there was another *DWF1* homolog, which may be expressed in vegetative tissues, while the targeted *DWF1* may be the only player in the flower head. Expression of *DWF1* was slightly lower in the buds and ray florets and lowest in disc florets. This is consistent with the lower expression in mutants with increased disc florets and the RNAi results of the transformants. So, the data show that a decrease in *DWF1* increases the percentage of disc florets.

To further prove that the effect results from altered BR levels, we performed exogenous hormone treatments with BL (active BR) and BZ (BR inhibitor). In a large experiment, the BZ, which should reduce BR concentrations, produced similar results as the *DWF1* RNAi and increased the percentage of disc florets in Chrysanthemum 1581. However, it did not increase the percentage of disc florets in V1 and V2, the varieties used in the RNAseq experiment. In an earlier experiment with fewer plants and two concentrations, BZ did increase the disc floret percentage for V2. While there appeared to be an increase for 1581, this could not be confirmed due to a limited number of plants. BL treatments were also conducted, but these did not affect the percentage of disc florets. However, they increased the ligule length of ray florets, thereby affecting the plants. This effect was also seen in experiments in *Gerbera* (Huang *et al.*, 2017). The lack of effect from the BL treatments is not wholly unexpected. BL is an important hormone, usually available in extremely low concentrations, and has a negative feedback mechanism to prevent high concentrations (Wu *et al.*, 2011; Peres *et al.*, 2019). Furthermore, BL is barely or entirely immobile, and therefore the application presumably needs to be done extremely local (Symons *et al.*, 2008). We attempted to get to the meristem by opening the involucral bracts and pipetting underneath to get the solution at the meristem, but it is possible that the BL did not reach the floret primordia cells. Another possibility is that the BL concentration was extremely high for the plants, causing an adverse reaction. Therefore, testing lower concentrations may result in a better effect. While the BZ treatments did not always cause a significant increase in disc florets, there were results in both experiments and for two different varieties, suggesting together with the RNAi experiments that lower BR concentrations result in a higher disc/ray floret ratio.

There has not been any literature suggesting BR might influence disc/ray floret ratio. However, BR is essential for correct organ fusion, and BR mutants exhibit abnormal growth of leaves and flower organs due to organ boundary defects (Li and He, 2020). This is likely

caused because BR was shown to regulate several boundary genes (Wang *et al.*, 2002; Yin *et al.*, 2002). Interestingly, several of these were implicated to be involved in the development of the radiate capitulum type in the Asteraceae (Wen *et al.*, 2022). Furthermore, BR plays an essential role in male fertility by regulating major genes responsible for correct stamen development (Ye *et al.*, 2010). Among others, *AG* and *SPOROCYTELESS* (*SPL*) can be directly targeted by *BRASSINAZOLE RESISTANT 1* (*BZR1*) and *BR1-EMS-SUPPRESSOR 1* (*BES1*) (Sun *et al.*, 2010; Ye *et al.*, 2010; Chen *et al.*, 2019). Also, male papaya flowers were reported to have lower expression levels of auxin/BR genes, and it was postulated that lower levels in these flowers are involved in pistil abortion (Liao *et al.*, 2022).
This indicates that BR can be involved in correct floral organ development. However, we found that lower levels of BR are associated with more disc florets, which develop functioning anthers. Therefore, it is likely that BR is most important to stimulate ray floret development in Chrysanthemum.

Several studies have shown that *CYCLOIDEA2* (*CYC2*) plays a vital role in determining floret identity. *CYC2* is a TCP transcription factor involved in the outgrowth of organs and was first found to have an important function in a symmetrical outgrowth of snapdragon petals(Clark and Coen, 2002). Several studies in sunflower, Senecio and Gerbera have shown that *CYC2* is more highly expressed in ray florets and that overexpression causes a conversion to more ray floret like disc florets (Chapman *et al.*, 2012; Tahtiharju *et al.*, 2012; Juntheikki-Palovaara *et al.*, 2014; Chen *et al.*, 2018; Shen *et al.*, 2021). If there is a change in identity is not completely clear. There is a connection between TCP transcription factors and BR. For instance, *TCP1* can regulate the expression of *DWF4*, thereby modulating BR biosynthesis in Arabidopsis (Guo *et al.*, 2010). A similar mechanism involving CYC2 and BR may be involved in floret identity. This would suggest that CYC most likely regulates BR. However, our data shows that a reduction in BR levels is enough to increase the disc/ray floret ratio in Chrysanthemum.

Overall conclusion and recommendations

The data have shown that BR and possibly *PDF2* play an important role in determining the disc/ray floret ratio, two factors never associated with floret identity before. Lower levels of both result in an increase in the percentage of disc florets. They may act in the same pathway since both have an important function in regulating the growth of organs in the L1 layer (Savaldi-Goldstein, Peto and Chory, 2007; Kamata *et al.*, 2014; Ogawa *et al.*, 2014). For root hair differentiation, researchers have proposed a model combining BR and *GL2* to work

together in the same pathway (Li *et al.*, 2022). A similar mechanism could work for BR and *PDF2* in floret differentiation.

For *PDF2,* it is possible to try and identify allelic variants that influence the floret ratio. Because this specific *PDF2* homolog is also present in a QTL/GWAS region on chromosome 1, suggesting that allelic variation is already available. Developing markers to distinguish these variants could assist in breeding programs to select for parents that will produce offspring with an increased amount of ray florets. Additionally, BZ treatments can temporarily increase the disc/ray floret ratio without affecting genetics. With an increase in disc florets, seed set can be increased, resulting in better chances of producing new varieties with desired traits. Combining these strategies could increase the number of seeds for the progeny with increased ray florets.

Material and methods

Plant material and media

Chrysanthemum morifolium '1581' was used for transformation. Tissue culture plants were grown in a climate room at 20 degrees for 16 hours with fluorescent tube light. All medium concentrations are calculated to one litre. Multiplication medium contained 0.5 MURASHIGE SKOOG (MS) basal salt mixture and full MS vitamins, 3 percent sugar and 8 grams lab M agar, pH5.8 and 0.1mg Indole-3-acetic acid (IAA). Inoculation media contained 3 percent sugar, full MS, 1mg 6-Benzylaminopurine (BA), 1mg IAA and 100uM acetosyringone. Cocultivation medium contained 3 percent sugar, full MS, 1mg 1-Naphthaleneacetic acid (NAA), 7g Plantagar and 100uM acetosyringone. The selection medium contained 3 percent sugar, full MS, 1mg BA, 1mg NAA, 25 mg kanamycin, 500 mg Carbenicillin and 7-gram Plantagar. Transformed plants were grown until they developed roots and consequently moved to soil in a growing chamber at 20 degrees Celsius. Plants were kept underneath a plastic sheet and in long-day conditions (16 hours light) for one week and then moved to short-day conditions with 10 hours light. Plants were fed with Hyponex 1g/L fertilizer once a week.

Cloning

RNAi and overexpression constructs were generated using a Goldengate approach (Weber *et al.*, 2011), in which different modules were coupled together in the binary vector pICSL4723. For the overexpression construct, the following modules were combined: 35S:NPTII in pICH47732, 35S:GFP in pICH47742 (to assess transformation success), PcUbi in pICH47751,

Full-length CDS in pICH47761, terminator tChrRbcS in pICH47772, End-linker pELE5. Level 1 constructs were prepared using BsaI; level 2 constructs using BpiI. The primers used for generating the modules that were newly prepared for this study can be found in Suppl. Table 1. For the RNAi constructs, a fragment-intron-inverted fragment module was cloned into pICH47761. Fragments consisted of 300-400 bp of coding sequence and were selected in a region of low homology with other sequences. The intron contained 417 bp of the intron from vector pK7GWIWG2 (Karimi et al., 2002), excluding Bpi/BsaI sites. The three fragments were PCR-amplified from the plasmid template (intron) and from Chrysanthemum cDNA (gene fragments) and connected using the GoldenGate approach with inner overhangs specific for the intron fragment and outer overhangs specific for the level 1 vector. All other modules in the RNAi construct were identical to the ones in the overexpression construct. After level 2 assembly, the resulting constructs were checked using restriction digestion and sequencing and transformed into Agrobacterium strain AGL0.

Transformation

On day one, AGL0 was inoculated in 15ml LB with kanamycin, gentamicin, rifampicin and 100uM acetosyringone and grown overnight in the shaker at 28 degrees Celsius. Day 2, Agrobacterium was centrifuged for 20 minutes at 3500 rpm and resuspended in inoculation medium to an OD600 concentration of 0.5. Young leaves were cut while submerged in inoculation medium to squares with sides of about 0.5 cm without including larger leaf nerves. Leaf explants were incubated in inoculation medium including Agrobacterium for 15 minutes, then rinsed in inoculation medium without Agrobacterium. Subsequently, leaf explants were dried by dabbing on filtration paper and then moved with the bottom-up on cocultivation medium. Next, leaf explants were inoculated in the climate chamber with 16 hours of light at 21 degrees Celsius underneath two layers of filtration paper. Controls with bacterium and without kanamycin, without bacterium and kanamycin and without bacterium with kanamycin were included. On day 4, leaf explants were moved from cocultivation medium to selection medium, and this medium was refreshed every three weeks. New appearing shoots were checked using UV light to detect (Green fluorescent protein) GFP signal to distinguish transformed shoots. These shoots were moved to multiplication medium to develop roots. Most shoots did not emit a GFP signal and did not appear to carry a construct. These presumably receive energy from part of the callus carrying a construct with anti-biotic resistance and, therefore, can be regarded as escapes. These non-GFP shoots did not survive on the selection medium and were discarded. A control plate was added with medium without kanamycin and

without inoculation with Agrobacterium for each transformation. These explants were treated identically to the transformed plants with the same hormone levels, producing control plants used for expression analyses and phenotyping.

Phenotyping

All flowers of each plant were collected when they were fully ripened and sliced in half. Disc and ray florets were counted for one of the slice surfaces. The percentage disc on this slice was calculated for each flower. These measurements essentially estimate the percentage of disc florets at the diameter of a circle. These measurements could be converted to an estimate of the true amount of florets by using the formula to determine the surface of a circle using the diameter. However, this does not change significantly, and these calculations produce artificial values. For *PDF2* and *DWF1*, clones were produced through cuttings. For these second plants, in addition to counting the slice, disc and ray florets of several flowers were also counted for the entire capitulum.

Stage determination using microscopy

Flower buds of several sizes per stage, focusing on stages 2 and 3 were collected and dissected for two controls and p11 (PDF2) and d35 (DWF1). These were analysed, and images were taken using a stereo microscope with enlargements ranging from 16 to 50 times. A ruler was used to determine the size in millimetres for each enlargement. For several dissected inflorescences, a series of images was taken focusing on different layers, and these were compiled using Photoshop to get a sharp image of the entire inflorescence.

qPCR

All samples were taken around 11:00 AM. Collecting samples for qPCR of different tissues was done on two biological samples. For transformants in the first round, single biological samples of stage 0/1 buds were taken. For further analysis of *PDF2* and *DWF1* expression, a mix of stage 0-3 buds was sampled in triplicate. Some samples failed, and for these, only a duplicate remained. RNA was isolated using the CTAB method for RNA extraction in liquid nitrogen, working in small volumes with 450ul CTAB. cDNA synthesis was performed using Superscript IV reverse transcriptase of ThermoFisher scientific and random hexamer primers according to the manufacturer's protocol. qPCR was performed using power SYBR^{TM} green PCR master mix (article nr 4367659 ThermoFisher scientific) in a reaction volume of 20ul, with 4µl 1mM primers and 6ul of 5 times diluted cDNA. qPCR was done in a bio-rad cycler

according to the ThermoFisher scientific protocol. Technical duplicates were used. Two reference genes, *CmPGK* and *CmSAND* (Qi *et al.*, 2016), were used. *CmSAND* was used as an extra control, *and CmPGK* was used for all calculations and graphs.

Brassinolide and brassinazole treatments

Epibrassinolide and Brassinazole were ordered from Sigma. BL and BZ stock was solved in 96 percent ethanol to a concentration of 5mM and was stored at -80^{0}C in aliquots. These were diluted in miliQ and used fresh every treatment to a total volume of 10ml. 0.2% silwet was added for better surface tension. Controls were prepared similarly, but by adding 96 percent ethanol without hormone in the same dilution. 100ul of the solution was applied for every individual treatment by pipetting the solution to the apical meristem. This treatment was repeated three times a week (Monday, Wednesday, and Friday). Treatment started one week after the start of a short day and ended when the plants were fully opened. The percentage disc florets of seven flowers were measured at each plant's slice surface. An average was calculated per plant and considered one biological replicate. A T-test was performed to distinguish significant differences. The first experiment was conducted with 10uM (BL10, BZ10) and 50uM (BL50, BZ50) for both BL and BZ. For 1581, six plants for each treatment were used; control, BZ and BL 10uM were used; due to limited plants, the 50uM was not performed for this variety. For V1, 12 controls, nine plants BZ10, six plants for BZ50, nine plants BL10, and five plants BL50. V2 had nine control plants, ten plants for both BZ10 and BL10, and seven plants for BZ50 and BL50. For the second experiment, BZ concentration was 50mM. V1 17 plants were treated with BZ, and 20 control plants were treated with water. 1581 18 BZ and 17 control plants, V2 had 31 BZ treated and 30 control plants. In addition to measuring the percentage disc on the slice, all florets on the entire flower were also measured for the second flower.

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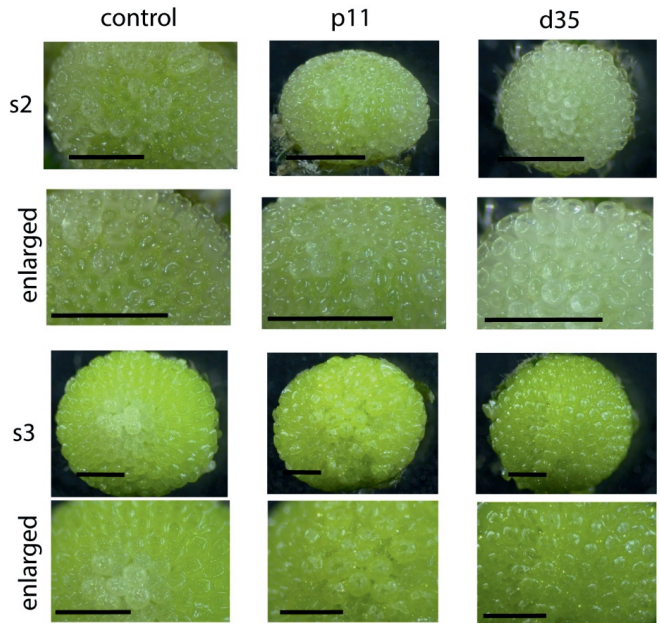
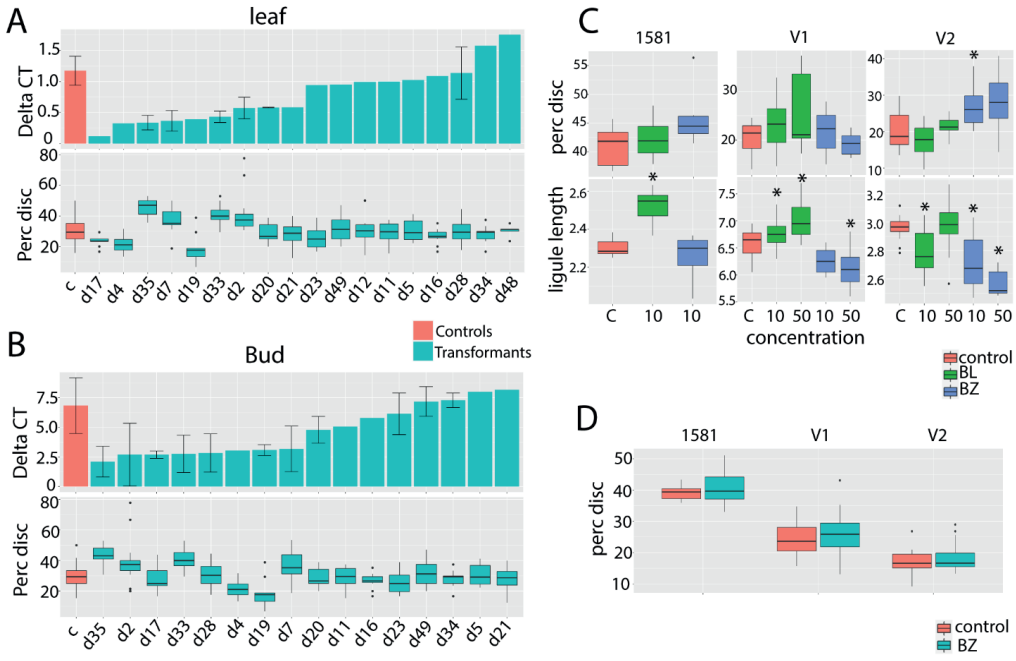
We are grateful to our colleagues from the Plant Breeding department for providing the ChrRbcS terminator sequence and advising on the transformation protocol. Additionally, we are grateful to Lowi Braas for taking special care of the plants used for brassinosteroid treatment.



Supplementary figure 1. Inflorescence development of flower buds of transformants. Floral buds were dissected in stages 2 and 3 to determine if floret identity is already determined in this stage for the mutants. Disc florets are already distinguishable as radially symmetrical, while ray florets display zygomorphic symmetry. Data are shown for a representative image of the controls, p11 and d35, in which p11 is a *PDF2* and d35 is a *DWF1* transgenic line. The number of disc florets for p11 and d35 seemed to increase compared to the control. The bar is 1mm.



Supplementary figure 2. DWF1 transformation results and BZ and BL results. (A) QPCR result for 18 lines in leaf with corresponding percentage disc floret results. (B) QPCR result for 16 lines in buds with corresponding percentage disc floret results. (C) The percentage disc florets and ligule length of ray florets for hormone-treated plants. Both BZ and BL are in two concentrations. (D) The percentage disc florets for control and BZ treated plants, measured on the slice.

Supplementary table 1. Primer sequences used in this chapter. The first columns indicates whether it was used for cloning or qPCR. The second column is the primers name, including 'gene name' and purpose. The last column is the primer sequence.

application	primer	primer sequence
cloning	intron FW p RNAi	CTGTGGTCTCAGCTGTTATGTTCAGTGTCAAGC
cloning	intron RV p RNAi	CTGTGGTCTCAGCAGAAGCAACCTCATGGAAGGTAAAA CACTCTAAATCCTCTTC
cloning	GIR F1 p RNAi	CTGTGGTCTCAGGAGTGAGGCTCCAAACCAACAAC
cloning	GIR R1 p RNAi	CTGTGGTCTCACAGCCATCGAGGACACCCAACCA
cloning	GIR R2 p RNAi	CTGTGGTCTCACTGCCATCGAGGACACCCAACCAA
cloning	GIR F2 p RNAi	CTGTGGTCTCAAGCGTGAGGCTCCAAACCAACAAC
cloning	HTH F1 p RNAi	CTGTGGTCTCAGGAGTGTTGAGGGACATACAGCGT

cloning	HTH R1 p RNAi	CTGTGGTCTCACAGCGCTACCACCCACACGTTTTCG
cloning	HTH R2 p RNAi	CTGTGGTCTCACTGCGCTACCACCCACACGTTTTCG
cloning	HTH F2 p RNAi	CTGTGGTCTCAAGCGTGTTGAGGGACATACAGCGT
cloning	FW2 t ChrRbcS	TGTGGTCTCAGGAGTCATAAGCCCGATGGCTACTAAGT TTTAC
cloning	RV2 t ChrRbcS	TGTGGTCTCAAGCGCAATTAATGCGTCTACCGTAGTTGC TACAG
cloning	GIR1 R1B p RNAi	CTGTGGTCTCAAGCGCATCGAGGACACCCAACCA
cloning	HTH R1B p RNAi	CTGTGGTCTCAAGCGGCTACCACCCACACGTTTTCG
cloning	RSL RNAi F1	CTGTGGTCTCAGGAGGTGCGGTTTATGTGGTTCG
cloning	RSL RNAi R1	GACTGGTCTCACAGCAAGGTTGCGTGTGAATGGTG
cloning	RSL RNAi R1B	CTGTGGTCTCAGCAGAAGGTTGCGTGTGAATGGTG
cloning	RSL RNAi R2	CTGTGGTCTCACTGCAAGGTTGCGTGTGAATGGTG
cloning	RSL RNAi F2	CTGTGGTCTCAAGCGGTGCGGTTTATGTGGTTCG
cloning	DWF1A F1	CAGAGGTCTCAGGAGAGCATACACTACCGTCGTC
cloning	DWF1A R1	GACTGGTCTCACAGCATGCCCACTGTAACCCATG
cloning	DWF1A R1B	CTGTGGTCTCAGCAGATGCCCACTGTAACCCATG
cloning	DWF1A R2	GTCTGGTCTCACTGCATGCCCACTGTAACCCATG
cloning	DWF1A F2	GTCTGGTCTCAAGCGAGCATACACTACCGTCGTC
cloning	GIR1 OE For	CTGTGGTCTCAGGAGATGAGCCGAAGAAACGGTG
cloning	GIR1 OE Rev	CTGTGGTCTCAAGCGTAACTCTTTTTTGTTTTCTTGAGG
cloning	PDF2 RNAi F1	CTGTGGTCTCAGGAGTGTTGAAGTTGTCCGAGCGA
cloning	PDF2 RNAi R1	GACTGGTCTCACAGCGACACAATTGCCTGGATCG
cloning	PDF2 RNAi R1B	CGTTGGTCTCAAGCGGACACAATTGCCTGGATCG
cloning	PDF2 RNAi R2	CTGTGGTCTCACTGCGACACAATTGCCTGGATCG
cloning	PDF2 RNAi F2	CTGTGGTCTCAAGCGTGTTGAAGTTGTCCGAGCGA
cloning	TINY OE FRAG 1F	CTGTGGTCTCAGGAGATGGCTAGTGGTCACAAAAC
cloning	TINY OE FRAG 1R	CTGTGGTCTCAGCAACTCATGTCCTCCTTG
cloning	TINY OE FRAG 2F	CTGTGGTCTCATTGCAAACATCCAGTGTACC
cloning	TINY OE FRAG 2R	CTGTGGTCTCAAGCGCTAGGCAAGAAAACTATCGA
cloning	WIP2 OE FRAG 1F	CTGTGGTCTCAGGAGGCATCAAGTTTTTGACAACATG
cloning	WIP2 OE FRAG 1R	CTGTGGTCTCAGTGATTCGGGACCTCTTCTG
cloning	WIP2 OE FRAG 2F	CTGTGGTCTCATCACTAAGAGGTAGACAACC
cloning	WIP2 OE FRAG 2R	CTGTGGTCTCAAGCGTCATTGCTCAATTTCAGAAACAC
qPCR	WIP2-52295-F	GCAATACCAAAGCTTGCCACA

qPCR	WIP2-52295-R	GACAAGCCGTTCATGTGTCAC
qPCR	Blm-GIR-33154-F	AGCTCTTGGTGGCGAAAGATT
qPCR	Blm-GIR-33154-R	GGTGGTAGTCCGAAGCTTGAT
qPCR	67771RSL2-F	CTGCTCACCCCTACTTTGAGG
qPCR	67771RSL2-R	AGAAGAGGAAGCGGTTGAGTG
qPCR	DWF1-45082-F	CTTTTGGCGTTGCTTGTACGA
qPCR	DWF1-45082-R	GGAAGAAGGTGTGGGTCGATT
qPCR	Spacer-1-fw_RNAi	GCATCTAAACCCCTATGGCCA
qPCR	Spacer-1-rv_RNAi	AGGTGGCACTTGTTGGTATGA
qPCR	TINY2-2_fw	GCTAGAGCTCATGATGTAGCTGCA
qPCR	TINY2-2_rv	GGGGATATAGAAGCAGGGCG
qPCR	PDF2-827F	CATGGCTGCAACTGCTAGTTC
qPCR	PDF2-901R	AACCTTCTGCGGTCAGTTTCA
qPCR	HTH-677F	GAAGTGCAAGGCAAACGAGC
qPCR	HTH-728R	TTGACCCATTGTGGCTAGCG
qPCR	TINY2-ox-fw	TTGAGACGTTGGATGGTCCG
qPCR	WIP1-ox-fw	CATTTGGGAAAGGGCATGG
qPCR	TINY2-2_fw_ox	GCTAGAGCTCATGATGTAGCTGCA
qPCR	TINY2-2_rv_ox	GGGGATATAGAAGCAGGGCG
qPCR	GIR1-4-fw_ox	CTCATGTACATCATGCTATCCGAG
qPCR	GIR1-4-rv_ox	GTTCTCACGTACCACATCAAGCAA
qPCR	GIR1-2-RV	CCAGAGGCCACGTCTATGATG
qPCR	GIR1-2-FW	TGCTCTTGCATTTAGGGCACT
qPCR	DWF1-2-F_RNAi	GTAACAATGGGTCAGCTCACGAG
qPCR	DWF1-2-R_RNAi	GCGAGCTTCCTTCAAGTCCA
qPCR	RSL2-2-FW_RNAi	GTGCCTGCATTAGCCACTTGA
qPCR	RSL2-2-RV_RNAi	ATACTCGACTCCTCCTCCTTCGA
qPCR	Ref_EF1a_Fw	CCATTCAAGCGACAGACTCA
qPCR	Ref_EF1a_Rv	TTTTGGTATCTGGTCCTGGAG
qPCR	Ref_PGK_Fw	TGCCACATACAAGAATAACCAACG
qPCR	Ref_PGK_Rv	GGGCTACGGGCAAGAGTACA
qPCR	Ref_SAND_Fw	CGTTGCTCACTACGAGTTCAC
qPCR	Ref_SAND_Rv	GCAGATGGGTCAACAGGTAA

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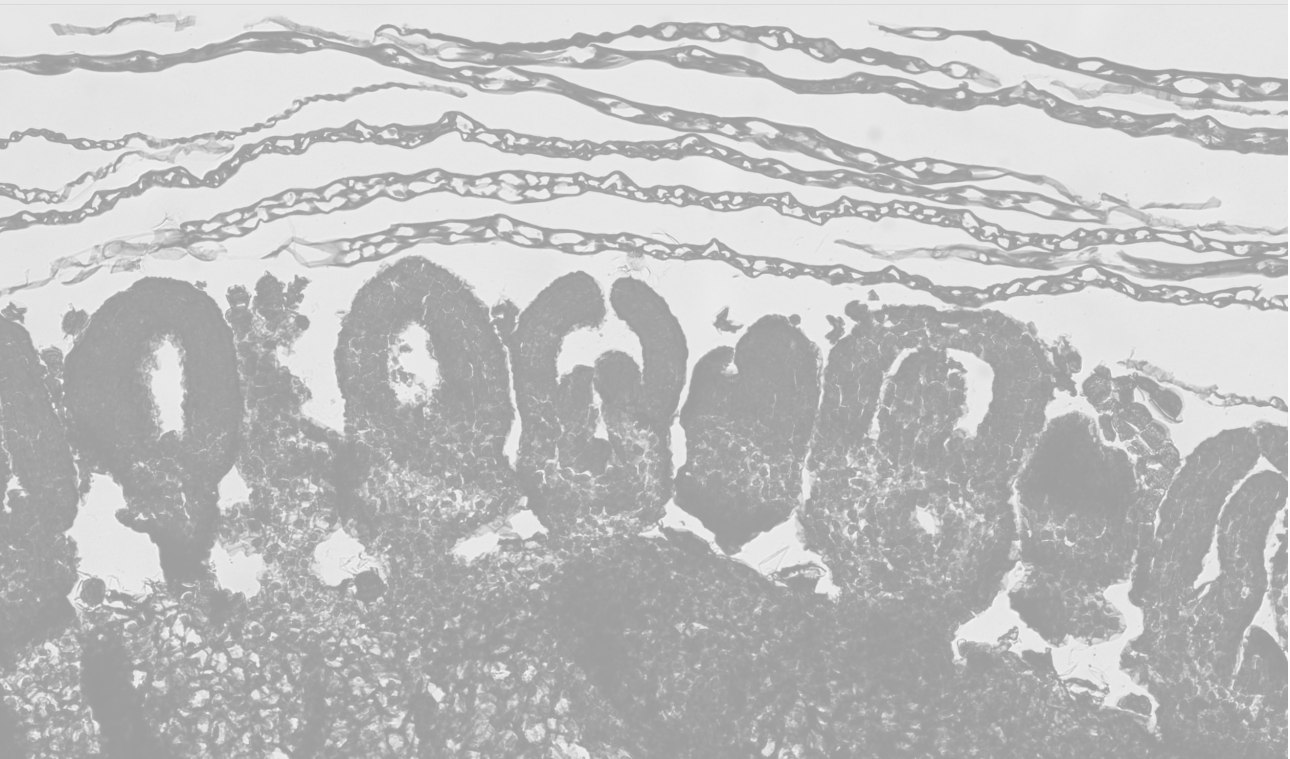
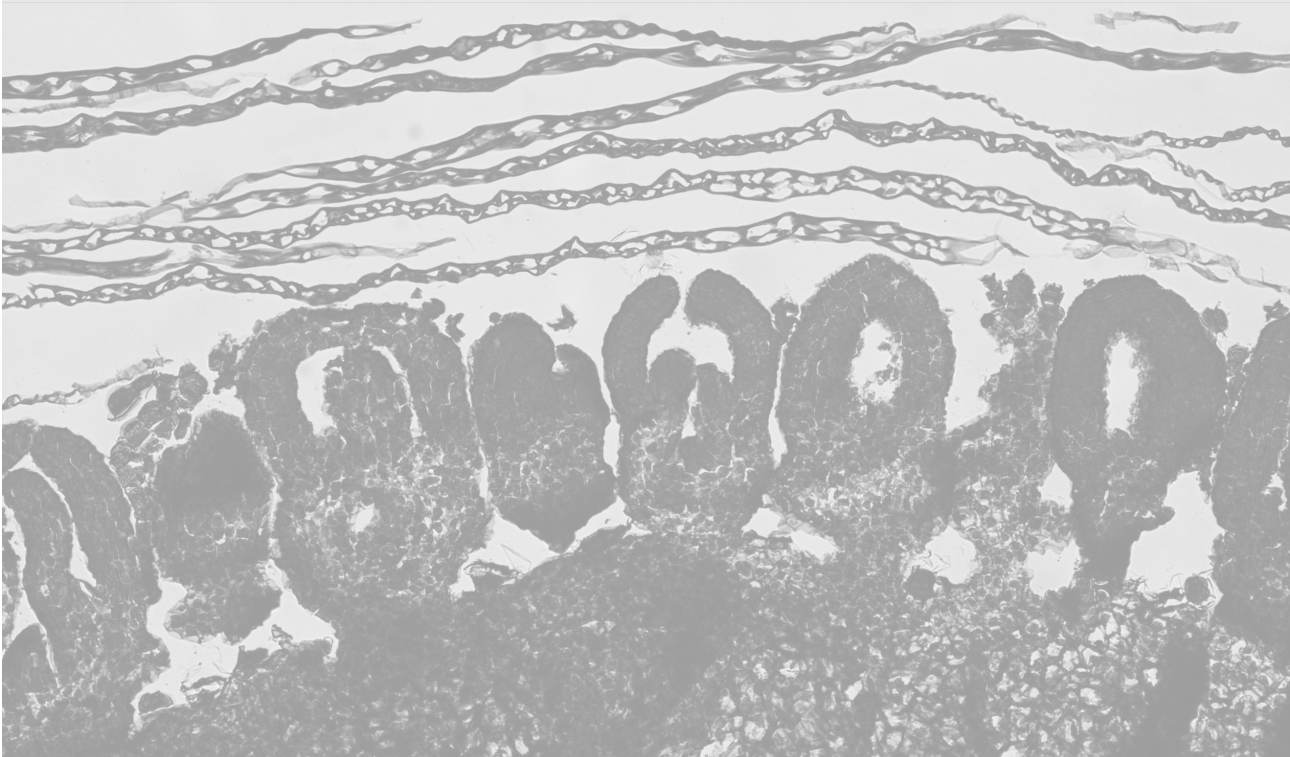


Chapter 6

General discussion



The cultivated Chrysanthemum has a diverse range of flower types, but breeding efforts are hampered in flower types with increased ornamental value because of low seed set. We showed in this thesis that low seed set results from a decreased percentage of disc florets and discovered that differences in fertility between floret types could be partially explained by the fact that ray florets produce lower quality stigmas. Subsequently, we identified candidate genes that regulate the disc/ray floret ratio using transcriptome analysis of mutants with an increased percentage of disc florets and through a genetic approach using QTL analysis and GWAS. We then performed a functional analysis for part of these factors to validate their supposed effect on the disc/ray floret ratio. In this chapter, the results of the performed morphological, genetic, molecular, and hormonal analyses are discussed and placed in the context of results from other studies. Additionally, recommendations are provided for further research in Chrysanthemum and other Asteraceae species and for breeders to improve seed set in varieties with high ornamental value.

Differences between the morphology of disc and ray florets

The first research question addressed in this thesis was what causes low seed in varieties with high ornamental value, and we hypothesised that this was caused by a low ratio of disc/ray florets. Therefore, we analysed different flower types with various disc/ray floret ratios and confirmed the positive relationship between the percentage of disc florets and seed set upon hand pollination. Thus, even though excess pollen was used for the hand pollinations, seed set is lower in ornamental flowers than in daisy-type flowers, suggesting that female fertility is different between disc and ray florets. This was of great importance for the rest of the thesis as it evoked the interest in gaining knowledge about the mechanism that influences the disc/ray floret ratio. Depending on the mutations causing double-flowered phenotypes in other Asteraceae, ray florets could have similar fertility problems in species such as *Gerbera* or Dahlia. Unfortunately, the majority of the morphological analyses of Chrysanthemum and other Asteraceae were performed many years ago, and since Chrysanthemum is not a model crop, recent studies are scarce. Anderson and Asscher in 1989 reported fertility problems during their research on self-incompatibility in Chrysanthemum and already encountered male and female fertility problems (Anderson *et al.*, 1989). Actually, most research in plants related to fertility focuses on the development of stamen and pollen, processes that are often severely impacted by heat stress (Hedhly, Hormaza and Herrero, 2005; Giorno *et al.*, 2013; Müller and Rieu, 2016). On the contrary, reduced female fertility has been given much less attention. This led us to investigate problems in the female reproductive organs of Chrysanthemum ray florets

in comparison to disc florets. Only one study focussed on fertility in Chrysanthemum specifically, including an investigation of female reproductive organs during an inbreeding process. They reported problems with pollen viability and embryo abortion and the most significant problem was with pollen ingrowth (Wang *et al.*, 2018). We determined that ray florets produced more often stigma with altered morphology, analogous to non-mature stigmas. Additionally, there was a variation in stigma receptivity between flower heads, which was floret-type independent, and negatively affected the number of crosses producing seeds. An explanation could be that ray florets evolved to attract pollinators. For radiate species such as Chrysanthemum, the original flowers only have a row of ray florets at the rim of the capitulum (Anderson, 2007). The development of functional reproductive organs, which costs energy, does not necessarily make sense for these limited amounts of florets. Therefore, there was likely no or limited natural selection on functional reproductive organs of ray florets. The differences in reproductive organs in ray florets between Asteraceae species could be consequential to differences in selection pressure. However, while this might explain differences in fertility between disc and ray florets, it does not help elucidate the mechanisms through which they are developed.

Little is known about the development of the open two-lobed Chrysanthemum stigma, which is morphologically distinct from that of species such as Arabidopsis and tomato, which both develop a single closed stigma (de Folter, 2020). We show that lower stigma quality results in lower pollen capture and, subsequently, seed set. The main factor associated with lower stigma quality was the degree of opening of the stigma lobes. Chrysanthemum florets, like other Asteraceae, produce a bifurcate stigma with reflexed lobes and stigmatic papillae along the rims of the lobes (McInnis *et al.*, 2006; Allen, Lexer and Hiscock, 2010). The opening of the stigma lobes typically happens during development as the style elongates (chapter 2 Fig. 2D), but in the lower quality stigmas, the lobes are barely opened, resembling the morphology of a developing stigma at an earlier stage. Because of this resemblance, we hypothesise that these lower-quality stigmas did not mature properly. Stigma maturation is generally characterised by high levels of peroxidase and esterase activity, also referred to as receptivity (McInnis *et al.*, 2006). We also tested stigma receptivity and found little correlation between the opening of the stigma lobes and receptivity, suggesting that different factors are involved in the regulation of stigma lobe opening and receptivity. It would be interesting to further characterise the processes involved in stigma maturation. Transcriptomic comparison of developing stigmas with high and low quality (opening of the lobes) could provide a better understanding of the processes involved. Additionally, studies in other species have shown that

several hormones, such as ethylene, are involved in organ maturation (Kai *et al.*, 2019). Therefore, it is possible that exogenous ethylene treatments could induce proper maturation of lower quality stigmas and thereby rescue low seed set in certain cultivars. Our study elucidated that considerable variation in the stigma quality of both disc and ray florets exists in the Chrysanthemum germplasm. Characterising the stigma and selecting varieties with high-quality stigmas as mother plants may help to select ornamental varieties with better seed set in the future.

Identification of genetic loci influencing seed set

To facilitate the selection and breeding of more fertile ornamental varieties, different approaches were applied. In this section, the results and implications of the use of a genetic approach involving QTL analysis and GWAS are discussed. A genetic approach for identifying causal loci for a particular trait in Chrysanthemum used to be complex due to Chrysanthemum's hexaploid outcrossing nature. Until recently, it was, therefore, nearly impossible to compose a genetic map encompassing all chromosomes and identifying the different homeologs required for a decent QTL analysis. There is still no genome sequence for *Chrysanthemum morifolium,* but several diploid genome sequences have become available in the past few years (Song *et al.*, 2018; Nakano *et al.*, 2021; van Lieshout *et al.*, 2021; Wen *et al.*, 2022). This, and the development of several tools for polyploid genetic analysis (Bourke, van Geest, *et al.*, 2018; Bourke, Voorrips, *et al.*, 2018) have enabled genetic analyses to identify loci related to the seed-set problem. However, despite the recent progress in genetic tools, the identification of allelic effects for the two quantitative loci on linkage groups 1 and 4 that were identified in Chapter 4 for the disc/ray floret ratio, proved rather tricky. In the end, we had to conclude that the population size of 87 was too small to rely on the predictions, and we validated the loci identified by the bi-parental QTL analysis using GWAS analysis, which also narrowed down the regions of interest. Interestingly, these regions contained a few hundred genes, including *PROTODERMAL FACTOR 2* (*PDF2*) on linkage group 1 and *CYCLOIDEA2* variants a-f (*CYC2a-f)* on linkage group 4. *PDF2* was additionally identified by the transcriptomic approach, and *CYC*-like genes have been associated with disc/ray floret identity in previous studies, as will be further discussed in the next paragraph. Furthermore, exploring other genetic sources of plants not previously used in these analyses would be interesting and optimising the GWAS analysis by measuring the percentage of disc florets instead of the global phenotyping that was now applied and which was relying on flower-type descriptions. These are strategies for gaining more knowledge on the loci controlling disc/ray floret ratio. Finally, regarding seed

set, it would be interesting to extrapolate seed set scores from available breeding data and directly identify loci controlling this trait using a GWAS approach.

The analyses in Chapters 2 and 4, where many phenotypic characteristics were scored, also identified correlations between different traits. Interestingly, both in the analysis on seed set and stigma quality (Chapter 2) and the QTL analysis (Chapter 4), we discovered indications that stigma quality and green flower colour are linked. The large-scale analysis based on existing breeding data (Chapter 2) revealed a correlation between green flower colour and low stigma quality, mainly for ray floret stigma morphology. The QTL analysis also identified a region at linkage group 3 with a single allele responsible for green colour and negatively affecting ray floret stigma receptivity. The most likely explanation for the apparent coupling of these traits is that they are linked by proximity. This is especially possible if a selection of green colour was conducted separately from other genetic material to improve the intensity of the green colour. Interestingly, a connection between malformed pistils and a green flower colour was also identified by Liu et al. (2021) in an unstable mutant, also suggesting a correlation between these traits (Liu, Luo, *et al.*, 2021). The QTL region of chromosome 3 contains *STAY-GREEN* (*SGR*), a Mg-dechelatase, regulating chlorophyll degradation(Ohmiya *et al.*, 2017). This gene probably causes the green colour phenotype, but if and how it could influence stigma morphology or receptivity remains unknown. An interesting approach would be to knock out or to down-regulate *SGR*. Since SGR is responsible for chlorophyll degradation, knocking it out should result in green flower colour. It is then possible to determine if stigma quality has also been affected. Several studies have performed differential expression analysis between white and green flowered mutants in Chrysanthemum, and several DEGs were identified (Ohmiya *et al.*, 2017; Fu *et al.*, 2021; Luo *et al.*, 2021). It would be interesting to analyse these genes in relation to stigma fertility and check if any are located in the identified region on chromosome 3.

Identification of genes that can regulate the disc/ray floret ratio

The second approach that we took to develop tools for breeding more fertile ornamental varieties was a molecular one, which focused on the identification of genes that can regulate the disc/ray floret ratio. The RNA-seq that we performed to compare the transcriptomes of mutants with a higher disc/ray floret ratio with that of their non-mutated counterparts was powerful but could mainly uncover downstream effects of the causal mutation. Extreme differences were not expected, as both samples produce disc and ray florets, albeit in a different ratio. Therefore, genes that are, for example, more highly expressed in disc florets will be

identified as upregulated genes, regardless of their role in the specification of floret identity. It is nearly impossible to determine if these differentially expressed genes are causal for the phenotype or downstream and simply floret specific. Interestingly, the differential expression analysis did not indicate differences in *CYC2* expression, except in the later stages. This was unexpected since it is one of the main targets for floret identity research in Asteraceae (Chapman *et al.*, 2012; Garcês, Spencer and Kim, 2016; Huang *et al.*, 2016; Chen *et al.*, 2018; "News and Views CYCLOIDEA3 Is Targeted by Disparate Transcription Factors in Patterning Flowers in Gerbera," 2020). However, mutations in the coding region can influence protein function without affecting expression level, and mutations in the *CYC2* genes may thus not have been picked up in the differential expression analysis. Additionally, the mutant phenotype could also result from differences in proteins interacting with CYC2. Noticeably, M1 exhibited an increased percentage of disc florets and a conversion of white to yellow ligules. A recent paper focusing on co-evolution of flower colour and floret shape discovered that CYC2g activates *CAROTENOID CLEAVAGE DIOXYGENASE 4a (CCD4a)*, a gene coding for an enzyme that breaks down carotenoids (Chu-Jie *et al.*, no date). Thus, a mutation in *CYC2g* could both result in more disc florets and in yellow colour in the ligules, suggesting that in the M1 mutant, *CYC2* may be mutated. Furthermore, we have strong indications that *CYC2* causes the variation in disc/ray floret ratio in at least part of the varieties used for the GWAS analysis and in the population used for the QTL analysis, as already indicated in the previous paragraph. The genetic analysis indicated a QTL in chromosome 4 in a region that contains *CYC2a-f*. Therefore, it is highly likely that the variation is caused by one of the *CYC2* allele variants. In Sunflower, Gerbera and *Senecio*, overexpression of *CYC2* increased ligule length in ray florets (Broholm *et al.*, 2008; Kim *et al.*, 2008; Chapman *et al.*, 2012; Juntheikki-Palovaara *et al.*, 2014; Chen *et al.*, 2018; Fambrini *et al.*, 2018). Moreover, in both *Gerbera* and *Senecio*, overexpression disrupted stamen development in disc florets. Lower expression of *CYC2* in Gerbera only reduced ligule length in ray florets but did not affect disc floret development (Broholm *et al.*, 2008). Furthermore, in sunflowers, overexpression of *CYC2,* induced by the insertion of a transposable element in the promotor, results in capitula that contain only ray florets (Chapman *et al.*, 2012). Interestingly, another Sunflower mutant with a truncated CYC2 protein developed a functional pistil and stamen in the normally sterile ray florets (Chapman *et al.*, 2012). Data from *Senecio* indicated that in addition to *CYC*, *RADIALIS* (*RAD*) was involved in ray-floret development (Maria Pereira Garcês, R Spencer and Kim, 2016). However, in our transcriptome analysis, both *CYC* and *RAD* genes were only differential in later developmental stages. The Chrysanthemum CYC2 function has still been quite elusive

since several different variants exist. Overexpression of the highest expressed variants has some influence on corolla length (Huang *et al.*, 2016; Chen *et al.*, 2018). There are reports of different splice variants with different expression patterns during development, and overexpression of both splice forms inhibited the development of stamen and corolla in Arabidopsis, suggesting their function in developing these organs (Liu, Sun, *et al.*, 2021). A recent paper showed that these *CYC2a-f* variants had different expression patterns and, therefore, likely different functions in different developmental stages (Wen *et al.*, 2022). This expression pattern may suggest which *CYC2* variant should be targeted for further research. In conclusion, the function of the *CYC2* gene in Chrysanthemum floret specification/development is still unclear, but its conserved role in ray floret development in other Asteraceae species strongly indicates a differential role in Chrysanthemum floret formation as well. The fact that *CYC2* genes were not identified as differentially expressed genes in the early stages of mutants M1 and M2 (Chapter 3) suggests that they were either not the causal factors for the enhanced disc/ray floret ratio or may act upstream. In particular, in M1, where ligule colour is changed along with disc/ray floret ration, the latter scenario is plausible. Sequencing the *CYC2* variants in this QTL region would be very interesting to identify causal mutations responsible for floret identity.

While the *CYCs* were not differentially expressed in the early stages of floret development, our analysis did reveal several genes that were lower expressed in the mutants with a higher disc/ray floret ratio. Functional characterisation was performed for several of these genes (Chapter 5), which yielded interesting results in the case of homologs of the class IV homeodomain-zipper (HD-ZIP IV) transcription factor *PDF2* and the brassinosteroid biosynthesis gene *DWARF1 (DWF1)*. The differential expression analysis indicated that several HD-ZIP IV genes could be involved in floret identity and the subsequent characterisation of a *PDF2* homolog using RNAi showed that lower levels of *PDF2* may increase the percentage of disc florets. Although the functional analysis was inconclusive (see Chapter 5), the presence of *PDF2* in the QTL region on chromosome 1 makes it an interesting candidate. In Arabidopsis, *PDF2* is a transcription factor with roles in epidermal patterning of the embryo and later during development (Nakamura *et al.*, 2006; Ogawa *et al.*, 2014). Interestingly, most floral tissues develop from the L1 layer, including the stigma (Aida *et al.*, 2020). Research has shown that L1 cells promote and restrict organ growth by sending growth signals to the other two layers (Savaldi-Goldstein and Chory, 2008). In Arabidopsis, HD-ZIP IV mutants grow abnormal floral organs with homeotic conversions, where petals were transformed into sepaloid organs and stamens into infertile and/or carpelloid organs (Kamata

et al., 2014). In *pdf2* mutants, *APETALA 3* (*AP3*) expression is normal in early flower primordia (Kamata *et al.*, 2013, 2014) but diminished in developing organs, causing defects and conversions during the outgrowth of floral organs.

The identification of several BR-signalling genes in the list of DEGs suggested that BR may play a role in the specification of disc/ray floret identity or in a differential development of the florets. BR is generally known to regulate and repress organ boundary genes for correct organ boundary development (Li and He, 2020). Other research has shown that BR is essential in regulating the growth of organs in the L1 layer (Savaldi-Goldstein, Peto and Chory, 2007). *BRI1-EMS-SUPPRESSOR1* (*BES1*) and *BRASSINAZOLE RESISTANT1* (*BZR1*), the two main BR-regulated transcription factors (Wang *et al.*, 2002; Yin *et al.*, 2002) regulate shoot organ boundaries in Arabidopsis by suppressing CUP-SHAPED COTYLEDON genes *CUC1*, *CUC2*, *CUC3*, and *LATERAL ORGAN FUSION 1* (*LOF1)* (Gendron *et al.*, 2012). Differently from *CUC* and *LOF* genes, *LATERAL ORGAN BOUNDARY* (*LOB*) was upregulated in response to increased BR levels in the *bzr1-1D* mutant (Lee, Geisler and Springer, 2009). Interestingly, several genes were suggested to be involved in capitula types or disc/ray floret ratio in Asteraceae evolution (Wen *et al.*, 2022). Specifically, *NO APICAL MERISTEM* (*NAM)/CUC-like* and *LOB30* were unique to forming radiate capitula (forming both disc and ray florets) compared to capitula forming only disc florets (discoid) or ray florets (ligulate). *BES* regulates *TOPLESS* (*TPL*) to form a *BES-TPL* complex that represses *CUC* expression (Espinosa-Ruiz *et al.*, 2017). Thus, BR may regulate the disc/ray floret ratio via the regulation of the boundary genes.

In addition, differentially regulation of boundary genes as a result of low BR concentrations may affect the development and outgrowth of the florets. Boundary genes have essential functions in reproductive organ development in other plant species. In Arabidopsis *LOB* overexpression lines, anthers and pistils did not develop normally; they rarely produced pollen grains and were female sterile (Shuai, Reynaga-Peña and Springer, 2002). Since *LOB* defines boundary cells, expanded gene expression causes overgrowth of boundary regions with an improper fusion of organs. Another study in Arabidopsis has shown that *CUC* genes prevent *SPATULA*-mediated carpel fusion (Nahar *et al.*, 2012). These genes are necessary for adequately developing floral organs within their spatial boundaries (Aida *et al.*, 1997; Bowman *et al.*, 1999). Therefore, they could be responsible for the difference in stigma quality between floret types. Scanning the DEG list of Chapter 3 for homologs of these boundary genes reveals that several are differentially regulated. Homologs of *CUC2*, *LOB DOMAIN CONTAINING*

PROTEIN15 (*LBD15*) and *LBD19* had higher expression in M2 and *LATERAL ORGAN FUSION 2* (*LOF2)* was more highly expressed in both mutants.

Our data suggest a role for HD-ZIP IV transcription factors and Brassinosteroid-related genes, in addition to the already likely candidate *CYC2*. The remaining question is how these factors might influence the disc/ray floret ratio and whether they interact together. While the differential expression of HD-ZIP IV TFs was more prominent in M1, the alteration of BR signalling genes was more prominent in M2, indicating that separate mechanisms may have induced a higher disc/ray floret ratio in each mutant. However, the fact that several HD-ZIP IV and BR-signalling genes are differential in both mutants suggests that both factors may influence each other. BR-signalling may be upstream of both HD-ZIP IV TFs and CYC-like TFs in the regulation of floret identity, as there are examples of BR-inducing TCP transcription factors (Guo *et al.*, 2010)in Arabidopsis, where BR is also known to regulate root hair differentiation through HD-ZIP IV TF *GLABRA2* (*GL2*) (Li *et al.*, 2022). However, since most of these mechanisms contained different genes and were conducted in Arabidopsis, this remains highly speculative.

Hormonal control of Chrysanthemum capitulum, disc and ray floret development

In contrast to some other Asteraceae species that contain florets with an intermediate identity, the situation in Chrysanthemum is more black-and-white with either disc- or ray-floret development. However, the fact that the ratio between disc and ray florets are quite variable in cultivated Chrysanthemum, ranging from a single rim of ray florets to a flower with only a few disc florets in the centre, suggests that a gradient of a particular factor may exist in the capitulum (flower head), which act as a determinant for the identity of the initiated florets. Beyond a certain threshold, disc florets may be developed instead of ray florets. The black-and-white situation in Chrysanthemum could be the result of a regulatory feedback system, such as existing in floral organ boundary specification in Arabidopsis (Sundström *et al.*, 2006), where the onset of expression of a key TF directly represses the previously active key genes. Factors that are commonly involved in the establishment of this kind of gradients are known as morphogens (Christian, 2012) and are often represented by mobile transcription factors or hormones. There is evidence that an auxin gradient influences floret identity in Gerbera (T. Zhang, Wang, et al., 2021; Zoulias et al., 2019), and we discovered here that in Chrysanthemum, Brassinosteroids could influence the floret ratio, suggesting that a gradient may be established in the capitulum. It is possible that, like auxin, BR is highest at the capitulum's margin and lower at the center. The type of floret is then dependent on a BR

threshold. Moreover, lower levels would, in this case, result in more disc florets. However, no evidence exists that these hormone levels remain during floret maturation. Interestingly, we did find a link between the percentage of disc florets and disc floret stigma quality, and this could suggest that BR levels also affect later stages of growth. However, it seems more likely that this link is a consequence of diverged breeding between flower types with a higher and lower disc floret ratio. It would be interesting to further investigate this link in different varieties or populations. It was surprising that we did not find evidence of enrichment of auxin-related genes in the differential expression analysis. The mutants in our studies appeared to have defects in BR-signalling rather than in auxin signalling. It is possible that in Chrysanthemum, an auxin gradient is not involved in the same manner as Gerbera. However, auxin is of key importance in many processes, and the absence of mutants with a disrupted auxin gradient may also indicate that the disruption of auxin signalling is so detrimental to plant development that mutants never get selected during breeding. Moreover, Oh et al. discovered that ARF6, PIF4 and BZR1 could physically interact with each other to cooperatively regulate common target genes (Oh *et al.*, 2014), enabling the plant to respond to diverse internal and external stimuli (van Mourik *et al.*, 2017). This could explain the slight variation in the disc/ray floret ratio we observed depending on environmental signals such as day length or elevated temperature.

Other hormones that could be involved are ethylene and cytokinin. Ethylene can influence flower gender in several species, such as cucumber (J. Pan et al., 2018; D. H. Wang et al., 2010), and cross-talk between BR and ethylene has been reported (Jiroutova, Oklestkova and Strnad, 2018). Specifically, recent data has shown that BR can induce ethylene to initiate more female flowers in several cucurbits species, including cucumber (Manzano *et al.*, 2011; Papadopoulou and Grumet, 2019). In *Jatropha curcas,* female flowers were increased in response to cytokinin treatments, and transcriptome analysis showed upregulation of the main BR response genes, indicating positive crosstalk between the cytokinin and BR signalling pathways (Pan *et al.*, 2014). A similar mechanism with either of these hormones could be responsible for floret identity in Chrysanthemum.

The fact that the hormone treatments with Brassinolide (BL), an active form of BR, did not influence the disc/ray floret ratio was not surprising because BR is almost immobile (Hayat & Ahmad, 2011), and it was impossible to administer the hormone solution directly on the developing floret primordia. Additionally, the absence of an effect could be a consequence of the tight feedback mechanisms that counter high BL levels. BZ might be more mobile or be

less restricted to mechanisms modulating BR levels. BR is tightly controlled with negative feedback mechanisms and synthesised in very low concentrations.

While the BZ treatments did indicate some effect on both the '1581' and V2 genetic backgrounds, there was never an effect on the floret ratio of V1. This may indicate that V1 has better internal mechanisms to regulate BR levels. Furthermore, more BR-related genes were differential in M2, and these plants were slightly shorter compared to V2, which might be expected taking into account that dwarfism is a known phenotype of BR deficiency (Peres *et al.*, 2019). Further experiments should be conducted to optimise the BR treatments. Lower concentrations may give better results. Additionally, it would be interesting to determine if overexpression of BR-biosynthesis genes results in fewer disc florets. In addition to testing altered BR levels, it would be interesting to test BR in combination with auxin, ethylene or cytokinin because of the before mentioned cross-talk between the hormones. Moreover, these BZ treatments could help breeders temporarily induce more disc florets for a better seed set while the progeny retains the genetics for more decorative flower types.

Concluding remarks

Our analysis showed that lower seed set in varieties with higher ornamental value is due to decreased disc/ray floret ratio. In addition, we determined that ray florets have a lower stigma quality, influencing pollen capture and consequential seed set.

Both QTL analysis of the bi-parental population and the GWAS analysis identified two regions on chromosomes 1 and 4. Interestingly, these were also identified previously in a daisy-type population (van Geest *et al.*, 2017). These identified regions contained several interesting genes that could be investigated further. Most notably, *PDF2* on chromosome 1 and several *CYCLOIDEA2* (*CYC2a-f*) variants on chromosome 4. CYC2, which has long been investigated for its involvement in floret ratio in several Asteraceae species, is interesting because its' presence at this locus could indicate that part of the current day variation in disc/ray floret ratio is controlled by alleles of this gene.

The differential expression analysis indicated potential roles in regulating floret ratio for HD-ZIP IV transcription factors, in particular *PDF2* and Brassinosteroid-associated genes. The functional analysis seemed to confirm that lower concentrations of *PDF2* increase the percentage of disc florets. However, this remains inconclusive due to an incomplete analysis of transformants. We simulated the BR effect in a transgenics approach by downregulating

DWF1, an early BR biosynthesis gene and by conducting exogenous treatments with BR inhibitor brassinazole. Both increased the percentage of disc florets.

Based on all these findings, a model was generated to explain the role of the identified factors in the regulation of the disc/ray floret ratio (Fig. 1), which focuses on the levels of BR, *CYC2* and *PDF2*. The fact that *CYC2s* were not differentially expressed in early bud stages in both our mutants, while BR signalling was probably reduced, suggests that CYCs act rather upstream of BR in the regulation of Chrysanthemum disc/ray floret identity or that *CYCs* do act downstream, but are only in a later stage responsible for differential outgrowth and development of the flower organ primordia. In M1, the mutant that also changed colour, a mutation in *CYC2* genes may cause the disc/ray floret ratio phenotype, which is more downstream-regulated by HD-ZIP IV TFs. In the M2 mutant, the differential expression of BR-signalling genes is more prominent, and *CYCs* may play a less prominent role.

The transcriptome analysis results and candidate genes identified in this study are very interesting for further research in floret identity in Asteraceae species. Furthermore, the loci identified can help breeders to select parents and to increase seed set in varieties with more ray florets. We have shown that these varieties with more ray florets have lower seed set because ray florets have a lower quality stigma. However, there is variation in stigma quality; hence, these traits can be uncoupled, offering prospects for future improvements of seed set in highly valued Chrysanthemum varieties.

Figure 1. Model summarising the results of this thesis with respect to the factors influencing disc/ray floret ratio. Left is a schematic overview of a Chrysanthemum with a low disc/ray floret ratio and the right one with a higher disc/ray floret ratio. Underneath are shown the levels of Brassinosteroid (BR), *PROTODERMAL FACTOR 2* (*PDF2*), *CYCLOIDEA 2* (*CYC2*), and several boundary-related genes *LOB*, *CUC* and *LOF*. In this thesis, we validated the effect of lower levels of BR and *PDF2* on increasing the percentage of disc florets. *CYC2* data is mainly based on evidence from literature. The information for boundary genes is mostly circumstantial, based on literature and the transcriptome analysis.

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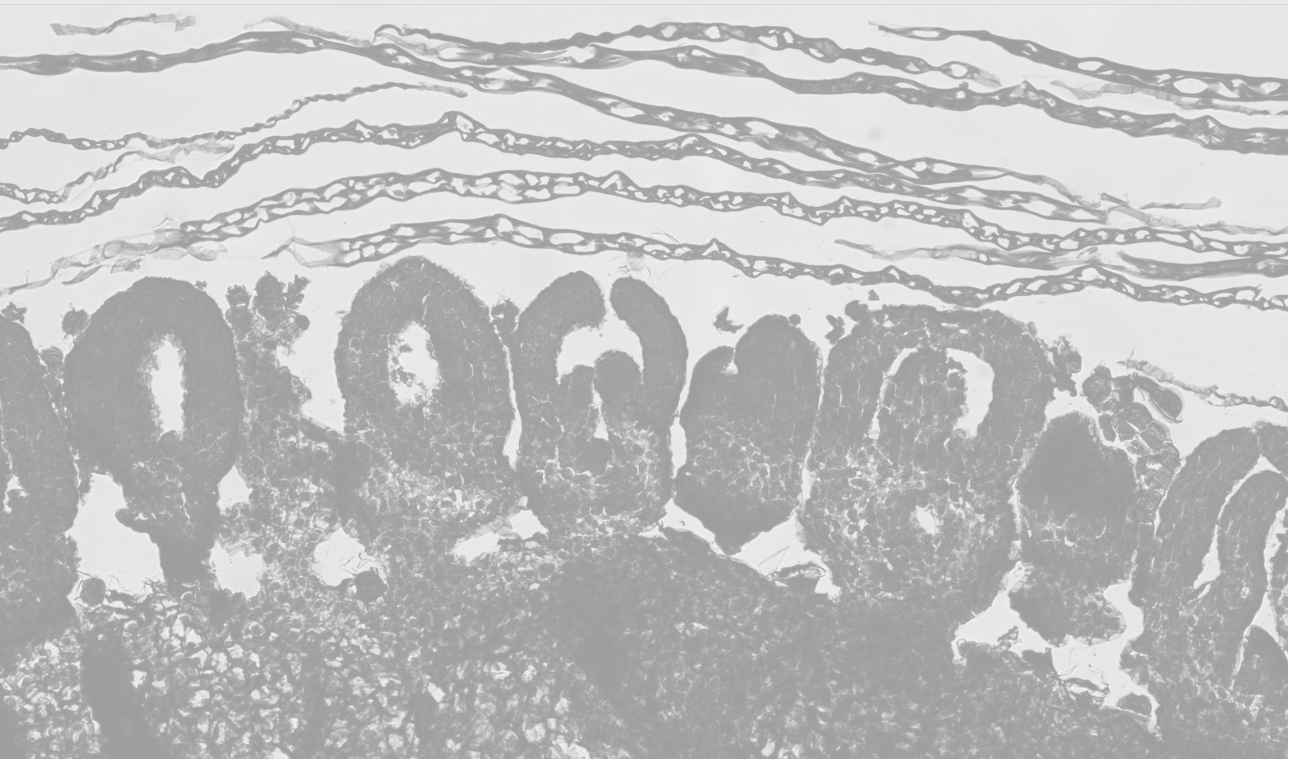
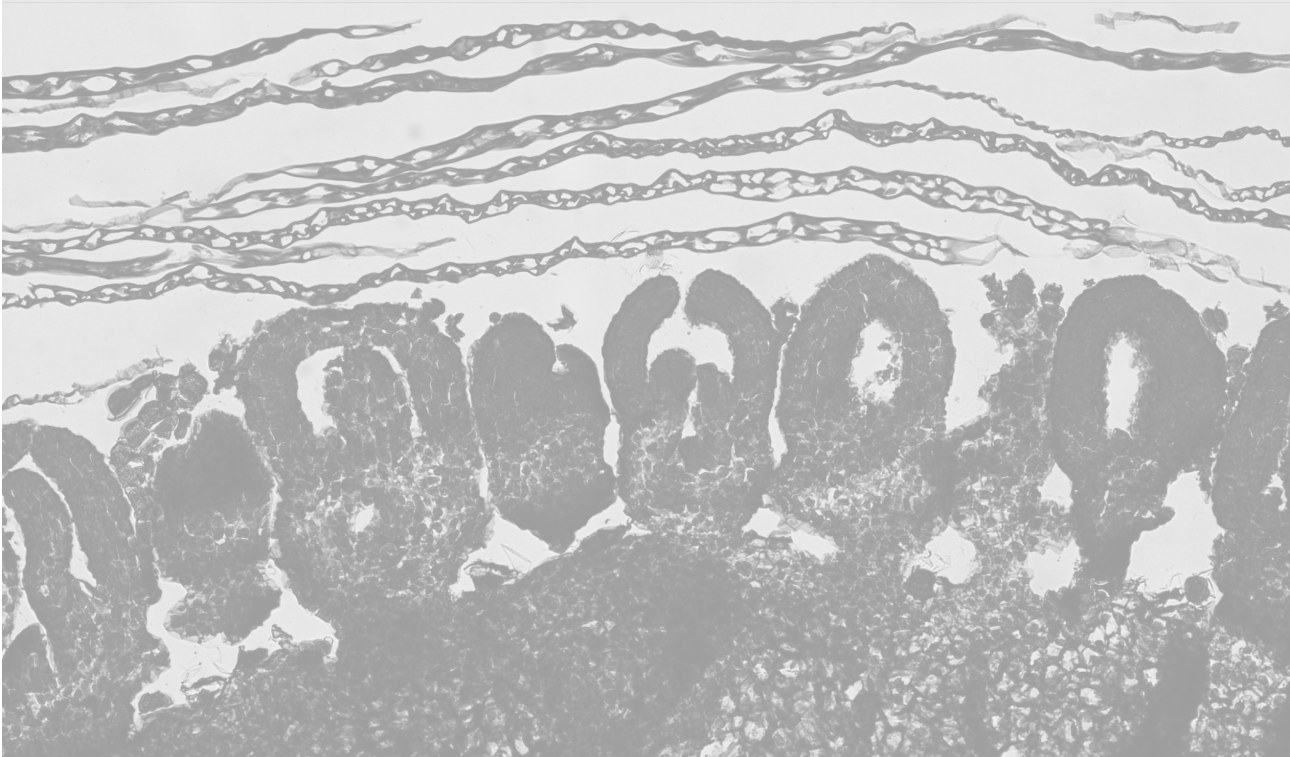


Appendix

Summary English
Samenvatting Nederlands
Acknowledgements
About the author
EPS education statement



General summary (English)

Chrysanthemum is a major crop and the world's second most important cut flower. It has a composite flower, an inflorescence structure where many flowers/florets mimic a single flower. These florets include the showy female ray florets and hermaphroditic disc florets. A variance in the ratio between these floret types influences the appearance of this ornamental flower and its seed set.

In **Chapter 2**, we investigated the effect of the disc/ray floret ratio on seed set. We discovered that a low disc/ray floret ratio was one of the main factors influencing low seed set in varieties with high ornamental value. We subsequently investigated differences in fertility between the ray and disc florets and discovered a generally lower stigma quality of ray florets. This lower quality was mainly caused by a lower degree of stigma lobe opening, a phenotype reminiscent of immature stigmas. There was also variation in stigma receptivity, but this was not floret dependent. Low stigma quality resulted in lower pollen capture and consequential reduced seed set. Interestingly, about twenty percent of the crosses did not produce a single seed, and this was mainly influenced by low stigma receptivity. Nevertheless, overall, the interaction of disc/ray floret ratio with stigma morphology impacted seed set most significantly. For this reason, we focussed further on factors influencing the disc to ray floret ratio.

We identified two mutants with an increased disc/ray floret ratio compared to their respective 'normal' varieties and performed transcriptomic analysis during key developmental stages (**Chapter 3**). Floret differentiation was first visible at stage 2, and we, therefore, investigated buds of the two stages prior to visible differentiation (s0 and s1) and the two subsequent stages (s2 and s3). During data analysis, we focused on the genes similarly differentially expressed (DEG) in both mutants. Of all genes analysed, 145 genes were upregulated, and 245 were downregulated at the same stages for both mutants. Interestingly, the list of differentially expressed genes contained several homeodomain-leucine-zipper type IV transcription factors (HD-ZIP IV) and several genes related to Brassinosteroid (BR) signalling. 42 genes were selected for further examination based on their expression in the earliest developmental stages and on the function of homologs in other species. 24 DEGs could be verified in independent samples of at least one of the mutants. From these genes, a further selection was made for functional analysis.

In addition to a transcriptomic analysis to identify potential candidates influencing the disc/ray floret ratio, we performed genomic analyses to identify relevant loci (**Chapter 4**). This

involved QTL analysis for a bi-parental population between a green pompon type (mostly ray florets) mother and a pink daisy type (mostly disc florets) father, segregating for several flower-related traits. Based on this analysis, we identified two regions affecting the disc/ray floret ratio on linkage groups 1 and 4. Furthermore, we identified a QTL for disc floret stigma receptivity on linkage group 3. Further analysis showed that receptivity was negatively affected by a single homeolog from the paternal side. Interestingly, this same locus was also responsible for green flower colour within this population. Since the QTL region was too large to narrow down candidate genes, we additionally performed a genome-wide association study (GWAS) on a large set of individual plants segregating for disc/ray floret ratio. This analysis distinguished between daisy flower type (high disc/ray floret ratio) and other flower types with lower disc/ray floret ratios and identified a region on chromosome 4, which matched the earlier identified QTL on linkage group 4. Furthermore, there was a non-significant but distinct string of markers with higher scores on the earlier identified region on chromosome 1. The regions identified using GWAS were smaller than the QTL regions and only contained limited amounts of genes, enabling the identification of potential candidate genes. We compared the genes in this region to the differentially expressed genes. Interestingly, the region on chromosome 1 also contained *PROTODERMAN FACTOR 2* (*PDF2*), an HD-ZIP IV transcription factor, which was also differentially regulated. The region on chromosome 4 contained several variants of *CYCLOIDEA2* (*CYC2a-f*), a TCP transcription factor often implicated in determining ray floret identity in several Asteraceae species.

In **Chapter 5**, we present the functional analyses of *PROTODERMAL FACTOR2* (*PDF2*), *GLABRA2-INTERACTING REPRESSOR* (*GIR1*), *DWARF1* (*DWF1*), *TINY2* (*TNY2*), *HOTHEAD* (*HTH*), *RICESLEEPER2* (*RSL2*) and *WIP DOMAIN PROTEIN2* (*WIP2*) using RNAi knock-down and overexpression approaches. RNAi was used as a strategy because Chrysanthemum is a hexaploid outcrossing species, and it would be challenging to develop complete knock-out mutants. Data for *RSL2* and *WIP2* were inconclusive because the downregulation/overexpression approach was not successful. Moreover, for *GIR1*, *TINY2* and *HTH*, no significant change in the disc/ray floret ratio was observed in relation to expression, which may be due to the fact that both controls and transgenic lines displayed a substantial variation in the disc/ray floret ratio. Interestingly, we showed that downregulation of *PDF2* did increase the percentage of disc florets in three lines. However, these data are still preliminary since we did not manage to characterize all transformants for this gene. Additionally, to test the effect of reduced BR signalling, we downregulated *DWARF1*, an early BR biosynthesis gene, which resulted in a significantly increased percentage of disc florets. In line with these

results, exogenous application of brassinazole, a BR inhibitor, also increased disc floret number. All these findings are in accordance with the differential expression analysis, strongly suggesting that BR has essential functions in controlling the disc/ray floret ratio in Chrysanthemum.

Based on the findings in this thesis, we propose a mechanism whereby *PDF2* and brassinosteroids play important roles in determining the disc/ray floret ratio. *PDF2* and BR have not been implicated in floret determinacy before, and these findings provide interesting leads for further studies, not only in Chrysanthemum but also in other Asteraceae species. Additionally, the QTL and GWAS analyses have identified several loci influencing floret ratio and stigma receptivity. These can be included in marker sets to assist breeders in the optimal selection of parents to achieve a high seed set. The GWAS analysis also revealed a region containing *CYC2*, indicating for the first time that variation in disc/ray floret ratio in Chrysanthemum varieties are likely caused by allelic variation of this gene. More practically, our findings suggest that breeders can apply brassinazole treatments to reversibly increase seed set in their breeding programs without permanently affecting progeny genetics.

Algemene samenvatting (Nederlands)

Chrysant is een economisch belangrijk gewas en de op één na belangrijkste snijbloemsoort ter wereld. Chrysanten zijn samengesteldbloemig, wat betekent dat een heleboel bloemen samengepakt zitten op één bloemhoofd en zij samen één bloem nabootsen. Er zijn twee bloemsoorten (florettypes) aanwezig: de opvallende lintbloemen (vrouwelijk) en hermafrodiete buisbloemen. Variatie in de ratio (verhouding) tussen deze twee florettypes heeft grote invloed op zowel het uiterlijk van de bloem als de zaadzetting.

In **hoofdstuk 2** onderzochten we het effect van de ratio van buis-/lintbloemen op de zaadzetting. Hierbij bleek dat een lage buis-/lintbloem verhouding resulteerde in een lagere zaadzetting. Op basis van deze bevindingen verwachtten we verschillen in fertiliteit tussen buis- en lintbloemen. Na verder onderzoek bleek dat de stamperkwaliteit lager was in lintbloemen dan in buisbloemen, vooral doordat de twee stempel lobben niet goed geopend waren. Deze verminderde kwaliteit stampers waren minder goed in staat om stuifmeel op te vangen wat vermoedelijk leidde tot de lagere geobserveerde zaadzetting bij bloemhoofden met veel lintbloemen. Er was ook variatie in stempel ontvankelijkheid, alleen bleek dit niet afhankelijk van het type floret. In twintig procent van de kruisingen werd geen zaad gezet en dit werd verklaard door slechte stigma ontvankelijkheid. Verder werd het grootste deel van de variatie verklaard door de interactie tussen de buis-/lintbloem ratio en stamperkwaliteit van deze floretten. Op basis hiervan is besloten verder te focussen op factoren die de floret ratio bepalen.

In **hoofdstuk 3** is met behulp van transcriptoom analyse inzicht verkregen in genen die mogelijk een rol spelen bij het reguleren van de buis-/lintbloem ratio. Hiervoor werden twee variëteiten gebruikt waarvan mutanten geïdentificeerd waren met meer buisbloemen. Het transcriptoom in de bloemknoppen van deze twee variëteiten werd vergeleken met dat van hun mutanten. Hiervoor hebben we eerst onderzocht in welk stadium de eerste differentiatie van buis of lintbloemen bepaald werd. Dit bleek in stadium 2 te zijn en daarom zijn zowel de twee stadia daarvoor (stadium 0 en 1) als stadium 2 en 3 gebruikt voor analyse. Bij deze analyse is vooral gefocust op genen die in beide mutanten hetzelfde reageerden ten opzichte van de wild-type controles. De analyse identificeerde 145 genen die verhoogd tot expressie kwamen en 245 die lager tot expressie kwamen. In deze set bevonden zich verschillende interessante transcriptiefactoren zoals homeodomain-leucine-zipper type IV-transcriptiefactoren (HD-ZIP IV) en brassinosteroïde gerelateerde genen. Van de 42 genen

die verder onderzocht zijn, konden er 24 bevestigd worden in nieuwe, onafhankelijke experimenten. Hieruit is een beperkte selectie gemaakt voor verdere functionele analyse.

Vervolgens hebben we in **hoofdstuk 4** met een op genetica gebaseerde aanpak getracht potentiële kandidaat genen of locaties op het genoom te identificeren. Hierbij hebben we een QTL (quantitative trait loci)-analyse gedaan op een populatie afkomstig van een kruising tussen een groen pompon type (vooral lintbloemen) met een roze enkelbloemig type (vooral buisbloemen). Deze populatie varieerde voor verschillende bloem gerelateerde eigenschappen. Met behulp van de QTL-analyse identificeerden we twee locaties op het genoom die waarschijnlijk invloed hebben op de buis-/lintbloem ratio. Deze regio's zaten op gelinkte genetische groepen (linkage groups) 1 en 4. Daarnaast bevond zich een QTL voor zowel groene kleur als stigma receptiviteit van buisbloemen op genetische groep 3. Hierbij was er één homoloog van de vader die dominant was voor groene kleur en een negatief effect had op stigma kwaliteit. Naast de QTL-analyse is er ook een GWAS (genome-wide association study) uitgevoerd op een reeks chrysantenrassen. Voor de GWAS vergeleken we enkelbloemige types met andere bloemtypes met meer lintbloemen. Deze analyse bevestigde het reeds in de QTL gevonden locus op chromosoom 4 en daarnaast was er een niet significante, maar wel opvallende, piek gelijk aan de QTL-locatie op chromosoom 1. Hiermee werd de waarschijnlijkheid dat deze twee loci inderdaad betrokken zijn bij het bepalen van de floret ratio verhoogd. De GWAS leverde een verbetering in resolutie op, waardoor het mogelijk werd om naar kandidaat genen te kijken. De genen op deze locaties zijn vergeleken met de differentieel gereguleerde genen uit hoofdstuk 3. Op chromosoom 1 bevindt zich onder andere *PROTODERMAL FACTOR 2* (*PDF2*), een HD-ZIP IV transcriptiefactor, die ook eerder in de differentiële expressie analyse werd gevonden. Daarnaast bevat de locus op chromosoom 4 verschillende varianten van *CYCLOIDEA 2* (*CYC2a-f*), een TCP transcriptiefactor die in veel studies geassocieerd wordt met lintbloem floret identiteit in samengesteldbloemigen.

In **hoofdstuk 5** hebben we een functionele analyse uitgevoerd op genen die gekozen zijn op basis van de analyses uit hoofdstukken 3 en 4. Hierbij hebben we gekeken naar *PROTODERMAL FACTOR2* (*PDF2*), *GLABRA2-INTERACTING REPRESSOR* (*GIR1*), *DWARF1* (*DWF1*), *TINY2* (*TNY2*), *HOTHEAD* (*HTH*), *RICESLEEPER2* (*RSL2*) en *WIP DOMAIN PROTEIN2* (*WIP2*). We hebben de genexpressie verlaagd met behulp van RNA interference (RNAi) of verhoogd door een extra gen-kopie in te brengen. RNAi is gekozen in plaats van het volledig uitschakelen van genen door middel van CRISPR/Cas omdat chrysant hexaploid en uitkruisend is en het lastig is om alle zes allelen uit te schakelen en te

karakteriseren. De data voor *RSL2* en *WIP2* waren niet duidelijk omdat de expressie niet - of niet op de juiste wijze - aangepast bleek. Voor *GIR1*, *TINY2* en *HTH* zagen we niet het verschil in fenotype dat we verwacht hadden. Mogelijk stoorde het weefselkweekproces de experimenten aangezien de controle planten ook al veel variatie in floret ratio lieten zien. Bij *PDF2* RNAi zagen we in drie lijnen een verhoging van het aantal buisbloemen. Dit is het fenotype dat we verwacht hadden op basis van de differentiële expressie in hoofdstuk 3. Helaas is het bij dit experiment niet gelukt om alle lijnen te karakteriseren en daardoor is de rol van *PDF2* nog niet met zekerheid vast te stellen. Als laatste hebben we getest of een verlaagd niveau van het plantenhormoon brassinosteroïde (BR) het aantal buisbloemen kon verhogen door zowel genexpressie te verlagen als hormoonbehandelingen uit te voeren. We hebben ervoor gekozen om *DWARF1* (*DWF1*), een enzym dat essentieel is voor de eerste omzetting in de BR biosynthese, te verlagen in expressie door middel van RNAi. Daarnaast hebben we brassinazole behandelingen gedaan omdat dit BR remt. Beide aanpakken resulteerde in de verwachte toename van het aantal buisbloemen en bevestigen de betrokkenheid van BR bij het reguleren van de buis-/lintbloem ratio in chrysant.

Op basis van deze bevindingen verwachten we dat zowel *PDF2* als brassinosteroïden een belangrijke rol hebben in het bepalen van de buis-/lintbloem ratio. Beide factoren zijn niet eerder genoemd in relatie tot floret ratio in samengesteldbloemigen en zijn daardoor interessant voor vervolgonderzoek in verschillende gewassen. Daarnaast zijn de QTL's die geïdentificeerd zijn in dit onderzoek voor zowel de floret ratio als stamper ontvankelijkheid interessant voor veredelaars om te gebruiken voor het verhogen van zaadproductie in rassen met veel lintbloemen. Als laatste heeft de QTL-analyse een locus geïdentificeerd dat *CYC2* genen bevat. Het is heel waarschijnlijk dat een deel van de natuurlijke variatie in buis-/lintbloem ratio op dit moment al wordt veroorzaakt door *CYC2* allelvarianten en ook deze informatie kan tijdens veredeling gebruikt worden. Daarnaast laten de hormoonbehandelingen zien dat het mogelijk is om door middel van brassinazole behandeling meer buisbloemen te verkrijgen en zo de zaadzetting te verhogen.

Acknowledgements

"One does not, simply, ~~walk into Mordor~~ do a PhD", said Boromir in Tolkien's *Lord Of The Rings*. Or that's how I remember it, at least.

First, I would like to thank my amazing supervisors for their continuous support through every step. My co-promotor Marian, for always being the voice of reason and to steer away some of the (maybe too) enthusiastic ideas to manageable projects. The always inspiring Richard, for always seeing and focusing on the positives. I always know when you are (more) enthusiastic when you start speaking more quickly. And Gerco, for being the amazing group leader who knows his science but also remembers managing every other part of the process.

I would like to thank everybody at PDS for their warm welcome and for always including me, even though I was only at Wageningen around one day a week. All of you made me feel that I really belonged to the group.
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My family, with my mom (Lia), dad (Cees) and brother (Cor) for all the good walks and visits to my uncle Jobs 'landje.' My grandmother (Gre) for the many coffees we had. My 'in-laws,' Maria, Jan, Mark-Jan, Hanneke, Erik en Tessa. Our wonderful cats Luci (Luciferase) and Rubi (Rubisco) for their continued love, distraction and calming my nerves.
My friends for always being there. Especially Ronja en Laura, whom I can always call for anything. But also, Astrid, Stephan, Lotte, Dirk, Robert, Leon, Martin, Monika, Bart, Roy, Elwin and Iris.
I would like to thank Frank for inspiring me to do research. I will never forget that on the day we met, you expected me to also love research as much as you, and you were right! My supervisors during my bachelor's study for teaching and inspiring me, Maike, Rechien and Dorien.

And last, but not least, Paul for always being my rock. You have been there through it all. Both scientifically and emotionally. I might sometimes get cranky when you have feedback on my research or suggest that I need to be more 'philosophical,' but I really appreciate all our conversations during evening walks. And of course, for rescuing me when I fight too much with 'R.' Hopefully, we will now have some more time for each other, although as you said, "you never get off this train" [quote from 'The hunger games'].

About the author

Annemarie was born on October 22nd in Alkmaar. Being a late bloomer, the road ending at this thesis was winding, with a few detours along the way. In elementary school she spent her time watching butterflies beyond the classroom windows, which, as one would expect, impinged on recommendations for further education. However, her classroom musings had sparked an interest in animals and plants unrivaled by any of the lessons taken. The logical place to continue education was the VMBO at Clusius College in Castricum. Since she wanted to learn more about the biology of plants, she subsequently decided to attend the MBO Clusius College to study plant horticulture in Hoorn. This is where she first learned about plant breeding and immediately realized what her career choice should be. Becoming a plant breeder required yet more education but, undeterred, she persevered! So, she switched schools to the Wellant College in Aalsmeer to do the last two years of MBO study in one and then decided to continue HBO in a different direction as to acquire a more broad knowledge base. This is why she chose to study biology and medical laboratory research at InHolland and found that she also thoroughly enjoyed research. The informed reader will have connected the dots and identified Annemarie's interest in marker-assisted breeding! The job she found at Dekker Chrysanten as a research technician, applying several facets of research to assist in breeding was perfect and she enjoyed this for several years. Craving to know not only if, but how, plants behave the way they do she decided to do a PhD together with Dekker and the PDS group at Wageningen University. Gerco and Richard were immediately enthusiastic and after successfully applying for a TKI grant, the foundations for the next steps were there. She was but two qualifying exams (because she did not have a master degree) away from becoming a PhD student! These were passed and the quest towards the thesis in front of you had begun. Marian was included as a co-promotor from the second meeting onwards and although it was sometimes hard combining the work at Dekker and commitments in Wageningen, with the help of her wonderful supervisors, Annemarie managed to complete this thesis.

EPS education statement

Education Statement of the Graduate School
Experimental Plant Sciences
Issued to: **Annemarie Castricum**
Date: **19 October 2022**
Group: **Bioscience & Laboratory of Molecular Biology**
University: **Wageningen University & Research**

1) Start-Up Phase	*date*	*cp*
► **First presentation of your project**		
Chrysanthemum flower development	25 Jan 2018	1,5
► **Writing or rewriting a project proposal**		
Elucidation of the molecular mechanisms underlying floral organ development in relation to seed set in Chrysanthemum morifolium	30 Dec 2017	1,5
► **Writing a review or book chapter**		
► **MSc courses**		
Subtotal Start-Up Phase		3,0

2) Scientific Exposure	*date*	*cp*
► **EPS PhD student days**		
EPS PhD student days 'Get2Gether', Soest, NL	11-12 Feb 2019	0,6
EPS PhD student days 'Get2Gether', online	01-02 Feb 2021	0,4
► **EPS theme symposia**		
EPS Theme 1 Symposium 'Developmental Biology of Plants', Wageningen, NL	30 Jan 2018	0,3
EPS Theme 1 Symposium 'Developmental Biology of Plants', Leiden, NL	31 Jan 2019	0,3
EPS Theme 1 Symposium 'Developmental Biology of Plants', Wageningen, NL	05 Feb 2020	0,3
EPS Theme 1 Symposium 'Developmental Biology of Plants', online	28 Jan 2021	0,2
EPS Theme 4 Symposium 'Genome Biology', Wageningen, NL	13 Dec 2019	0,3
EPS Theme 4 Symposium 'Genome Biology', online	11 Dec 2020	0,2
► **Lunteren Days and other national platforms**		
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	09-10 Apr 2018	0,6
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	08-09 Apr 2019	0,6
Annual meeting 'Experimental Plant Sciences', online	12-13 apr 2021	0,5
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2022	0,6
► **Seminars (series), workshops and symposia**		
Seminars:		
Teemu Teeri, 'Pelargonidin in flowers - why not? Gerbera and petunia flowers block pelargonidin biosynthesis in a different way'	14 Mar 2018	0,1
Rosanna Petrella, ' Transcriptional and epigenetic regulation of STK during flower development in Arabidopsis'	13 Nov 2018	0,1
Emanuela Franchini, 'The role of ALOG family genes in inflorescence patterning in rice and Arabidopsis'	13 Nov 2018	0,1
Plantae community webinar 'Jiri Friml and Nicola Patron'	24 Jun 2020	0,2
Plantae community webinar 'Caroline Dean and Jorge Casal'	01 Jul 2020	0,2
Keygene Webinar ' Data Science for Genome Insighs'	09 Jul 2020	0,1
Webinar "Plantae presents: Alejandra I. Huerta and Paola Reyes-Caldas"	09 Sep 2020	0,2
Jared LeBoldus presents "Population Genomic Analyses Reveal Connectivity via Human-Mediated Transport across Populus Plantations in North America and an Undescribed Subpopulation of Sphaerulina musiva."	15 Sep 2020	0,2

	Vidali Lab presents "Chitin Triggers Calcium-Mediated Immune Response in the Plant Model Physcomitrella patens."	29 Sep 2020	0,1
	Plantum webinar Seed Technology	06 Oct 2020	0,1
	Plantae Presents - Claudia Köhler and Sunil K. Kenchanmane Raju	14 Oct 2020	0,2
	Plantae Presents - Elizabeth Vierling and Rodrigo Gutierrez	21 Oct 2020	0,2
	Plantae Presents Panel: Evo-devo as a discovery tool	28 Oct 2020	0,1
	Plantae Presents: Gustavo MacIntosh and Jyothi Vaddasery	04 Nov 2020	0,2
	Plantae: Creating Crops for the Future: Challenges, Technology and Sustainable Solutions	11 Nov 2020	0,1
	Plantae Presents: Carlos Ballaré and Haim Trebes	25 Nov 2020	0,2
	The Future of Tomorrow: CRISPR and Gene Editing Roundtable Discussion w/ Influential Youths of Today's Global Society!	27 Nov 2020	0,1
	Plantae Presents – Zach Lippman and Arjun Khakhar	09 Dec 2020	0,2
	Plantae Presents - Eric Schranz and Caroline Pont	10 Feb 2021	0,2
	Plantae Presents - Malcolm Bennett and Edith Pierre-Jerome	17 Feb 2021	0,2
	Plantae Presents - Usha Vijayraghavan and Ramesh Sonti	03 Mar 2021	0,2
	Plantae Presents - Special edition to celebrate the Plant Physiology focus issue on Dynamic Membranes	10 Mar 2021	0,1
	Plantae Presents - Sheng-Yang He and Jonathan Jacobs	17 Mar 2021	0,2
	Plantae Presents - Yuling Jiao and Lilan Hong	23 Mar 2021	0,2
	Plantae Presents - Special edition to focus on Plant Synthetic Biology	31 Mar 2021	0,1
	Webinar John Bowman. Why all eukaryotes have homeodomain genes: a view from land plants (Euro- Evo-Devo society)	12 Apr 2021	0,1
	Plantae Presents - Sophia Stone and Sjon Hartman	14 Apr 2021	0,2
	Plantae Presents - Jim Birchler, Evan Forsythe, and Tom Jacobs	21 Apr 2021	0,2
	Plantae Presents - Julia Bailey-Serres and Rashmi Sasidharan	28 Apr 2021	0,2
	4th EPSO Plant Science Seminar	17 Jun 2021	0,1
	UgenTec-Bejo High-throughput Genotyping & Seed Health Webinar	24 Jun 2021	0,1
	PSG inspiration session on the NPEC lunch lecture Rick van der Zedde	01 Jul 2021	0,1
	Photosynthesis 2030+ Webinar Serie	05 Oct 2021	0,1
	Workshops & Symposia:		
	Mini-symposium 'Genetic and genomic analysis of polyploids' Dick de Ridder, Peter Bourke, Chaozhi Zheng, Jeff Endelman and Ehsan Motazedi, Wageningen, NL	6 Nov 2019	0,2
	Polyploid Workshop - QTL analysis in multi-parental populations, Chris Maliepaard and Paul Arens, Wageningen, NL	12 Dec 2019	0,3
	Plant-RX symposium "Artificial intelligence in plant science and breeding" (FutureFood@uu.nl), online	24 Feb 2021	0,2
	NWO Insight Out – Inspiring Women in STEM Marion Koopmans, online	21 Jun 2021	0,2
	EPS Mini-symposium Mendel - 200 years, Wageningen, NL	08 Jun 2022	0,2
►	**Seminar plus**		
	Discussion with prof. Teemu Teeri	14 Mar 2018	0,1
►	**International symposia and congresses**		
	Plant Biology Worldwide summit, online	27-31 Jul 2020	1,5
	21st EUCARPIA general congress, online	23-26 Aug, 2021	1,2
	26th International Conference on Sexual Plant Reproduction (ICSPR2022), Prague, CZ	20-24 Jun 2022	1,3
►	**Presentations**		
	Poster: Counting petals: RNAseq in Chrysanthemum morifolium (EPS Lunteren meeting)	08-09 Apr 2019	1,0
	Poster: Counting petals: Identification of factors involved in floret identity in Chrysanthemum morifolium (EPS Lunteren meeting)	12-13 Apr 2021	1,0

Poster: Factors influencing the disc/ray floret ratio in Chrysanthemum (ICSPR2022) ratio in Chrysanthemum	20-24 Jun 2022	1,0
Oral: Counting petals: unravelling the molecular mechanism that determines disc/ray floret ratio in Chrysanthemum (EPS Lunteren meeting)	11 Apr 2022	1,0
► **IAB interview**		
► **Excursions**		
Excursion to Bejo Zaden B.V.	20 Oct 2017	0,2
EPS & Seed Valley Online Networking Event- Bejo Zaden	14 Dec 2020	0,2
EPS Online Company visit Rijk Zwaan	16 Jun 2021	0,2
Subtotal Scientific Exposure		19,7

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
EPS course 'Data Analyses and Visualizations in R', Wageningen, NL	08-09 May 2018	0,6
EPS course 'The Power of RNA-seq', Wageningen, NL	11-13 Jun 2018	0,9
EPS course 'Transcription Factors and Transcriptional Regulation', Wageningen, NL	10-12 Dec 2018	1,0
► **Journal club**		
Journal club cluster Plant Developmental Systems/Bioscience	2018-2021	3,0
Journal club Dekker Chrysanten research department	2018-2021	0,0
► **Individual research training**		
Subtotal In-Depth Studies		5,5

4) Personal Development	*date*	*cp*
► **General skill training courses**		
EPS Introduction Course	26 Sep 2017	0,3
WGS PhD Workshop Carousel, Wageningen, NL	15 May 2018	0,3
Workshop 'Stratego for women', Wageningen, NL	02 Oct 2018	0,2
Tijdsturfen, Hensbroek, NL	12 Nov 2019	0,2
WGS course 'Scientific writing', online	17 Aug-25 Sep 2020	1,8
WGS workshop 'Critical Thinking and Argumentation', Wageningen, NL	15 Apr 2021	0,3
WGS course 'Effective and Efficient Communication in Academia and Beyond', online	10-27 May 2021	0,8
WGS workshop 'The Last Stretch of the PhD Programme', Wageningen, NL	04 Feb 2022	0,0
WGS workshop 'Writing propositions for your PhD', Wageningen, NL	04 Feb 2022	0,0
► **Organisation of meetings, PhD courses or outreach activities**		
► **Membership of EPS PhD Council**		
Subtotal Personal Development		3,9

TOTAL NUMBER OF CREDIT POINTS*	**32,1**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.	

** A credit represents a normative study load of 28 hours of study.*

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